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Establishing a model to functionally target dopaminergic neurons and astrocytes in the mouse hypothalamus

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Abstract

The hypothalamus regulates some of the most basic physiological needs such as sleep, reproduction or food intake. It was found to contain the highest diversity of neurons in the brain. In fact, several neuronal subtypes producing neurotransmitters, -peptides and hormones contribute crucially to the achievement of functional specificity. Regarding structural organization, these neuronal subgroups form a number of hypothalamic nuclei that receive innervation from and also project to distinct areas in the brain. However, it seems that not only neurons play a role in this complex network: In fact, the contribution of astroglia is crucial for the maintenance of important processes regulated in the hypothalamus and also other brain regions. In the past years, researchers revealed a solid amount of knowledge regarding the complex interplay of various cells in the hypothalamus with the goal to link their underlying functional mechanisms to physiological or behavioural outcomes in an individual. However, a lot of processes involving the hypothalamus still remain unknown and require further investigation.

The goal of this thesis was to establish an efficient model to chemogenetically manipulate specific neuronal groups and astrocytes in hypothalamic nuclei and their extrahypothalamic projection sites. For this, I used transgenic mice expressing Flp- and Cre- recombinase under specific promoters infected with AAV- dependent viruses encoding DREADDs to target neurons in the periventricular nucleus and astrocytes in the ventromedial hypothalamus, respectively. I used immunohistochemical analysis to reveal successful infection and activation of targeted cells. All in all, I managed to develop an efficient method to manipulate target cell groups which can be used for further research to reveal connections between processes on the molecular level and the resulting physiological or behavioural outcome. I successfully showed that dopaminergic A14 and A12 cells can be activated using a Flp- dependent approach. I additionally discovered that chemogenetic stimulation of dopaminergic A14 cells not only leads to increased locomotion, but also decreases food consumption in mice. Stimulation of their projection targets, which are Somatostatin-cells in the lateral septum, also resulted in increased animal mobility. Regarding astrocyte manipulation, I found that DREADD expression was way more efficient when a recombinase- independent approach was used. With this strategy, it was possible to deliver both activating and inhibiting constructs which in turn were used to successfully manipulate cells in the ventromedial hypothalamus. In addition to that, cells in the arcuate nucleus and in the ependymal layer next to the third ventricle also showed an activation pattern that was connected to the virus injection, suggesting that this approach can be broadly used to affect various forms of cells, e.g. tanycytes or other ependymal cells.

Abstrakt

Der Hypothalamus reguliert einige der grundlegendsten physiologischen Bedürfnisse wie beispielsweise Schlaf, Fortpflanzung oder Nahrungsaufnahme. Er beinhaltet die höchste Diversität an Neuronen im ganzen Gehirn. Verschiedene neuronale Untergruppen, welche Neurotransmitter,-peptide und Hormone produzieren, sind unentbehrlich um diese funktionale Spezifität zu erreichen. Betrachtet man die strukturelle Organisation, fällt auf dass diese neuronalen Untergruppen eine Vielzahl an Nuclei formen, welche sowohl von anderen Hirnregionen innerviert werden als auch axonale Projektionen zu selbigen bilden. Tatsächlich scheinen aber nicht nur Neuronen eine wichtige Rolle in diesem komplexen Netzwerk zu spielen: Denn auch Astrocyten tragen maßgeblich zum Erhalt von wichtigen intra- und extrahypothalamischen Prozessen bei. Innerhalb der letzten Jahre gewannen Forschende eine Vielzahl an Erkenntnissen hinzu, um dieses Zusammenspiel verschiedener Zellen innerhalb des Hypothalamus mit den ihren zugrundeliegenden funktionellen Mechanismen mit physiologischen oder verhaltensbiologischen Reaktionen in Kontext zu setzen. Jedoch sind viele der Prozesse, welche im Hypothalamus ablaufen, noch nicht aufgeklärt sondern müssen noch erforscht werden.

Das Ziel dieser Arbeit war, ein effizientes Model für die chemogenetische Manipulation von spezifischen Neuronengruppen und Astrocyten in hypothalamischen Nuclei und deren extrahypothalamischen Projektionszielen zu entwickeln. Dafür habe ich transgene Mauslinien, welche Flp- oder Cre- Recombinasen unter spezifischen Promotern exprimieren, mit adeno- assoziierten Viren injiziert. Diese Viren fungieren als Vektor für DREADDs, mithilfe dieser Neuronen im periventrikulären Nucleus und Astrocyten im ventromedialen Nucleus gezielt manipuliert werden sollten. Mithilfe von immunohistochemischer Analysemethoden konnte ich dann die erfolgreiche Infektion und Aktivierung dieser Zellen überprüfen. Letztendlich konnte ich eine effiziente Methode entwickeln um Zielzellgruppen zu manipulieren, welche für weitere Forschungen benutzt werden kann um die Verbindung zwischen Prozessen auf der molekularen Ebene und den daraus resultierenden physiologischen oder verhaltensbiologischen Reaktionen herzustellen. Des weiteren konnte ich zeigen, dass dopaminerge Zellen der A12 und A14 Gruppe mithilfe eines Flp- Rekombinase- basierendem Ansatz aktiviert werden können. Außerdem konnte ich zeigen, dass die chemogenetische Stimulation von dopaminergen A14 Zellen in der Maus nicht nur zu vermehrter Bewegung führt, sondern auch die Nahrungsaufnahme verringert. Die Aktivierung der Projektionsziele dieser Zellen, welche Somatostatin- positiv und im lateralen Septum zu finden sind, hat auch zu einem Anstieg im Bewegungsverhalten geführt. Des weiteren konnte ich zeigen, dass die Exprimierung der DREADDs zur Manipulation von Astrocyten effizienter ist, wenn ein Rekombinase- unabhängiger Ansatz genutzt wird. Mit dieser Strategie war es möglich, sowohl aktivierende als auch inaktivierende Konstrukte in der Zelle zu exprimieren. Mithilfe dieser Konstrukte konnte ich dann gezielt Astrocyten im

ventromedialen Hypothalamus manipulieren. Zusätzlich konnte ich auch bei Zellen im Nucleus Arcuatus und im Ependym neben dem dritten Ventrikel ein Aktivierungsmuster beobachten, das mit der Virusinjektion zusammenzuhängen scheint. Dies legt nahe, dass diese Methode zukünftig weitreichend genutzt werden kann um verschiedenste Zelltypen zu erreichen, beispielsweise Tanycyten oder andere Zellen des Ependyms.

Abbreviations

3V, third ventricle;

AADC, L-amino acid decarboxylase;

AAVs, adeno- associated viruses;

AgRP, Agouti gene- related protein;

ARC, arcuate nucleus;

CNO, clozapine-N- oxide;

CSF, cerebrospinal fluid;

DAT, dopamine transporter;

DMH, dorsomedial hypothalamic nucleus;

DREADDs, Designer receptors exclusively activated by designer drugs;

ER, estrogen receptor;

GABA, gamma-aminobutyric acid

Gal, galanin;

GH, growth hormone;

Ghrh, growth hormone- releasing hormone;

GnRH, gonadotropin- releasing hormone

GPCRs, G protein- coupled receptors;

LPO, lateral preoptic area;

LS, lateral septum;

ME, median eminence;

MPO, median preoptic area;

MS, medial septum;

NDB, diagonal band nucleus;

Nmbr; Neuromedin B receptor;

Nmur, Neuromedin U receptor;

NPY, Neuropeptide Y;

PAMPs, pathogen- associated molecular patterns;

PeVN, periventricular nucleus;

Pnoc, prepronociceptin;

POMC, proopiomelanocortin;

PVN, paraventricular nucleus;

SI, substantia innominate;

Sst, Somatostatin;

Tac1, tachykinin precursor;

TNF-α, tumor necrosis factor;

VLPO, ventrolateral preoptic nucleus;

VMAT2, vesicular monoamine transporter 2;

VMH, ventromedial hypothalamic nucleus;

αMSH, α-melanocyte-stimulating hormone;

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Introduction

Structural and functional organisation of the hypothalamus

The hypothalamus is one of the oldest regions in the brain which is highly conserved between different species. Though making up only 4g of the 1400g of adult human brain weight, it contains a high variety of cell types and contributes crucially to the maintenance of the basic physiological needs of an organism: energy metabolism and expenditure, thermoregulation, sleep and wakefulness behaviour and social interactions such as aggression, parenting and reproduction (Saper & Lowell, 2014). Through interaction with multiple brain regions, the hypothalamus connects external stimuli with complex behavioural responses and therefor enables response and adaption to an individuals environment (Saper & Lowell, 2014).

Development of the hypothalamus

During early stages of brain development, the hypothalamus arises from the most anterior part of the neural tube (Saper & Lowell, 2014). Most hypothalamic regions, especially tuberal and posterior parts, originate from the diencephalon, only the preoptic area stems from the telencephalon (Xie & Dorsky, 2017). The hypothalamus can be divided in 3 subregions which consist of various nuclei. From a ventral point of view, the 3 areas are enclosed by the optic chiasm (anterior) and the mammillary body (posterior) (Saper & Lowell, 2014). The third ventricle divides the hypothalamus into a right and left side, both are flanked laterally by the optic tract. It was found that the activity of one side is enough to maintain all the essential hypothalamic functions (Saper & Lowell, 2014).

The three main regions and their nucleii

As mentioned before, the first region of the hypothalamus lies right above the optic chiasm and is usually referred to as the preoptic area (Saper & Lowell, 2014). This region contains the median and ventrolateral preoptic nuclei and the suprachiasmatic nucleus and was found to play a role in fever and thermoregulation, electrolyte balance, circadian rhythms and wake-sleep regulation, and sexual behaviour (Saper & Lowell, 2014). The middle region is called tuberal hypothalamus and includes the dorsomedial, ventromedial, paraventricular, supraoptic and arcuate nuclei (Saper & Lowell, 2014). It is connected to the pituitary stalk (infundibulum), which is part of the endocrine system. Feeding behaviour is controlled via integrative circuitry predominantly involving the arcuate nucleus, whereas several output circuits were associated with sexual behaviour, aggression and various endocrine and autonome responses (Saper & Lowell, 2014). The third and last region (posterior) hypothalamus) contains the mamillary bodies and the tuberomammillary, supramammillary and posterior

hypothalamic nuclei. Providing output circuitry to the hippocampus and the arousal system, this region seems to play a role in the regulation of wakefulness and also stress responses (Saper & Lowell, 2014).

All 3 regions with their various nuclei and areas contribute to the integrative role of the hypothalamus, which allows the comparison of diverse sensory inputs to basic setpoints in order to enable important decisions for maintaining basic life functions.

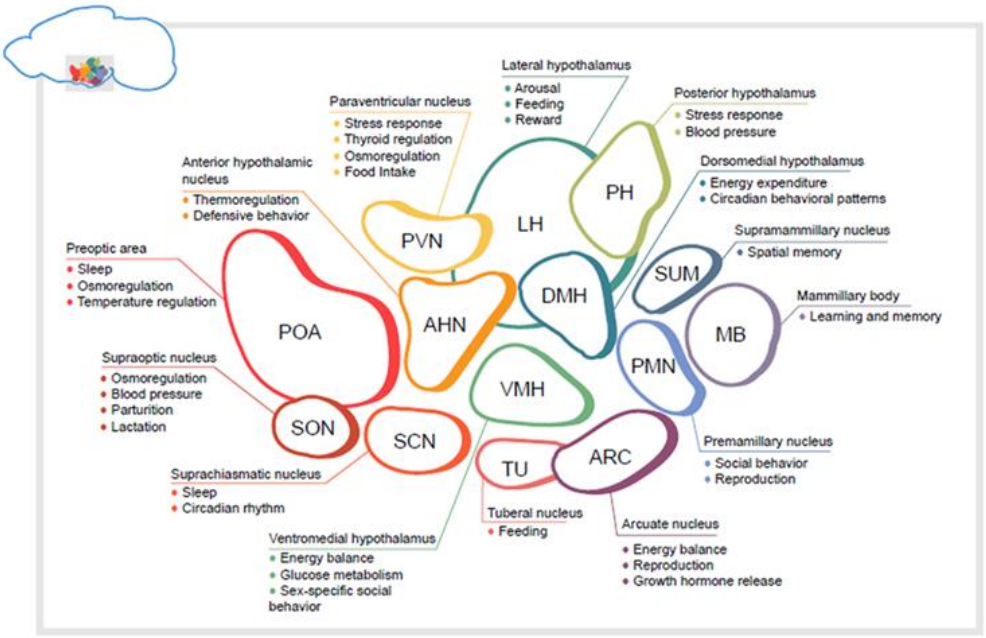

Figure 1.1: Schematic depiction of hypothalamic organisation
The hypothalamus can be subdivided into several areas and nucleii that cover tasks necessary for the maintenance of basic life functions up to higher cognitive responses (Hajdarovic et al. 2022)

Main functions of the hypothalamus

From a functional point of view, the hypothalamus is considered to have three major outputs. First of all, it exerts control on the autonomic nervous system. The main players for this are pre-autonomic neurons, which are located mainly in the parvicellular part of the paraventricular nucleus and the adjacent hypothalamic area, with a small subset also in the arcuate nucleus (Saper & Lowell, 2014). On one hand, they innervate both the parasympathetic and sympathetic preganglionic neurons and also cell groups in the brain stem, whereas some pre-autonomic neurons also project to the spinal cord. Subsets of these neurons were found to be activated by several physiological stimuli including leptin or lipopolysaccharide, indicating their rather functional than strictly anatomical organization (Seoane-Collazo et al, 2015; Saper & Lowell, 2014).

The second key output target, the endocrine system, can be again subdivided into 3 parts. The magnocellular system consists of large endocrine cells with secretory function which are predominantly found in the supraoptic and paraventricular nuclei (Saper & Lowell, 2014). The two

major cell populations are oxytocin and vasopressin neurons (Saper & Lowell, 2014). Via axonal projections along the pituitary stalk, they release these hormones into blood vessels in the posterior pituitary gland from where they can be distributed to the entire body (Saper & Lowell, 2014). The parvicellular system contains smaller neurons located in the wall of the third ventricle, mainly in the medial part of the paraventricular nucleus and in the arcuate nucleus (Saper & Lowell, 2014). Sending their axons to the floor of the third ventricle, the median eminence, these neurons secrete releasing or release-inhibiting hormones into the hypophysial portal vessels (Saper & Lowell, 2014). From there, the hormones are transported via the bloodstream to the anterior pituitary gland from where they control pituitary hormone release (Saper & Lowell, 2014). Lastly, the hypothalamus also controls the endocrine system by autonomic innervation of endocrine glands, an example for this is the autonomic innervation of the pancreas to regulate insulin and glucagon secretion (Saper & Lowell, 2014).

The most complex output target is the control of an individuals behaviour. While it was initially thought that the hypothalamus contains several centers that individually control specific behaviours such as aggression, feeding or reproduction, the current understanding is that the activation of distinct circuits increases the likelihood of an animal to behave in a certain way (Saper & Lowell, 2014). In hungry animals for example, the activity of cells associated with wakefulness and locomotion circuitry increases, resulting in an enlarged chance for the animal to encounter food (Saper & Lowell, 2014).

All in all, the hypothalamus relies on its complex molecular composition to maintain high functional specificity. Various subgroups of cells with different output targets are key players of the neural circuits that enable a huge variety of responses to internal and external stimuli.

Hypothalamic neurons

NPY/ Agrp and POMC neurons

Two distinct populations of neurons located in the arcuate nucleus were found to be crucially involved in the regulation of feeding behaviour and body weight: Proopiomelanocortin (POMC) neurons and Neuropeptide Y (NPY)/ agouti gene- related protein (AgRP) neurons (Broberger et al., 1998). POMC neurons release α-melanocyte-stimulating hormone (αMSH) which then binds to melanocortin-4 receptors (Broberger et al., 1998). This interaction leads to reduced food intake and increased energy expenditure in an individual. In contrast to that, NPY/AgRP neurons stimulate feeding behaviour by inhibiting POMC neurons through the release of AgRP, which is a natural antagonist of αMSH (Broberger et al., 1998). Both populations densely project to other hypothalamic areas, such as the paraventricular nucleus (PVN) and lateral hypothalamic area (Broberger et al, 1999, 1998; Atasoy et al, 2012).

Dopamine neurons

In the hypothalamus, dopamine was predominantly known as a hormone that is secreted into the hypophyseal portal system in order to inhibit the release of prolactin via the pituitary (Ben-Jonathan & Hnasko, 2001). Dopamine releasing neurons can be predominantly found in the tuberoinfundibular tract. However, the classical terminology of dopaminergic systems in the brain describes 9 distinct populations (referred to as A8-A16) in the whole brain and five of them are found in the hypothalamus (Björklund & Dunnett, 2007; Dahlström & Fuxe, 1964; Everitt et al, 1984). In fact, several centers for dopamine production exert a bandwidth of functions: (i) The A11 group in the periaqueductal grey matter is involved in pain modulation and is connected with spinal locomotor network, dysfunction in this area even seems to result in cataplexy (Koblinger et al, 2014); (ii) dopaminergic neurons found in the arcuate nucleus that modulate also parental care next to prolactin regulation (A12, Lyons & Broberger, 2014;; (iii) dopamine neurons located in the zona incerta (A13) that play a role in regulation of brown fat thermogenesis and nociception (Moriya et al, 2020; Folgueira et al, 2019). (iv) the A14 group in the periventricular nucleus (PeVN) that is connected to prolactin release via the pituitary (Björklund et al, 1973; Lehman et al, 1996); and (v) lastly, dopamine neurons of the anteroventral PeVN and preoptic area that control maternal care and oxytocin secretion, regulate shifts in estradiol sensitivity and suppress intermale aggression in males, all in addition to hypophyseal hormone release (Scott et al, 2015; Ben-Jonathan & Hnasko, 2001; Lehman et al, 1996). In order to precisely identify dopamine- producing neurons in the hypothalamus, the use of one single marker is not sufficient: Older approaches predominantly used tyrosine hydroxylase (TH), an enzyme involved in dopamine synthesis. However, recent single cell RNA sequencing data revealed that more than 15% of neurons in the hypothalamus are TH- positive, that would be far more than the actual amount of dopaminergic neurons present in the hypothalamus (Romanov et al. 2017). Thus, a more precise method is the use of TH in combination with other dopamine markers that include (i) L-amino acid decarboxylase (AADC), an enzyme that converts aromatic amino acids into their corresponding amines in the process of dopamine synthesis; (ii) the vesicular monoamine transporter 2 (VMAT2) that is responsible for the reuptake of monoamines from the cellular cytosol into synaptic vesicles and (iii) the dopamine transporter (DAT), which pumps dopamine out of the synaptic cleft into the cytosol (Björklund & Dunnett, 2007). Until recently, dopamine neurons were thought to be molecularly homogenous. This view was shifted after several neuropeptides and transcription factors were found to be differentially expressed throughout hypothalamic dopamine neurons. Based on the expression pattern of neuropeptides somatostatin (Sst), growth hormone-releasing hormone (Ghrh), tachykinin precursor (Tac1), prepronociceptin (Pnoc) and galanin (Gal) and transcription factors meis2 and onecut3, dopamine neurons of the hypothalamus can be organized into four groups (Romanov et al. 2017). Three out of those four groups display high homology, whereas the fourth group showed a unique

expression pattern that is highly characteristic for A14 dopamine neurons in the PeVN connected to the circadian circuitry: Onecut3, neuromedin U (Nmur) and neuromedin B (Nmbr) receptors are expressed together with axon guidance systems such as Robo1 and Dscam and their related GTPases (Romanov et al, 2017; Horvath, 1998). Moreover, neurons of the third group can be classified as A12 tuberoinfundibular dopamine neurons based on their expression of slc6a3 (Dat), Grhr and Tac1. However, this method of classification is limited as neurons of group 1 and 2 can not be assigned to groups of the classical dopaminergic system solely based on their molecular characterisation. In fact, deeper interrogation would require additional anatomic positioning. Regarding axonal projections, hypothalamic dopamine neurons were found to be involved in various intra- and extrahypothalamic circuits that modulate functional outcome. Neurons of the A11 and A13 group project to the spinal cord and the periaqueductal gray where they regulate nociception and locomotion (Koblinger et al. 2014). The A15 group was found to monosynaptically regulate magnocellular oxytocin neurons in the supraoptic nucleus and target the median eminence for prolactin secretion control (Scott *et al*, 2015; Ben-Jonathan & Hnasko, 2001; Lehman *et al*, 1996). Lastly, the A12 group is involved in the regulation of food intake as they interact with the previously described orexigenic NPY neurons and anorexigenic POMC neurons of the arcuate nucleus in a stimulating and inhibiting manner, respectively (Zhang & van den Pol, 2016).

Somatostatin neurons

Somatostatin in the hypothalamus is primarily known to have pituitary inhibiting function as it regulates the secretion of growth hormone (GH) (Clark et al., 1988). It is predominantly present in cell bodies and fibre like structures of neurons. Studies revealed that cell bodies were localized in the periventricular area at the level of the suprachiasmatic nucleus in a caudal direction, whereas somatostatin positive fibers were detected in the external layer of the median eminence from the beginning all the way down to the stalk (Luft et al., 1978). Their cell bodies sit in the periventricular anterior hypothalamic nucleus. It is in accordance with data that Sst and other neuropeptides/hormones (e.g. CRH) are widely expressed in neurons in numerous brain region including the ventromedial and arcuate nuclei, where dense plexuses of somatostatin positive fibers extend caudally into the ventral premammillary nucleus. This suggests a possible release of somatostatin from synaptic nerve endings, supporting its dual role as neurotransmitter and hormone (Luft et al., 1978).

Non neuronal cells in the hypothalamus

Astrocytes

Astrocytes display high diversity in their morphological appearance functional properties and their distribution. In the CNS, most protoplasmic astrocytes show highly developed and dynamic arborization (Khakh & Sofroniew, 2015). Astrocytes are highly reactive to their environment as they sense synaptic activity by expressing transporters, ion channels and neurotransmitter receptors, allowing them to control the concentration of ions, hormones and neurotransmitters in the extracellular space (Verkhratsky & Nedergaard, 2018). Additionally, they supply adjoining neurons with neurotransmitter precursors such as glutamine, which is required for GABA synthesis (Verkhratsky & Nedergaard, 2018). Astrocytes are part of the gliocrine system as they secrete various neuroactive molecules such as classical neurotransmitters, they bidirectionally communicate with neurons and, together with microglia, are crucially involved in synaptic formation . This interplay is referred to as multipartite system and evolved from the tripartite synaptic system, which originally included only astrocyte and neuron communication (Verkhratsky & Nedergaard, 2018; Araque et al., 1999). Astrocytes support synaptogenesis, synaptic maturation, maintenance and extinction (Araque et al., 2014). Furthermore, they modulate neuronal activity in several brain areas. Organized into networks, they communicate through gap-junctions that facilitate intercellular signalling via Ca^{2+} or Na^{+} waves (Verkhratsky et al., 1998). They were found to deliver glucose in an activity-dependent manner and transport glucose metabolites from capillaries to distal neurons of the CNS, including the hypothalamus (Rouach et al., 2008; Clasadonte et al., 2017). Astrocytes in the hypothalamus seem to be crucially involved in the regulation of nutrient and hormone sensing in the CNS (García-Cáceres C. et al., 2016; Kim et al., 2014). They are highly responsive to glucose and insulin signalling pathways, as they express insulin receptors and the astroglial glucose transporter (GLUT1; Chari et al., 2011; García-Cáceres et al., 2016). Mediating the transport of glucose from blood into the brain, hypothalamic astrocytes control the activation of POMC neurons, which is glucose- dependent (García-Cáceres et al., 2016).

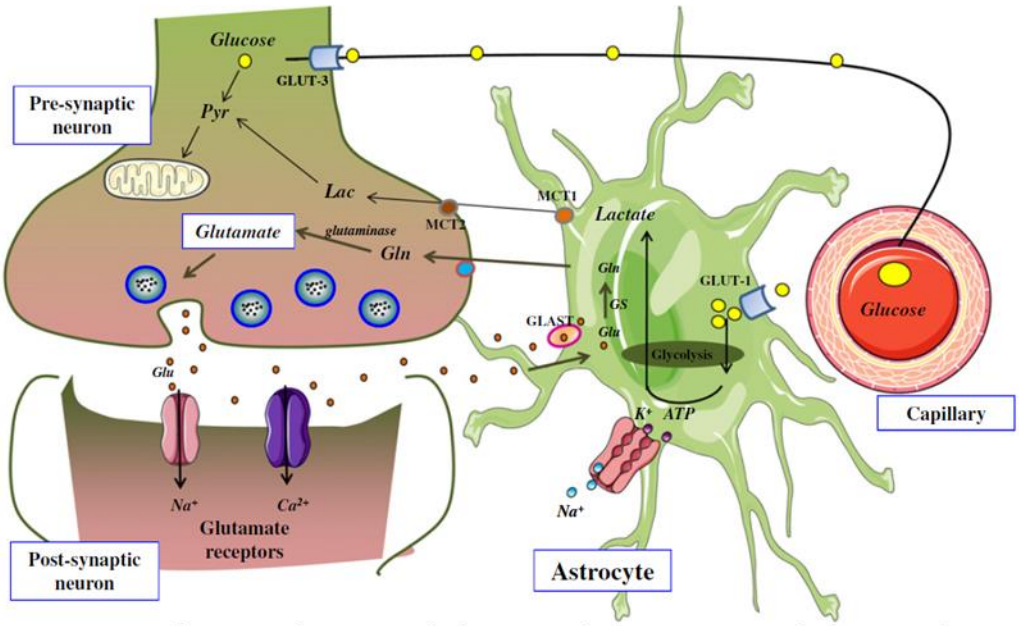

Figure 1.2: Schematic depiction of glucose and glutamate metabolism involving astrocytes and neurons

Astrocytes work together with neurons and blood vessels to regulate central and peripheral glucose levels (Cáceres et al, 2016).

As previously mentioned, these neurons exert anorexigenic effects as they reduce food intake and also control systemic glucose metabolism. Another astrocytic subtype that is suspected to influence the activity of their neighbouring neurons are gomori-positive astrocytes. Those cells contain cytoplasmic granules that derive from degenerating mitochondria and have a high affinity for Gomori's chrome alum hematoxylin and toluidine blue stain (Schipper, 1991; García-Cáceres et al., 2019). Mainly found in GLUT-2- rich areas of the arcuate nucleus, gomori-positive astrocytes display an elevated glucose-dependent oxidative metabolism (Young & McKenzie, 2004). Another example for the determination of astrocyte functionality and phenotype by the local environment and the present neurons is the stimulation of the periventricular nucleus (PVN) with norepinephrine or glutamate. This interaction induces the release of ATP via local astrocytes, leading to increased synaptic efficacy at glutamatergic synapses (Gordon et al., 2005 & 2009). Astrocytes can even directly influence the firing rate of neurons, an example for this are MBH astrocytes that are involved in bidirectional regulation of leptin- and-ghrelin- regulated feeding circuits. By releasing adenosine and the additional activation of adenosine A_1 receptors, those astrocytes alter the firing rate of AgRP neurons (Yang & Yang. 2015).

Tanycytes

The brain microenvironment is maintained by a plethora of factors, a crucial one is the blood- brain barrier. Preventing direct exposure of the cerebrospinal fluid (CSF) and the parenchyma to the blood, it requires a complex cellular organisation with highly coordinated interplay. In the median eminence, a highly specialized, polarized astroglial subtype referred to as tanycyte plays a crucial role in regulating hypothalamic key factors. Sealing the intracellular space with their tight-junction complexes located at their cell bodies, they prevent the unhindered diffusion of blood-borne molecules that extravasate from fenestrated capillaries in the ME into the CSF (Prevot et al., 2018; García-Cáceres et al., 2019). Their other pole mediates communication with ME microvessel loops in the ventromedial arcuate nucleus (ARC) or the blood-brain barrier vessels in the ARC itself (Prevot et al., 2018; García-Cáceres et al., 2019). Interaction on both poles is plastic as it can be restructured to modulate access of blood-borne metabolic signals to local neurons and to control the adaptive response to acute nutritional challenges (Langlet et al., 2013; Schaeffer et al., 2013). With their cell bodies lining the floor of the third ventricle in the tuberal hypothalamic region near the ARC, they mediate the exchange of metabolites such as leptin, ghrelin and also glucose between the third ventricle and the general circulation or the parenchymal area (Balland et al., 2014; Collden et al., 2014; García-Cáceres et al., 2019). Additionally, they regulate the secretion of neuropeptides into the hypothalamus- pituitary portal circulation and generate the active form of thyroid hormones. Because of their unique morphology and their stem cell properties, they are considered radial glia of the mature brain (Prevot et al., 2018; García-Cáceres et al., 2019).

Microglia

An important player when it comes to protecting the neural environment are microglia. They secrete neurotrophic factors such as BDNF and are crucially involved in brain development and the regulation of synaptic plasticity and connectivity via CX3CR1-dependent engulfing and phagocytosis of synaptic material (Kettenmann et al., 2013; García-Cáceres et al., 2019). Displaying a huge variety of surface receptors, they are able to sense pathogen-associated molecular patterns (PAMPs), ADP and ATP from injured cells and glycocalyx- bound sialic acids as well as cytokines, chemokines and neurotransmitters (Kettenmann et al., 2011).

The importance of astrocytes for maintaining basic functions and complex signalling in the brain

Astrocytic control of systemic energy metabolism

So far, non- neuronal cells have been fairly underestimated when it comes to understanding how the brain finetunes metabolic and behavioural parameters as a response to environmental cues. From a medical point of view, the focus was on connecting defects in neuronal signalling networks with metabolic and behavioural diseases. In fact, glia cells are far more involved in the maintenance of neuronal circuitry than it was estimated, and their misfunction often has a pathological outcome. In the hypothalamus, several neuron populations and various nuclei were found to be involved in the control of feeding behaviour. As mentioned before, AgRP and POMC neurons are important players in regulating food seeking, food intake and energy homeostasis (Broberger et al., 1998). When stimulated by a combination of high- fat and high- carbohydrate diet, these neurons produce and secrete advanced glycation end- products. Interestingly, these end- products are then taken up by microglia, leading to their activation (Gao et al., 2017). As a result, microglia increase their production of tumor necrosis factor (TNF)-α to enhance their own immune capacity (Kuno et al., 2005; García-Cáceres et al., 2019). Additionally, this leads to elevated mitochondrial stress levels in POMC neurons, which in the long run may cause their dysfunction (Yi et al., 2017; García-Cáceres et al., 2019). Astrocytes themselves also react to obesogenic diets: In the ARC, they were found to develop a reactive phenotype which results in changes in their reactivity and/or distribution (Horvath et al, 2010). This was found to influence the synaptic organization and responsiveness of POMC neurons to metabolic factors such as glucose or leptin (Fuente-Martín et al., 2012; Kim et al., 2014; García-Cáceres et al., 2019). As a consequence, the content of local synaptic messengers like endocannabinoids and the response of hypothalamic neurons to metabolic hormones might be affected (Koch et al., 2015; García-Cáceres et al., 2019). Loss of astrocytic lipid sensing via lipoprotein lipase induced by high fat diet can also have obesity promoting effects as it results in hypothalamic ceramide accumulation and body weight gain (Gao et al., 2017). Tanycytes are also crucial for maintaining metabolism homeostasis: In leptin receptor- deficient (db/db) mice, LepR- associated signaling pathway (STAT3, Akt, ERK) activation in tanycytes is ablated. Leptin then gets stored up in the median eminence (ME) and never reaches the MBH because it is not released into the third ventricle. Interestingly, leptin translocation can be rescued by independently activating the ERK signaling pathway in tanycytes, resulting in improved leptin signalling (Balland et al., 2014).

Astroglial control of reproduction

The hypothalamus harbors a specific neuronal subgroup: gonadotropin- releasing hormone (GnRH) neurons. Those neurons were found to secrete GnRH in order to coordinate the release of luteinizing hormone and follicle- stimulating hormone, both are crucial for the regulation of reproductive function (Párducz et al., 1993). In fact, astroglia also seem to play a role in this process: Following the estrus cycle, they show hormone dependent phasic morphological plasticity, which is estimated to regulate synaptic density (Párducz et al., 1993; Verkhratsky & Nedergaard, 2017). High estrogen levels for example lead to increased astroglial membrane coverage which in turn prevents formation of inhibitory synaptic inputs in neurons of the arcuate nucleus (García-Segura et al., 1994). In the rat hypothalamus, a reduction in the number of dendritic spines was observed after steroid- induced remodeling of astroglia (Mong et al., 1999). Plasma levels of 17b- estradiol and pulsatile secretion of gonadotropin hormones seem to be linked regarding the regulation of astroglial morphological plasticity (Dhandapani et al., 2003; Verkhratsky & Nedergaard, 2017). Additionally, astrocytes themselves can directly stimulate GnRH release by secreting several factors such as transforming growth factor, astrocyte- derived transforming growth factor-a or neuroactive steroid metabolites (Mahesh et al., 2006; Verkhratsky & Nedergaard, 2017). Lastly, the pulsatile release of GnRH from neurons was found to be regulated by oxytocin- induced astroglial PGE2 secretion (Parent et al., 2008).

Role of astrocytes regarding higher cognitive function

The contribution of astrocytes to cognitive processes like emotion, learning, memory and the generation of thoughts still remains widely unsolved. However, several findings and observations suggest that there is in fact a role for astrocytes, at least in combination with neuronal activity: Electrically silent astrocytes were observed to initiate Ca2+ signals in neighbouring cocultured neurons (Nedergaard, 1994). In fact, astrocytes can regulate the intrinsic excitability of neurons and even the strength of synaptic transmission (Araque et al., 2014; De Pittà et al., 2016). However, it is not clear whether there is direct contribution of astrocytes to cognitive processes or if their activity just indirectly allows or encourages those functions. Research into this topic is often technically limited: Brain slices obtained from newborn animals can contain immature astrocytes, or the experimental strategy is based on nonphysiological manipulation. Housekeeping and homeostasis function can be compromised when astrocytes are stimulated over a certain threshold, this can also result in nonphysiological neurotransmitter release generating artificial astrocyte- to- neuron signalling (Verkhratsky & Nedergaard, 2017). Nevertheless, interesting observations were made that support astrocytic involvement in cognitive functions. Astrocytic Ca2+ signalling seems to be highly connected to the state of arousal in mice. In intact animals, these signals can be strongly suppressed by anaesthesia (Thrane et al., 2012). In awake animals, astrocytic Ca2+ signalling is regulated by the “fight-

or- flight" neurotransmitter norepinephrine that is released when a reorientation in focus is required as a response to environmental cues (Ding et al., 2013; Gibbs et al., 2008). Over 90 per cent of spontaneous Ca2+ signalling is evoked by activation of astrocytic a1- adrenoceptors. This corresponds well with the elimination of this increased Ca2+ signalling by a1- adrenoceptor antagonists. Nevertheless, the exact functional background of this mechanism remains unclear, as it is possible that increased astrocytic Ca2+ signalling is connected to other processes like glycogenolysis and vascular response to prepare the brain for a sudden increase in the metabolic rate (Verkhratsky & Nedergaard, 2017). Astrocytic activity is also likely connected to the process of learning. It is known that complexity and extension of astroglial processes and also astroglial synaptic coverage is increased after exposure to an enriched environment (Jones & Greenough, 1996; Verkhratsky & Nedergaard, 2017). Hypertrophic astrocytes seem to boost synaptogenesis during the acquisition of motor skills (Anderson et al., 1994). Lastly, physical exercise enhances astroglial profiles and leads to an increased expression of glucose transporters (Rodríguez et al., 2013; Allen & Messier, 2013). However, only few studies were conducted to investigate the role of astrocytes in cognitive function in an in vivo approach. Still, it was observed that mice with conditional deletion of CB1 cannabinoid receptors in astrocytes did not show deficits in working memory after cannabinoid treatment, whereas CB1 receptor deletion in glutamatergic or inhibitory neurons did have negative impact (Han et al., 2012; Verkhratsky & Nedergaard, 2017).

Tools in neuroscience

Cre-lox System

One of the main tasks in neuroscience is to understand how the brains various cell types form neural circuits that regulate a large span of processes, ranging from maintaining basic metabolic function to regulating complex behavioural mechanisms. For this, several advanced molecular techniques have been established and developed over the past years. One widely used method for the targeted manipulation of genes at a DNA level is the Cre- lox recombination system. It allows the generation of transgenic mouse lines which can then be used to establish a model for the study of gene function or even for understanding the complex interplay of events that lead to the development of human diseases. Site- specific DNA recombination at sequences ranging from 25 to 150bp in length can be catalyzed by a large number of recombinases that are found in bacteria and yeast (Sauer, 1998). However, the Cre- lox system uses a recombinase from bacteriophage P1. The 38-kDa product of the *cre* (cyclization recombination) gene belongs to the Int family and has two main purposes in the life cycle of P1: After infection, it provides a backup mechanism for cyclizing P1 DNA and it enhances the stability of P1 plasmid in bacterial lysosomes (Segev & Cohen, 1981, Austin et al., 1981). This happens through resolvement of dimeric plasmids for increased partition fidelity at bacterial division. Cre recombinase recognizes a specific site on the P1 genome called loxP and in turn catalyzes reciprocal conservative DNA recombination between a pair of loxP sites (Sauer, 1987). LoxP (locus of X- over of P1) is a 34bp long fragment consisting of an 8-bp nonpalindromic core region flanked by two 13-bp inverted repeats (Sauer, 1987). The core fragment is crucial for reaching directionality of the loxP site and depending on the orientation of the two corresponding sites a different outcome regarding gene manipulation is achieved: Direct repetition of loxP sites results in excision of the DNA inbetween, whereas their inverse orientation leads to DNA inversion (Sauer, 1987). DNA strand breaking and rejoining happens at discrete locations within the core region, where cre recombinase forms a transient phosphotyrosine DNA – protein linkage with the DNA strand (Sauer, 1987). This requires no accessory factors or topological requirements within the DNA, which contributes to the suitability of Cre for targeted gene manipulation in eukaryotic cells (Sauer, 1987). In some cases, the introduction of a transgene leads to either morbidity or reduced viability that makes it difficult to obtain a transgenic mouse line by breeding (Lakso et al., 1992). The insertion of a STOP cassette between the transgene and its promoter solves this problem: By intercrossing with a second mouse line that expresses Cre, the STOP signal can be removed by Cre- mediated excision to activate the transgene (Lakso et al., 1992). In order to limit transgene expression to a specific tissue, Cre expression can be spatially or developmentally reduced so that the transgene is only activated in the intersection of the two expression patterns. Another possibility is crossing a mouse that expresses Cre ubiquitously with a mouse carrying a transgene with a tissue specific promoter. Transgene expression can also be

temporarily controlled: For this, the recombinase gene has to be placed under the control of a promoter that has the desired spatial and temporal expression pattern (Sauer, 1987). Another option is the use of Cre-ER system, that consists of Cre recombinase that is fused to a mutated ligand binding domain of the human estrogen receptor (ER) (Feil et al., 1996). In order to activate Cre, tamoxifen is administrated which binds the estrogen receptor causing its dislocation from the recombinase. Depending on the time point of the tamoxifen injection, Cre expression can be temporarily controlled (Feil et al., 1996). To control whether animals are homozygous, heterozygous or even negative for the expression of the desired transgene, western blotting is a frequently used method. For this, the use of specifically designed primers is required. Other options include the use of a reporter sequence like tdTomato or GFP, immunostaining of the target region and dual fluorescent in situ hybridization (dual fISH) with probe for reporter (Cox et al., 2012).

Flp/ Frt system

Another frequently used system for targeted gene manipulation is the FLP/ Frt- system. In contrast to Cre recombinase, which has a prokaryotic origin (see above), the FLP protein has evolved in a eukaryotic cell (Pringle et al., 1997). Originally encoded on the yeast 2 µm plasmid, the FLP gene was found to be involved in a recombination event on the same plasmid (Cox, 1983). This happens between two copies of a 599-bp inverted repeat which are present on opposite sites of the plasmid (Cox, 1983). FLP- mediated recombination between these FLP recombination targets (FRTs) results in the inversion of one plasmid section relative to the other, which is also possible between FRTs on different molecules (Vetter et al., 1983). Similar to the CreER system, FLP- mediated recombination can also be temporarily controlled by tamoxifen administration (FlpER) (Goodrich et al., 2018). Validation of the recombinase expression is analogous to the Cre lox system (see above).

Viral vectors

Understanding neural networks in the brain requires highly efficient and precise methods. Classical tools for gene manipulation like the previously described Cre/loxP and Flp/ FRT systems, the use of cis-regulatory elements or the generation of targeted knock- in transgenic mice have already produced promising results. Another elegant tool for neurobiological studies that allows the manipulation of specific neuronal subtypes but also non- neuronal cell types of the nervous system is the use of recombinant adeno- associated viral vectors for target gene delivery. Adeno- associated viruses (AAVs) are small, nonpathogenic members of the Parvoviridae family that display multiple natural serotypes that differ in capsid structure (Agbandje-McKenna & Kleinschmidt, 2011). The capsid is formed by 60 viral proteins (VPs) that build an 18- to 26-nm icosahedral particle (Agbandje-McKenna & Kleinschmidt,

2011; Bedbrook et al., 2018). AAVs have a relatively simple single stranded genome that is composed of two genes: The *rep* gene encodes four nonstructural proteins for replication, whereas the viral capsid proteins and the assembly- activating protein which plays a role in capsid assembly are encoded in the *cap* gene (Grosse et al., 2017). The genome is flanked by inverted terminal repeats (ITRs) which can form T- shaped hairpins (Bedbrook et al., 2018). Additionally, the ITRs contain cis- elements that allow genome replication, genome packaging and the generation of stable episomes (Bedbrook et al., 2018). In currently used rAAV vector systems, the transgene, which can be any sequence less than 4.7kb, replaces the cap and rep genes between the ITRs. rAAVs are well tolerated by the subject as they show a low frequency of integration into and minimal disruption of the host genome, this makes them suitable for gene therapy and safe for the use in clinical studies, respectively (Bedbrook et al., 2018). Cell- type specific expression of genetically targeted markers (fluorescent proteins), neuronal activity sensors (calcium and voltage indicators), actuators (chemo-, opto- and thermogenetic tools) and gene editing tools such as CRISPR- Cas9 can be efficiently expressed (Bedbrook et al., 2018). However, the low integration rate does not ensure persistent gene expression and rather complicates lineage tracing studies (Bedbrook et al., 2018). AAVs are frequently used for circuit studies in the central and peripheral nervous system. Terminally differentiated or nondividing cells, like neurons, are transduced with high efficiency by most serotypes (Alisky et al. 2000; Bedbrook et al., 2018). Astrocytes, oligodendrocytes, endothelial cells and ependymal cells can also be transduced (Van der Perren et al. 2011, Davidson et al. 2000). AAV- mediated specificity is mediated by three factors: the delivery method, the packaged cargo and the structure of the capsid protein (Bedbrook et al., 2018). Regarding the delivery, AAVs are most commonly injected intercranially by using a stereotaxic device to reach the right coordinates. The spread and transduction pattern of the virus is influenced by the injection volume, the rate, flow orientation, the viral titer and formulation buffer and the AAV serotype (Bedbrook et al., 2018). Transduced cells increase their expression level over time but reach a plateau by 4-6 weeks (Bedbrook et al., 2018). Widespread delivery within the brain, sinal cord and dorsal root ganglia can be achieved by injecting into the cerebrospinal fluid (McLean et al. 2014). Throughout the CNS, systemic injection in the lateral tail vein or the retroorbital venous sinus can provide widespread transgene expression (Deverman et al. 2016, Yang et al. 2014a; Bedbrook et al., 2018). The limiting factor in systemic delivery is the crossing of the blood- brain barrier (BBB). This can be solved by the use of either naturally occurring or engineered AAVs which can naturally pass the BBB or by temporarily opening or disrupting the BBB using chemical measures or ultrasound, respectively (McCarty et al. 2009, Wang et al. 2015). In order to modify transgene expression within cell types of the nervous system, transcriptional or translational regulatory elements and also recombinase recognition target elements can be directly included within the recombinant viral genome (Bedbrook et al., 2018). Those elements have to be relatively small as the AAV genome has a limited capacity, but

still allow cell- type specific expression in order to optimize transgene expression as desired, it is also necessary to consider specificity and strength of the used promoter: Some neuronal actuators like ChR2, NpHR and Arch need to be highly expressed, whereas high expression levels of other transgenes like GCaMP and Cre recombinase are not well tolerated (Bedbrook et al., 2018). In order to reduce off-target tissue expression after systemic delivery, the use of miRNA target sequences is an efficient method (Bedbrook et al., 2018). miRNAs are noncoding RNAs that bind to complementary sequences in protein coding mRNA which can result in gene silencing. By inserting tandem copies of short miRNA-target sequences (miRNA- TSs) into the 3' untranslated region of the AAV genome, transgene expression can be specifically regulated after transcription (Bedbrook et al., 2018). It was shown that transgene expression can be reduced in heart and liver by using miRNA- TS that is complementary to mir- 122 and mir- 1, which are enriched in liver hepatocytes and cardiac and skeletal muscle, respectively. The desired expression of the transgene in the CNS was still functional (Xie et al. 2011). Lastly, the structure of the capsid plays a crucial role for transduction efficiency. Capsids are very diverse by nature: more than 100 unique, full- length *cap* genes have been retrieved from human and nonhuman primate tissue which display the highest sequence divergence at the outer surface of the viral particle (Gao et al. 2004; Bedbrook et al., 2018). Nevertheless, artificial engineering in the laboratory can also generate capsid diversity, for example by recombination of capsid sequences to build chimeric capsids or also the incorporation of nucleotide changes by random mutagenesis (Gray et al. 2010, Maheshri et al. 2006). High- throughput sequencing data revealed several sequence elements that are crucial for maintaining functional properties like receptor binding, tropism, neutralization and blood clearance. This allows the development of functionally specific AAVs that can be strategically engineered to reach a broad and diverse range of cells (Bedbrook et al., 2018).

Tet- On/Tet- Off system

Another elegant tool that allows stringent control of target gene activity is the use of tetracycline – responsive promoters. Tetracyclines are antibiotics that can bind the bacterial 30S ribosomal subunit which results in the inhibition of bacterial protein synthesis and growth (Das, A. T., et al., 2016). In order to prevent that, bacteria have developed several resistance mechanisms which include tetracycline efflux, ribosome protection and tetracycline modification (Roberts, 2005). Gram negative bacteria use the membrane protein TetA for tetracycline efflux (Chopra, I. & M. Roberts, 2001). The TetA level is hereby controlled by a tetracycline- responsive repressor protein called TetR (Das, A. T., et al., 2016). The tetA and tetR genes are encoded on the bacterial Tet operon and share a regulatory region that controls their expression at the transcription level. Two tetO operator systems overlay the

two promoters Pa and PR1/ PR2 that control transcription of tetA and tetR, respectively (Bertram, R. & W. Hillen, 2008). In absence of tetracycline, TetA and TetR production is shut off. This happens by a complex mechanism taking place in the regulatory region: TetR dimerizes and binds to the tetO elements which results in the suppression of the PA and PR promoter activity. When tetracycline is now present, it binds to the TetR dimer inducing a conformational change that leads to the ablation of tetO binding. The activity of the PA and PR promoters is now no longer suppressed and transcription of TetA and TetR can take place (Hillen, W. and C. Berens, 1994). TetR is highly affine for tetracycline which ensures that TetA can be produced at already low tetracycline levels and a blockade in protein synthesis is prevented (Berens, C. and W. Hillen, 2003). In contrast, TetR has a very low affinity for non-operator sequences which makes it suitable for the regulation of transgene expression in organisms with much larger genomes than bacteria (Bertram, R. & W. Hillen, 2008; Das, A. T., et al., 2016). The Tet- Off system uses a tetracycline- controlled transcriptional activator (tTA) that consists of the 207-amino acid (207- aa) TetR which was fused to the 127- aa transcription domain of the herpes simplex virus VP16 protein (Gossen, M. and H. Bujard, 1992). Additionally, a promoter sequence consisting of 7 tetO sequences fused to a minimal TATA- box containing eukaryotic promoter was constructed (Ptet) (Gossen, M. and H. Bujard, 1992). When tetracycline is not present, the tTA dimers bind to the tetO sites of Ptet which leads to the activation of the transgene that is positioned downstream. Tetracycline or dox binding results in a conformational change in the TetR domain of tTA that ablates tetO binding and therefor switches gene expression off (Gossen, M. and H. Bujard, 1992; Das, A. T., et al., 2016). A disadvantage of the Tet- Off system is therefor that Tc or dox have to be administered continuously to stop gene expression and this longterm exposure is not beneficial for the organism (Das, A. T., et al., 2016). Additionally, tetracycline has to be actively removed by washing of the cells and replacing the culture medium, which is not convenient when large cell cultures are used and also in animal experiments (Das, A. T., et al., 2016). *In vivo,* the kinetics of induction are determined by the half- life of the effector (Das, A. T., et al., 2016). However, the Tet- On system was developed to enable active gene expression not by removal but by addition of dox. This system uses a TetR variant that binds tetO in the presence and not in the absence of the effector (Gossen, M., et al., 1995; Das, A. T., et al., 2016). In the so- called reverse- TetR (rTetR), a 4 amino acid substitution is responsible for the reverse phenotype, unfortunately this also leads to a significant decrease in effector sensitivity. As a result, only poor activation was observed and an increased effector amount was needed (Baron, U. & H. Bujard, 2000). However, several methods have been used to systematically modify the different components so that high expression levels are achieved while background activity is reduced. Several variants of the Tet- on system are convenient for in vitro cell culture approaches, whereas some complications can occur when used in an in vivo situation: Cellular and humoral immune responses against system components have been observed in rodents and non- human primates. Additionally,

toxic side- effects were reported in transgenic mice (Das, A. T., et al., 2016). However, the use of Tet-On in the CNS is mostly not disturbed by deleterious immune responses, which makes this application a promising tool in the field of neuroscience (McPhee, S. W., et al., 2006). Additionally, dox and the tetracycline-derivative minocycline (Mc) were found to display anti- inflammatory properties which could also be beneficial for the treatment of neurodegenerative diseases (Blum, D. et al., 2004; McKeage, K. & E. D. Deeks, 2010). However, in order to optimize the outcome of any *in vitro* or *in vivo* experiment, the limitations of both Tet- Off and Tet- on system should be considered.

DREADDs

The term 'chemogenetics' describes a method by which engineered proteins interact with small molecule chemical actuators (Strobel, 1998; Sternson and Roth, 2014; Roth, 2016). Several protein classes including kinases, non- kinase enzymes (Strobel, 1998) G protein- coupled receptors (GPCRs; Bishop et al., 1998; Cohen et al., 2005; Armbruster and Roth, 2005, Armbruster et al., 2007) and ligand-gated ion channels (Magnus et al., 2011; Zemelman et al., 2003) have been generated over the past years, the most frequently used are Designer Receptors Exclusively activated by Designer Drugs (Armbruster and Roth, 2005, Armbruster et al., 2007). These GPCRs belong to the human muscarinic receptor family and were engineered to be activated by the synthetic ligand clozapine- N- oxide (CNO) but not by their native ligand acetylcholine (ACh) (Armbruster and Roth, 2005; Armbruster et al., 2007). In humans, five families of GPCRs can be described based on sequence homology and pharmacological similarities: family A (rhodopsin), B (secretin), C (glutamate), adhesion and Frizzled/ Smoothen/ Taste2 (Fredriksson, R., et al., 2003). Characteristic for GPCRs is their seven- transmembrane topology with an extracellular N- terminus and an intracellular C- Terminus (Lambright, D. G., et al., 1996). After activation through ligand binding, GPCRs in turn activate G proteins by exchanging GTP for GDP in the Ras- like G domain that is part of the G proteins Gα subunit (Lambright, D. G., et al., 1996). The Gβ and Gγ subunits form a complex, that can also interact with effectors to activate the G protein, resulting in a signalling cascade (Sondek, J., et al., 1996; McCudden, C. R., et al., 2005). The alpha- subunit can be subdivided into different families that activate distinct downstream signalling pathways: Gαs stimulates the activity of adenylyl cyclase which results in increased intracellular concentrations of the second messenger cAMP, whereas Gαi reduces cAMP levels in the cell (Gilman, A. G., 1987). Lastly, Gαq/11 activates β-type phospholipase C (PLC-β) which in turn hydrolyzes membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3) (Simon, M. I., et al., 1991). IP3 induces calcium release through the calcium channel IP3 receptor from the ER whereas DAG activates protein kinase C (Berridge, M. J., 2016). The provoked response is determined by cell type, specificity of G protein coupling and duration and intensity of receptor

activation (Armbruster and Roth, 2005). Cell proliferation, differentiation, contraction and neurotransmitter release are common outcomes after activation of a GPCR signalling cascade (Armbruster et al., 2007). Regarding their pharmacology, GPCR activity can be described by various states ranging from “fully inactive” over “partially active” to “fully active”. Those states can be ligand-dependent or -independent (Roth and Marshall, 2012; Samama et al., 1993). Apparently, multiple inactive states exist that can be stabilized by ligands but can also occur in their absence. An active state can also be reached when no ligand is present, this happens by spontaneous isomerization. When this isomer then interacts with G proteins, this results in a state called “constitutive activity” (Samama et al., 1993). GPCRs can also form alternative signalling complexes by interacting with arrestins (Luttrell et al., 1999). Currently, several subtypes of DREADDs with different functions are in use. In order to enhance neuronal firing and activating the Gq signalling pathway in neuronal and non- neuronal cells, the excitatory DREADD hM3Dq is most frequently used (Armbruster et al., 2007; Roth, 2016). It is activated by CNO, which is usually administered through intraperitoneal injection. It was shown that CNO resides at least 60min in vivo in mice, therefor its effects can be robust and prolonged (Bender et al., 1994). Thus, it is beneficial to use the lowest effective dose so that only peak concentrations activate the DREADD, unless longterm signalling activation is needed. In non- rodents, CNO was also found to show potential for back- metabolism to clozapine and other clozapine metabolites which can be pharmacologically diverse and therefor interfere with the experimental outcome (Davies et al., 2005; Roth, 2016). Other non- CNO chemical attenuators include compound 21, which displays a minimal off- target activity and is highly selective for activating hM3Dq instead of muscarinic and other GPCRs (Chen et al., 2015; Roth, 2016). Well suited for translational studies is perlapine, which is normally used for insomnia treatment in Japan. This drug has to be tested in control animals at its lowest possible dose before being used for studies including DREADDs as it shows a modest affinity for biogenic amine receptors (Davies et al., 2005. Those off- target actions could interfere with the studied phenomena. In order to express hM3Dq in cells, several options are available: In genetically engineered mice, the DREADD can be expressed under control of the tetracycline (tet- off) promoter and also via Cre- mediated recombination. Cell- type- specific expression is mediated by specific promoters or the use of viral vectors (Roth, 2016). DREADDs can also be used in an inhibitory manner for neuronal silencing. Currently, three of the so- called Gi- DREADDs are in use: hM2Di, hM4Di and KORD (Armbruster et al., 2007). CNO, compound 21 and perlapine can be used to activate hM2Di and hM4Di, whereas KORD is activated by salvinorin B (Chavkin et al., 2004; Vardy et al., 2015). hM4Di and KORD were found to inhibit neuronal activity via two mechanisms: First, by inducing hyperpolarization through Gβ/γ-mediated activation of G-protein inwardly rectifying potassium channels (GIRKs) (Armbruster et al., 2007, Vardy et al., 2015; Roth, 2016) and second, via inhibiting the presynaptic release of neurotransmitters (e.g., synaptic silencing) (Stachniak et al., 2014, Vardy et al., 2015; Roth,

2016). To time, one DREADD coupled to the Gs pathway is in use. It was created by swapping the intracellular regions of the turkey erythrocyte β adrenergic receptor for equivalent regions of a rat M3 DREADD (Roth, 2016). This Gs- DREADD (GsD) displays a small degree of constitutive activity in transfected cells (Guettier et al., 2009; Roth, 2016). It was shown that GsD can activate Gαolf, the major Gs-like Gα protein in some regions of the brain (Farrel et al., 2013; Roth, 2016). Several groups used this DREADD to study Gs- like signalling for various behaviours like locomotor sensitization (Farrell et al., 2013), circadian rythmns (Brancaccio et al., 2013), reward (Ferguson et al., 2013) and ethanol consumption (Pleil et al., 2015).

iBark

DREADDs allow the targeted manipulation of cells throughout the whole brain. However, this method has been frequently used in neuronal cell types, whereas little work was done with astrocytes. Astrocytes do express GPCRs, allowing them to respond to neurotransmitters or neuromodulators in the extracellular space which stem from local or from long range projections (Kofuji and Araque,2021b; Porter and Mc-Carthy, 1997). However, astrocytic GPCR signalling does differ from the neuronal GPCR signalling pathways and it is not yet fully understood how this contributes to neural and brain circuit function. Even though the use of chemogenetic tools for stimulation of astrocytes has shown quite some progress, there are still limitations: The lack of physiological context regarding cellular responses makes it hard to distinguish direct and secondary distributions to behaviour and other complex responses (Yu et al., 2020a). To have a better understanding of the effects and consequences of astrocytic activating GPCR signalling pathways, it would be beneficial to have a method to halt this activity as a complementary approach. Similar as in neurons, ligand binding to Gq GPCRs induces intracellular Ca2+ signalling through the conversion of Gaq-GDP to Gaq-GTP, resulting in phospholipase C (PLC)-dependent IP3 generation and Ca2+ release from the intracellular endoplasmatic reticulum (ER) stores. It was found that the GPCR kinase 2 (GRK2)/b-adrenergic receptor kinase 1 (bARK1) can sequester Gaq- GTP by its regulator of G-protein signaling homology (RH) domain (Carman et al., 1999). Based on this, a 122- residue inhibitory peptide bARK-rgs (ibark) was found to disrupt astrocytic Gq-GPCR Ca2+ signalling (Nagai et al., 2021,). This can be used to attenuate Ca2+ signalling in astrocytes not by using an inhibitory GPCR signalling pathway as for example Gi in neurons, instead this approach is based on actively interrupting an activating Gq pathway. This method has several advantages: spontaneous Ca2+ signalling, ligand independent GPCR function and other GPCRs are not affected (Jiang et al., 2016). iBark can be delivered brain- wide and it can also be used for intersectional studies. However, this approach still needs improvement: IBark does not block Gby mediated pathways that can also be activated by Gq GPCRs. With current strategies, iBARK expression might not always be

consistent enough to bind to all available Gaq- GTP. Another fact to consider is that further controls and evaluations are needed to asses specificity and efficiency but also possible downstream implications should be assessed (Nagai et al., 2021). However, iBark can be a promising approach to be used in mechanistic studies. Microinjections using AAVs (see above) into brain areas of interest might provide the possibility to give a better understanding of how astrocyte GPCR signalling effects their multiple cellular interaction partners (Nagai et al.,2021). In vivo behavioural functions that are associated with astrocytic GPCR signalling can also be studied extensively. Astrocytic contribution to synaptogenesis, neurovascular coupling and circuit dysfunctions are just some of the various processes that could be explored using this promising approach (Heller & Rusakov, 2015).

Materials and methods

Animals

The mice were kept under a 12- hour light/dark circle at 22°C and a humidity of 50%. Food and water were present ad libitum. DAT-Flp mice (#035436) (Kramer et al., 2021) were used for examining dopaminergic neurons in the periventricular nucleus of the hypothalamus, whereas GLAST-CreER mice (#012586) (Nathans, 2010) were used for the characterisation of astroglia in the ventromedial hypothalamic nucleus. Sst-IRES-Cre mice (#013044) (Josh Huang, 2011) No statistical methods were used to predetermine sample size and the experiments were not randomised and did not require data collection in a blinded fashion. Tissue collection and experiments on live animals conformed to the EU directive and were approved by the Austrian Ministry of Science and Research. The 3Rs (Replacement, Reduction and Refinement) were systematically applied throughout the study and particular effort was taken to minimise animal suffering during experiments.

Genotyping

Transgenic animals were determined using a PCR- based screening kit (Accustart II, Quantabio). The following primers were used for verification:

Table 2.1: Primers for genotyping

line	**type**	**sequence**
Bac-Glast/cre ERT	Int Pos. Ctrl. forward	CAA ATG TTG CTT GTC TGG TG
	Int Pos. Ctrl. reverse	GTC AGT CGA GTG CAC AGT TT
	Transgene forward	ACA ATC TGG CCT GCT ACC AAA GC
	Transgene reverse	CCA GTG AAA CAG CAT TGC TGT C
DAT- Flp	Mut forward	CATGCAGAAGGACAGACACT
	Mut reverse	AGGATGTCGAACTGGCTCAT
Sst-IRES-Cre	Mut forward	TCA GGT ACA TGG ATC CAC TAG TTC T
	WT forward	GAG GTC TGC CAA CTC GAA C
	Common	AGT CAA ACG CTT GCT CTT CA

Virus microinjections in vivo

Mice of all three lines were injected with the corresponding viruses to allow targeted manipulation of neurons or astroglia (Figure 2.2). To reduce postoperative pain and also to prevent infections, the animals received Metacam (2mg/ml, dosage 0,3mg/kg) and Baytril (25mg/ml, dosage 10mg/kg) via subcutaneous injection, respectively. Subsequently, the mice were anesthetized using an anasthesia cocktail consisting of 0,45ml Ketamine (100mg/ml), 0,25ml Xylacin (20mg/ml) and 4,3ml 0,9% Saline. The dosage was adjusted depending on the weight of the animal: 10µl/ g mouse, for older or heavier animals the volume was reduced by 10% and for animals weighing less than 10g the cocktail was diluted 1:2 to receive higher precision. The mice were placed in a stereotaxic frame (RWD) and received an additional inhalation anaesthetic with isoflurane (5%, 1l/min) to prevent them from waking up during the injection and surgery. Virus injections were performed uni- and bilaterally using a volume of 100-400nl of virus. The injection was performed at a speed of 50-100nl/min using a micropipette coupled to a stereotaxic injector (RWD). The injection targets were the ventromedial hypothalamic nucleus (GLAST- CreER), the periventricular nucleus of the hypothalamus (DAT- Flp) and the lateral septum (Sst- Ires- Cre) at the following coordinates:

Table 2.3: **Injection coordinates**

	anterior/ posterior	**medial/ lateral**	**dorsal/ ventral**
Ventromedial hypothalamic nucleus (VMH)	-1.15	± 0.6	-4.6
	-1.3	± 0.55	-5.6
	-1.5	± 0.65	-5.6
	-1.6	± 0.6	-5.6
	-1.6	± 0.6	-5.7
Periventricular nucleus of the hypothalamus	-0.8	± 0.2	-5.2
Lateral septum	0.25	± 0.5	-3.25

After the injection was completed, the micropipette was held in place for 5 min before it was slowly retracted from the injection site to limit virus spread.

Induction of Tamoxifen- responsive gene tracing

In order to activate target gene expressing in GLAST- CreER mice, tamoxifen has to be administered via subcutaneous injection. This was done 1-2 days after virus injection to give the animals time to recover. Tamoxifen (Sigma)) was dissolved in corn oil (Sigma) to reach a concentration of 20mg/ml for adult mice, 5-10mg/ml for pups and 10- 20mg/ml for pregnant females. This was done by incubating the solution in a light protected vial at 50-60°C for at least half an hour with frequent vortexing until the tamoxifen crystals were completely dissolved. The mixture can be stored at 4°C in the dark and used up to 7 days.

Immunohistochemistry

14- 21 days after the injection, the animals were anesthetized with pentobarbital (50 mg/kg, i.p.) and transcardially perfused using a fixative composed of 4% PFA in 0.1 M phosphate buffer (PB; pH 7.4) followed by a rinse with physiological saline containing heparin (10 units/ml, Sigma H0777). The perfusion was carried out during daytime. Whole brains were postfixed in the same fixative at 4°C overnight and then the fixative was replaced with 10% sucrose (in 0.1 M PB, pH 7.4) containing 0.01% sodium azide (Merck) and 0.02% bacitracin (Sigma) over 2–3 days at 4 °C. For immunohistochemistry, 35 µm thick free-floating cryosections were cut on a cryostat (Leica), washed in PBS and blocked in a cocktail composed of 5% normal donkey serum (NDS; Jackson), 2% bovine serum albumin (BSA; Sigma), and 0.3% Triton X-100 (Sigma) in PB for 2 h at 22–24°C. Specimens were then exposed to a specific combination of primary antibodies (Table 2.3) together with Hoechst 33342 (Concentration 1:1000; Sigma) in PB solution containing 2% NDS, 0.1% BSA, and 0.3% Triton X-100 for 72h at 4°C or 16h at 22-24°C.

Table 2.3: List of antibodies

Antibody	Source	Concentration	Supplier
cFos	rabbit	1:500	Synaptic Systems
NeuN	mouse	1:400	Millipore
mCherry	chicken	1:1000	EnCor Biotechnology
GFAP	guinea pig	1:1000	Synaptic Systems

Immunoreactivities were revealed by carbocyanine (Cy) 2-, 3- and 5- tagged secondary antibodies diluted in 2% BSA in PB (1:300, Jackson ImmunoResearch), at 22–24°C for 2 h. Brain sections were mounted on fluorescence- free glass slides and left to dry for at least 30 min before coverslips were applied with Entellan (in toluene; Merck).

Laser- scanning microscopy and image analysis

For acquisition of immunofluorescent images, a LSM 880 laser- scanning microscope was used at 10x, 20x or 40x primary magnification. The emission spectrum was limited for each dye as follows: Cy2 (505–530 nm), Cy3 (560–610 nm), and Cy5 (650–720 nm). To determine colocalization, images were analyzed in ZEN3.0 software. Optical sections covering the regions of interest were used for quantification. All images were acquired with the same laser power, pinhole, digital and master gain on the LSM 880 laser- scanning microscope.

Home- cage activity monitoring

To investigate the role of somatostatin neurons in the LS regarding animal activity, I used a cre-dependent AAV virus to induce hM3Gq expression for conditional activation (Krashes et al, 2011). SSt-Ires- Cre mice (n=3, 9 months old) were bilaterally injected into the lateral septum using a stereotaxic device (RWD). 14 days after recovery, the animals were single housed in a PhenoTyper cage (Noldus) with 4 days of habituation and 5 days of animal activity monitoring. Home cage activity was recorded 24 hours/day while food and water were available ad libitum. At days 2 and 5, CNO was administered to the drinking water (5mg/kg) to stimulate hM3Dq. The following measurements were acquired: total activity (%), velocity (cm/s), walking duration (%), and wheel running (number of wheel rotations). At the end of the experiment, animals were sacrificed, and their brains were used for immunohistochemical analysis. Data were analysed using EthoVision 17.

Results

Flp- dependent Activation of cells in the periventricular nucleus

Throughout the whole brain, 9 distinct populations of dopaminergic neurons can be found and five of those subgroups (A11- A15) belong to the hypothalamus. Those neurons can be identified by their expression of genes that encode enzymes that play a role in the dopamine metabolism, e.g. the dopamine reuptake transporter DAT. This membrane transporter is expressed by specific neuronal subpopulations which either do or do not produce dopamine ((Korchynska et al., Stagkourakis *et al*, 2018, 2020; Romanov *et al*, 2017). Expression of the corresponding gene Dat1 together with the marker genes Th and Slc18a2 can be used to identify A14 neurons in the periventricular area (PeVN; Romanov *et al*, 2017).) DREADD- mediated, chemogenetic manipulation of those dopaminergic A14 neurons revealed long axons that project outside the hypothalamus to the lateral septum (LS), using dopamine as a neurotransmitter. For those experiments, excitatory DREADDs were induced via cre-dependent viral targeting. As Cre recombinase was expressed under the control of the DAT- promoter, DREADD expression in the PeVN was limited to dopaminergic target cells, i.e the A14 group when the virus was specifically injected into the PeVN area (Korchynska et al., 2021). The next step for further investigating the PeVN- LS circuitry and downstream targets is the injection of a second virus into the LS in order to allow bidirectional manipulation. This intersectional approach is based on the combination of two site specific recombinases, most commonly used are Cre and Flp together (Dymecki et al., 2010). As cre- dependent targeting of A14 neurons in the PeVN has already proven successful, I wanted to test whether those cells could also be efficiently targeted using Flp instead of Cre. This would facilitate the targeted manipulation of both A14 cells in the PeVN and their interaction partners in the LS, e.g. SSt+ cells, by using a combination of both recombinases. For this, I injected a flp- dependent AAV virus coexpressing an excitatory DREADD (hM3D(Gq)) together with mCherry as a reporter in the PeVN of homo- and heterozygous Dat- flp mice. As the PeVN is closely located to the third ventricle, we adjusted the injection coordinates to more lateral so that accidental injection into the ventricle is prevented. I waited two weeks to ensure optimal target gene expression and then gave clozapine N- oxide (CNO) to induce ligand- mediated DREADD activation which in turn activates the cells (Armbruster et al., 2007). Subsequently, the animals were transcardially perfused and cryosections were stained in an immunohistochemical approach to show infected cells (mCherry signal) and their activation (cFos). I additionally stained for TH in order to confirm the dopaminergic identity of cells. Several cells in the target region showed an mCherry signal, suggesting that they were successfully infected with the virus (Figure 3.1a-b)

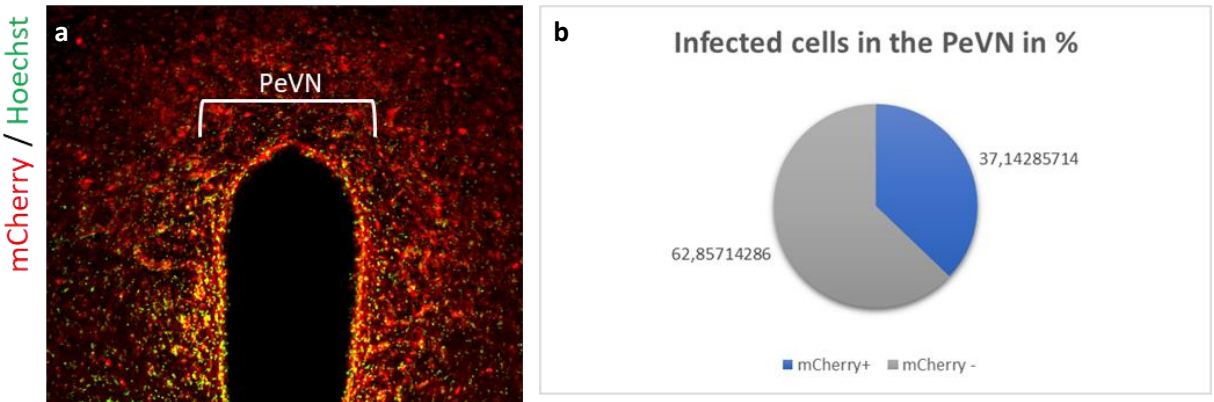

Figure 3.1: AAV- infected cells in the periventricular hypothalamic nucleus
a, immunohistochemical detection of mCherry positive cell bodies in the periventricular hypothalamic nucleus (PeVN) surrounding the third ventricle (3V)
b, quantification of mCherry positive cells in the target region

Additionally, I found infected cells that stained positive for TH and cFos (Figure 3.2) This indicates that the Flp- recombinase based approach can in fact be used to target and activate cells which likely belong to the dopaminergic A14 group.

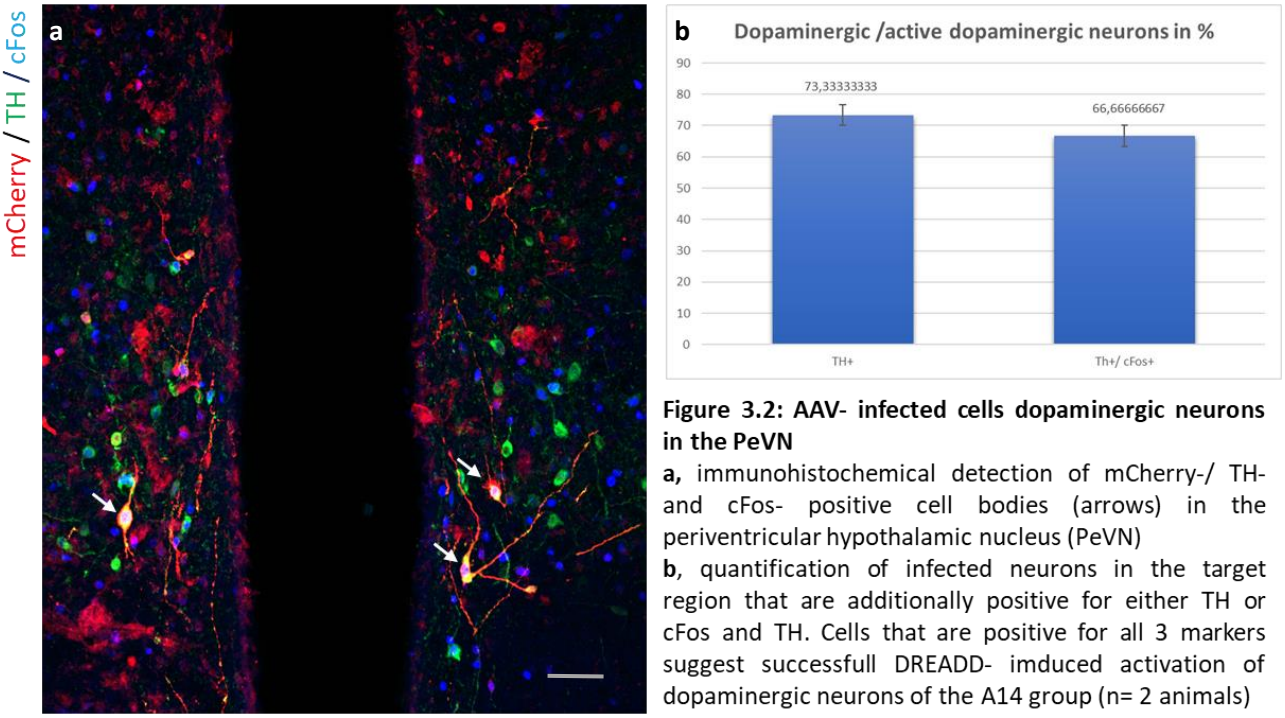

Figure 3.2: AAV- infected cells dopaminergic neurons in the PeVN
a, immunohistochemical detection of mCherry-/ TH- and cFos- positive cell bodies (arrows) in the periventricular hypothalamic nucleus (PeVN)
b, quantification of infected neurons in the target region that are additionally positive for either TH or cFos and TH. Cells that are positive for all 3 markers suggest successfull DREADD- imduced activation of dopaminergic neurons of the A14 group (n= 2 animals)

I also found mChery-/ cFos- and TH- positive cells in the dorsal region of the arcuate nucleus (Arc, Figure 3.3). This suggests that not only cells of the dopaminergic A14 group located in the PeVN were infected with the AAV- virus, but also dopaminergic neurons of the A12 group which is located in the Arc. In order to determine virus efficiency, I compered infected dopamine neurons (mCherry and TH positive) to uninfected dopamine neurons (TH positive, mCherry negative) regarding the additional presence of a cFos signal (Figure 3.3b). Whereas only 7,1% of A12 neurons without AAV- infection were active, 85,7% of dopaminergic A12 neurons showed activation after being infected with the AAV- construct, suggesting that activation is in fact DREADD induced. Altogether, this indicates that the Flp-dependent AAV- based approach is a useful tool for the targeted activation of dopamine neurons not only in the PeVN but also other hypothalamic regions. Interestingly, homozygous animals showed more cells with an mCherry signal when compared to heterozygous ones, suggesting that the virus is more efficient in those animals. We therefore tried to breed more homozygous animals, however this approach had unexpected limitations since this genotype is more likely to display health issues.

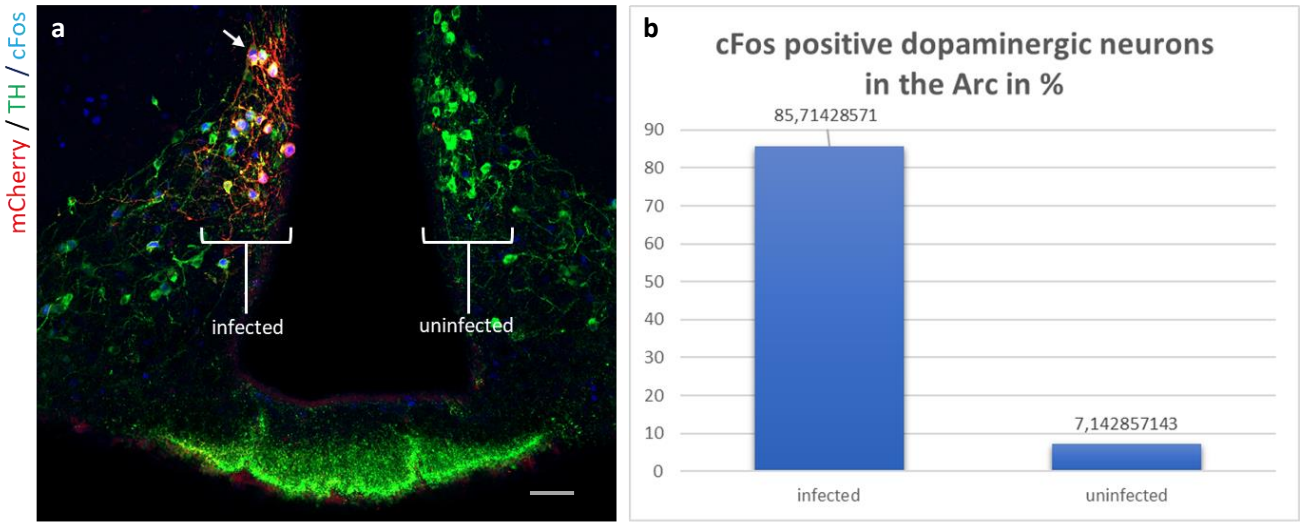

Figure 3.3: AAV- infected cells dopaminergic neurons in the arcuate nucleus
a, immunohistochemical detection of mCherry-/ TH- and cFos- positive cell bodies (arrows) in the arcuate nucleus (Arc).
b, quantification of active dopaminergic neurons in the Arc. Data is based on the comparison of AAV- infected TH- positive cells with uninfected TH- positive cells. Activation was determined by the presence of an additional cFos signal (n=1)

Cre- dependent Activation of cells in the lateral septum in somatostatin cre

The previously described A14 dopamine neurons of the PeVN were found to innervate somatostatin (Sst)- positive neurons in the lateral septum (LS), which is highly connected to locomotor speed and acceleration (Wirtshafter & Wilson, 2019; Korchynska, 2022). Furthermore, A14 dopamine neurons receive dense excitatory input from the suprachiasmatic nucleus (ScN), which is the central circadian pacemaker. Recent research showed that this LS- PeVN- ScN network seems to regulate animal locomotion behaviour in a diurnal fashion. In fact, chemogenetic activation of A14 dopamine neurons led to increased locomotion, whereas their inhibition resulted in the suppression of animal mobility (Korchynska et al., 2022). Based on these findings, I wanted to test whether DREADD- induced activation of Somatostatin neurons in the LS also affects locomotion behaviour. For further combination of Cre and Flp recombinase, we injected a cre- dependent AAV virus coexpressing an excitatory DREADD (hM3D(Gq)) together with mCherry as a reporter in the LS of Sst- Cre mice (animal=3). We next examined animal behaviour in a home cage situation focusing on food & water intake, wheel use and resting time. While food consumption was not affected, the overall locomotion was dramatically increased after target cell activation: we observed a 67%+-30% (SEM+-SD) decrease in locomotor activity when the CNO was added to the water to activate SST neurons in the LS. These results are in line with the previously mentioned findings. After the Experiment, mice were perfused and cryosections were stained for mCherry to identify infected cells and cFos to monitor cell activity (Figure 3.4). All three animals showed colocalization of mCherry and cFos in the lateral septum, indicating that DREADD- induced activation of cells was successful.

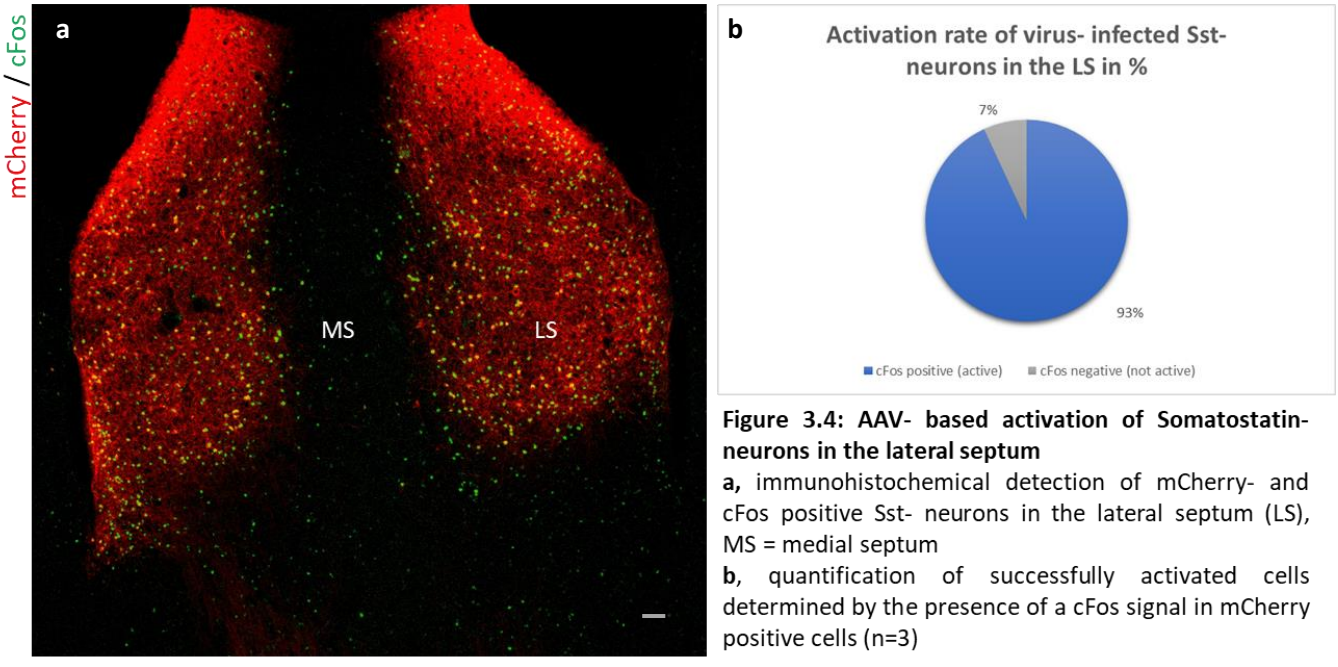

Figure 3.4: AAV- based activation of Somatostatin- neurons in the lateral septum

a, immunohistochemical detection of mCherry- and cFos positive Sst- neurons in the lateral septum (LS), MS = medial septum

b, quantification of successfully activated cells determined by the presence of a cFos signal in mCherry positive cells (n=3)

I also found that the infected neurons of the lateral septum project to regions in the hypothalamus, like the median magnocellular preoptic nucleus (MCPO), the lateral preoptic area and the ventrolateral preoptic nucleus. Other projection targets include the substantia innominata and the diagonal band nucleus (Figure 3.5).

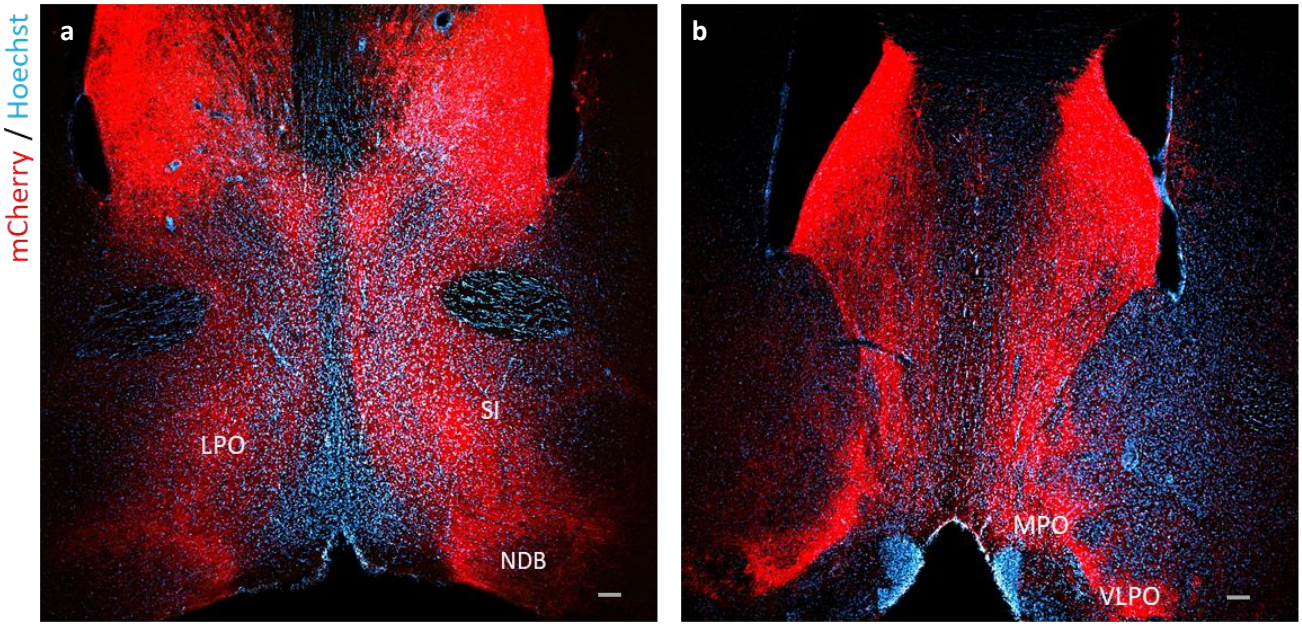

Figure 3.5: projection targets of Sst- neurons of the lateral septum
a-b, mCherry positive cell bodies show long projections to regions inside the hypothalamus, e.g the median preoptic area (MPO), the lateral preoptic area (LPO) and the ventrolateral preoptic nucleus (VLPO). Extrahypothalamic targets include the substantia innominata (SI) and the diagonal band nucleus (NDB)

Cre- dependent activation of dopaminergic A14 cells decreases food consumption

In parallel to changes in locomotory functions, I with the lab members (Prof. Roman Romanov and lab-assistant Patrick Rebernik) performed new experiments where we measured the food consumption during 3h period of the active animal phase and contribution of A14 cells. For that we utilized DAT-CRE mice that were earlier infected with AAV viruses to express excitatory DREADDs in the DAT-expressing cells in the periventricular area of the hypothalamus. We showed that chemogenetic stimulation of A14 DA cells in with CNO injection suppresses eating (Fig. 3.6). The circuit mechanism remains unclear, however, this result simulates NMS action in similarly designed experiments, where authors analysed 3-h food intake of the first quarter of a dark period (Nakahara et al., 2010). Taking into account that exogeneous NMS activated those hypothalamic dopamine neurons (Romanov et al 2017), this behavioural testing reinforces the putative role of dopamine periventricular cells in possible modulation of eating behaviour via neuropeptides related (i.e. Neuromedin S) circuits.

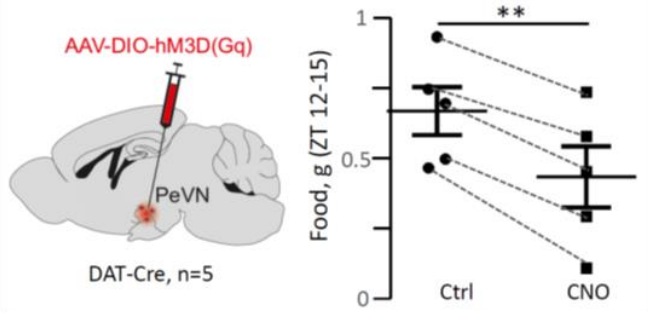

Figure 3.6: unpublished preliminary data. Chemogenetic stimulation of A14 neurons led to decrease in food consumption

1mg/kg CNO was i.p. injected at ZT12 and food consumption was analysed during the first quarter of a dark period (ZT12-15) to be comparable with earlier observed results (Nakahara et al., 2010). For each animal, the food consumption was repetitively measured 3 (CNO) and 5 (ctrl) times at different days to find an average value.

Activating astroglia in the ventromedial hypothalamic nucleus

In order to chemogenetically manipulate astroglia, the experimental strategy was similar to the one that was previously used for neuronal targeting: mice were injected with AAV viruses coexpressing an excitatory DREADD (hM3D(Gq)) together with mCherry as a reporter. While there are many different tools for manipulating the activity of specific neuronal populations, similar tools for specific astrocyte manipulation are missing. For this part of my thesis, i.e. in order to activate astroglia, i tested three viral constructs that differed in virus serotype and promoter in the ventrolateral region of the VMH. The VMH can be found in the tuberal hypothalamus and is associated with a plethora of functions, including social behaviours like reproduction or parenting, thermoregulation and glucose metabolism etc. Additionally, several studies identified cells of the VMH, especially in the ventrolateral part, as a key region for aggression behaviour. In the following experiments I wanted to test whether the establishment of aggression stems from an astroglial background. I used the mouse brain atlas online tool to determine the exact injection coordinates in order to reach the target region (Paxinos & Franklin, 2001). The goal was to efficiently and specifically target astrocytes in the VMH, meaning to reach a sufficient number of cells expressing the activating DREADD but at the same time limit gene expression to astroglia but not neurons. In this work, I provide a comparative analysis of different AAV-constructs to functionally target astrocytes. For this, I used Bac- Glast- Cre- ER mice that express CreERT under the control of the glial high affinity glutamate transporter *Slc1a3* (solute carrier family 1, GLAST). This strain allows the targeted manipulation of glia and neural progenitor cells that can be temporarily controlled by tamoxifen administration (Nathans, 2010). The first two AAV constructs carry the

sequence encoding the activating DREADD fused to mCherry flanked by two loxP sites under the control of the hSyn and the hEF1- alpha promoter (Virus 44361-AAV8 and v98-9, respectively). Using these viral constructs, target gene expression was actively induced by intraperitoneal injection of tamoxifen which in turn activates Cre recombinase that subsequently recognizes and cuts the loxP sites, catalysing reciprocal DNA recombination (Sauer, 1987). The third virus was not cre- dependent as it carries the hM3D(Gq)_mCherry construct solely under control of the glial fibrillary acidic protein (GFAP) promoter (Figure 3.7). GFAP is a main component of the glial cytoskeleton as it is the major protein constituent of glial intermediate filaments in differentiated astrocytes of the CNS. By using the GFAP promoter, we wanted to confine DREADD expression to astroglia. Further, we expected to reach an increase in gene expression levels with this viral construct compared to the previously described cre- dependent approach: According to previous experiments in the lab, the efficiency of cre-dependent recombinase can be limited due to relatively low gene expression level. Additionally, this virus does not require the use of transgenic animals but can be used also in wildtypes, which was beneficial for the experimental progress. To ensure optimal target gene expression for both viruses, I waited at least two weeks after virus injection or tamoxifen injection (in wildtype or transgenic Bac-Glast- Cre- ER animals, respectively) and then gave clozapine N- oxide (CNO) to induce ligand- mediated DREADD activation which in turn activates the cells (Armbruster et al., 2007). After 40-60min, we estimated to reach a peak in cell activation which is when the animals were transcardially perfused. Cryosections were stained in an immunohistochemical approach to show infected cells and their activation.

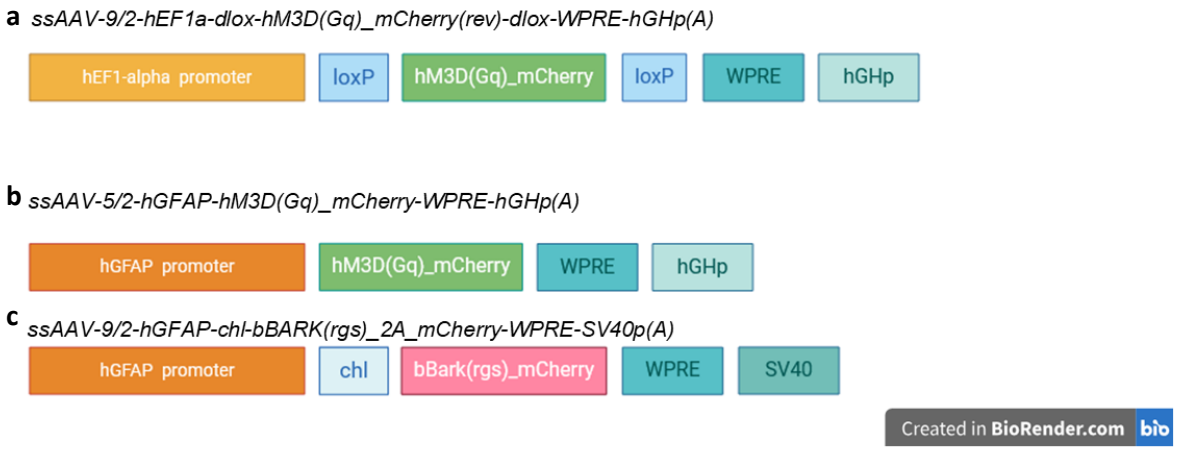

Figure 3.7: viral constructs used for chemogenetic manipulation
a, cre- dependent construct encoding activating DREADD. This construct carries an hM3D(gq)_mCherry DREADD flanked by loxP sites under the control of the hEF1-alpha promoter.
b, cre- independent construct encoding activating DREADD. This construct carries an hM3D(gq)_mCherry DREADD under the control of the hGFAP promoter.
c, cre- independent construct encoding iBark for inactivation. This construct carries a bBark(rgs)_mCherry sequence under control of the hGFAP.

Use of cre- dependent activating DREADDs in transgenic animals

The first two animals were injected with virus 44361-AAV8. I stained the cryosections for cell nuclei and used antibodies against mCherry and GFAP to confirm successfully infected cells and astrocyte identity, respectively. To monitor cell activity, I used Anti- cFos. When looking at 20x images, I found colocalization of the mCherry and cFos signal in a few cells, suggesting that astrocytes were successfully infected. Nevertheless, most mCherry and cFos positive cells lacked a GFAP signal, hinting that the virus infected not only astrocytes as planned (Figure 3.8a). In fact, it seems that with this virus, cells of neuronal identity were mainly infected and activated. I used 20x fluorescence microscopy images to quantify the amount of mCherry- and GFAP- positive cells and found that the majority of infected cells (76,9%) were in fact neurons and not astroglia as planned (Figure 3.8b)

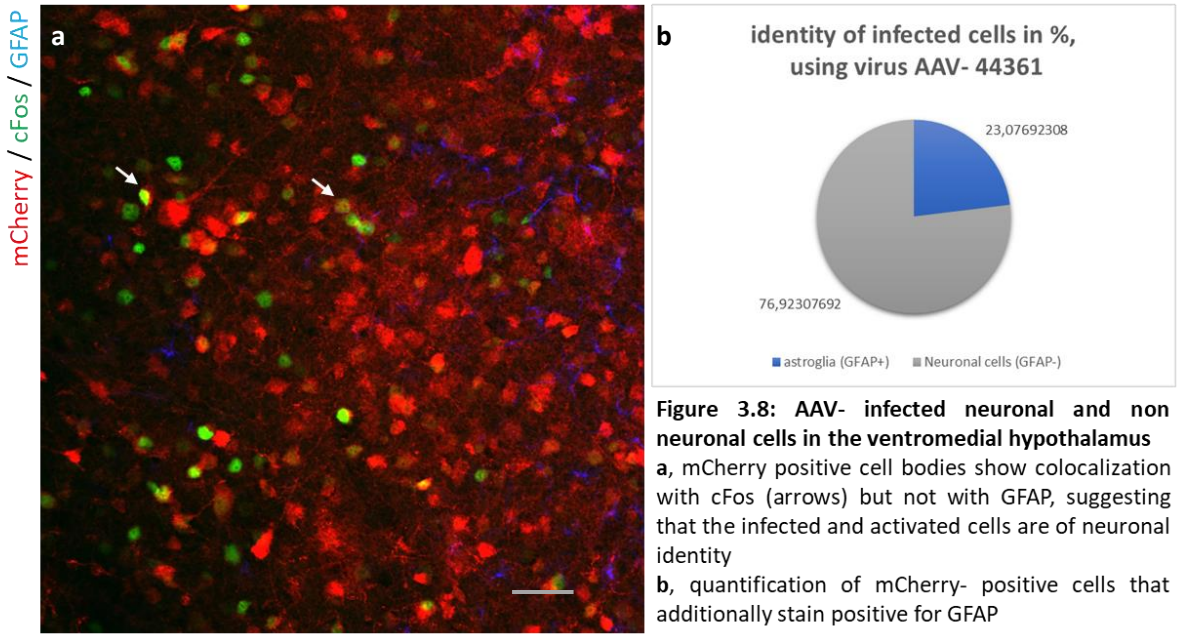

Figure 3.8: AAV- infected neuronal and non neuronal cells in the ventromedial hypothalamus
a, mCherry positive cell bodies show colocalization with cFos (arrows) but not with GFAP, suggesting that the infected and activated cells are of neuronal identity
b, quantification of mCherry- positive cells that additionally stain positive for GFAP

Therefor, we questioned the efficacy of virus 44361-AAV8 for astrocyte- specific activation and replaced it with the second virus v98-9. This virus uses a different promoter, as it consists of the eukaryotic translation elongation factor 1 α (EF-1α) promoter instead of the human synapsin promoter. This new promoter should to show increased transfection efficiency and transgene expression compared to other promoters (Wang et al., 2017). Additionally, the serotype was changed from AAV-8 to AAV-9/2, as these serotypes were found to be more suitable for astrocyte transduction. (Vagner et al., 2016). I used again two animals for immunohistochemical analysis: cryosections from the first one I stained with the previously described antibody combination in order to determine the amount of infected and activated cells. I also used an antibody against neuN to mark cells of neuronal

identity in order to check whether with this virus the rate of infected astroglia is increased compared to the old one (Figure 3.9a). For quantification of infected cells, I used 20x images of the VMH to count cell nuclei that showed colocalization with the mCherry signal. Cells that showed an additional nuclear NeuN signal were counted as neurons. When compared to the previously used virus 44361-AAV8, I observed more astrocyte- specific infection (27,2% compared to 23,1% with the previously used cre-dependent virus), nevertheless the majority of infected cells still were of neuronal identity (72,7%, Figure 3.9b)

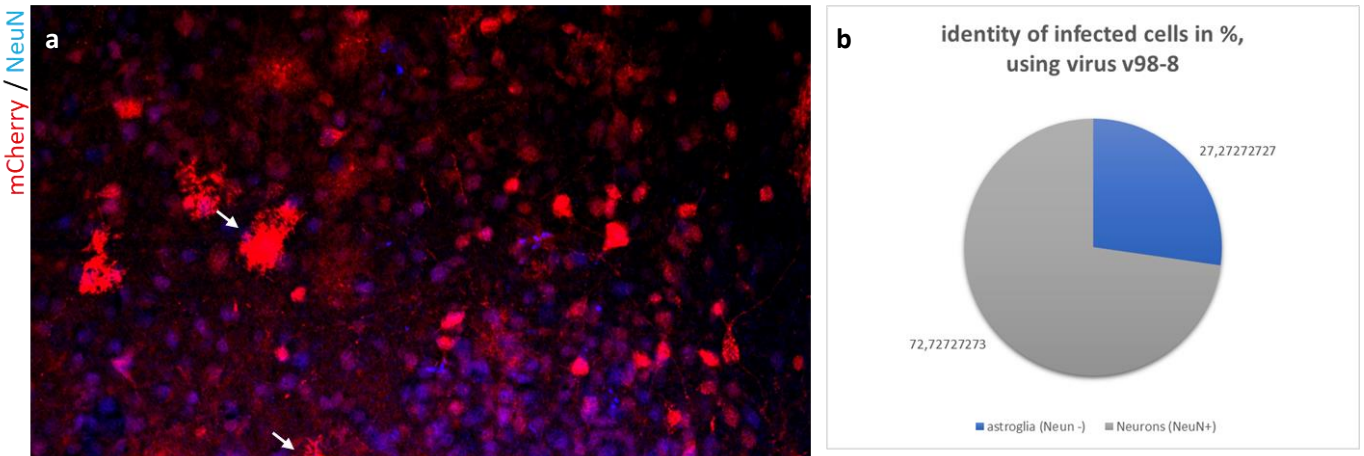

Figure 3.9: AAV- infected neuronal and non neuronal cells in the ventromedial hypothalamus with virus v98-8
a, mCherry positive cell bodies show typical astroglial phenotype (arrows), cell nucleii were additionally stained with NeuN to reveal neuronal identity
b, quantification of mCherry- positive cells that additionally stain positive for NeuN

All in all, the cre- dependent approach for astroglial targeting lacked specificity and was not very efficient: Both viruses mainly infected neurons but not astrocytes, even though changing the serotype and the promoter slightly improved the situation. Nevertheless, more or less no meaningful activation of astroglia was observed for both cre- dependent AAV viruses.

Activating DREADD under gfap promoter for wildtype animals

To increase the amount of infected astrocytes I next tested the third virus which was not cre-dependent and therefor also suitable for wildtype animals. As previously shown, both cre- dependent viruses were not astrocyte- specific. By switching to the cre- independent virus v 97- 5, we expected an increase in infected cells and also higher specificity for astrocytes. I injected 2 animals with the GFAP virus and stained the cryosections with the same antibody combinations that were also used for the animals injected with the cre- dependent virus: Anti- cFos, Anti- mCherry and either Anti- GFAP or Anti Neun. First of all, I observed a visibly higher number of virus infected cells and also the virus spread was bigger than with the cre- dependent v98-9 virus, even though I used the same injection settings for both experiments.

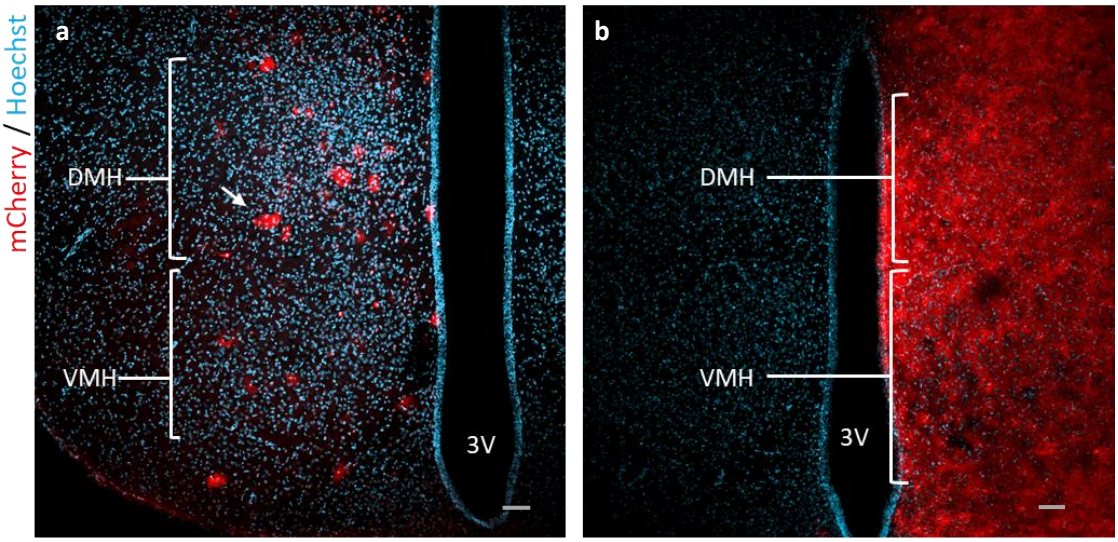

Figure 3.10: increase in infected cells after replacing cre- dependent AAV8-44361 virus with cre- independent virus v97-5

cells of tuberal hypothalamus were stained for nucleii with Hoechst, virus- infected cells are mCherry- positive, DMH = dorsomedial hypothalamic nucleus, VMH=ventromedial hypothalamic nucleus, 3V= third ventricle

a, virus- infected cells (arrows) after injection with cre- dependent AAV8-44361 virus

b, virus- infected cells after injection with cre- independent virus v97-5

As done before with the cre- dependent v98- 9 virus, I used 20x images of the VMH of both animals to quantify the number of active astroglia in the VMH compared by the presence or absence of a nuclear cFos signal. Cells with an additional NeuN signal were again labelled as neurons. When comparing the data of both experiments, I found a slight increase in total cell activity in the VMH of animals injected with the GFAP virus V97-5 compared to the cre- dependent virus v98-8 (Figure 3.11)

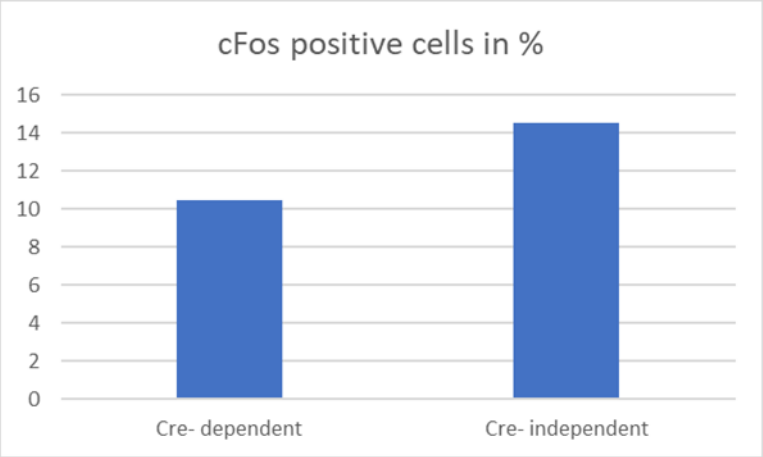

Figure 3.11: increase in cFos- positive cells after replacing cre- dependent AAV8-44361 virus with cre- independent virus v97-5

cFos positive nucleii were counted for animals injected with cre- dependent AAV8-44361 virus and cre- independent virus v97-5, (n=4)

I additionally counted cells showing a nuclear NeuN signal to find out how many of the active cells were neurons and not astroglia: In fact, most of the activated cells were neurons but the increase in the number of active cells was due to an increased amount of astroglia. With the cre- dependent virus only 1% of active cells were non neuronal cells, using the cre- independent virus increased this number to 4% (Figure 3.12).

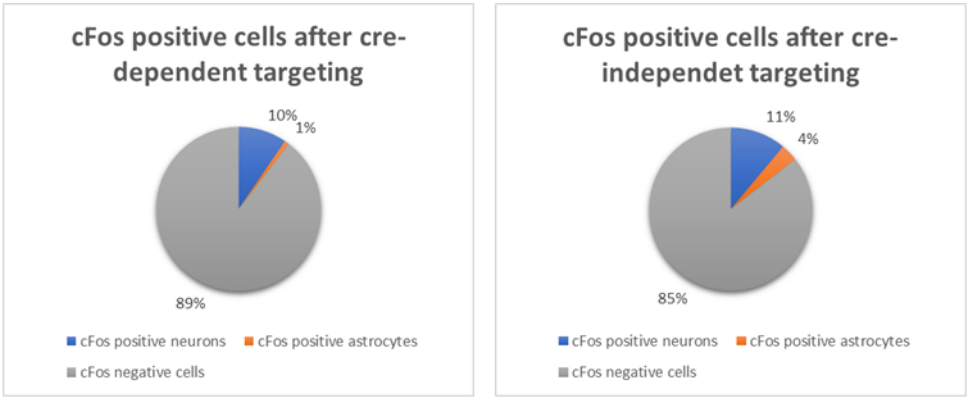

Figure 3.12: increase in cFos- positive neurons and astrocytes after replacing cre- dependent AAV8-44361 virus with cre- independent virus v97-5

cFos positive nucleii were counted for animals injected with cre- dependent AAV8-44361 virus and cre- independent virus v97-5, cells that were additionally positive for NeuN were counted as neurons, (n=4)

I also wanted to know whether cell activation is connected to the virus injection or if it is a result of other background activities. For this, I quantified the amount of cFos positive cells in the injected VMH vs. the uninjected VMH of the same animal. I found that the amount of active cells is increased in the injection site, this is also in line with the fluorescence microscopy images. Nevertheless, cell activity is apparently not solely DREADD- induced, as in the uninjected region some cells also stained positive for cFos (Figure 3.13).

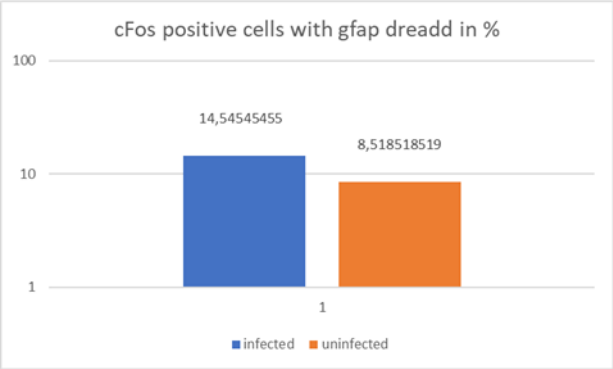

Figure 3.13: amount of cFos- positive cells found in injected VMH compared to the uninjected VMH of the same animal
After virus injection followed by DREADD- induced activation, the number of cFos- positive cells was increased compared to the uninjected VMH of the same animal.

Interestingly, I observed a pattern in both animals that seems to be linked to the virus injection: In the cell layer lining the wall of the third ventricle next to the dorsomedial hypothalamic nucleus (DMH) and the VMH, a group of ependymal cells stained positive for cFos (Figure 3.14a). Additionally, I also found an increased amount of cells with a nuclear cFos signal in the ependymal layer at the level of the arcuate nucleus (Figure 3.14b). In both cases, this pattern was present only on the site where the virus was injected into the VMH and the cells were also mCherry positive, suggesting they are virus infected. In one animal, ependymal cells at the level of the ARC lacking the mCherry signal also stained positive for cFos.

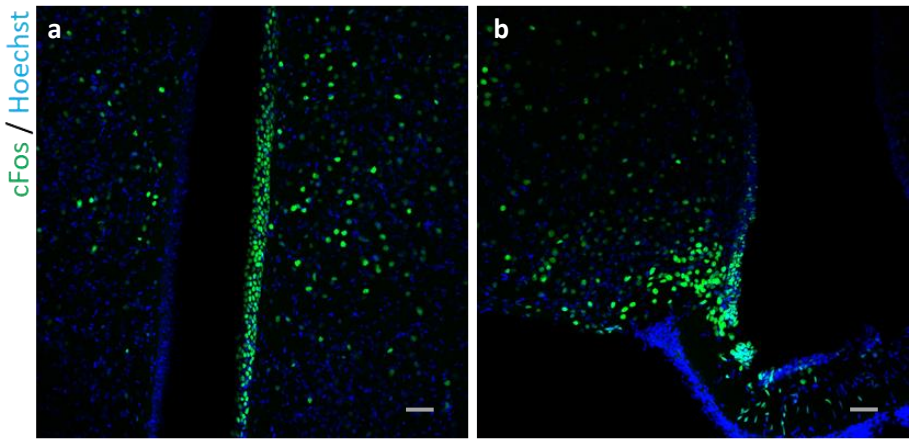

Figure 3.14: cFos- positive cells found in other areas after virus injection
After virus injection followed by DREADD- induced activation, cFos- positive cells were not only found in the injection target but also in distinct other regions including
a, ependymal layer at the level of dorsomedial hypothalamus (DMH) and
b, arcuate nucleus (ARC)

Inhibition of astroglia with iBark

In order to inhibit astroglia in the ventrolateral area of the VMH, I stereotaxically injected two wildtype BAC-glast CreER mice with an AAV virus v748-9 carrying a *bBark(rgs)_mCherry* construct under the control of a GFAP promoter. This sequence encodes a 122- residue inhibitory peptide (ibark) fused to mCherry as a reporter, which can be used to attenuate the activating Gq pathway in astrocytes and therefore result in the disruption of Ca2+ signalling. Therefore, for this virus we expect a significant decrease in the number of cFos positive cells, as it was observed in previous research (Nagai et al., 2021). Again, expression of the iBark gene is under control of the GFAP promoter to predominantly reach high expression levels in astroglia. As previously described for the animals injected with an activating AAV- construct, I stained cryosections with Anti- mCherry, Anti- GFAP and Anti- cFos. Similar to the construct carrying the activating DREADD under control of the GFAP promoter (v97-5), I observed a visibly higher level of infected cells and also a bigger virus spread than with the Cre-dependent virus. I quantified the amount of cFos positive nuclei in the VMH of both animals and found a significant reduction in comparison to animals that were injected with the constructs carrying the activating DREADDs (Figure 3.15)

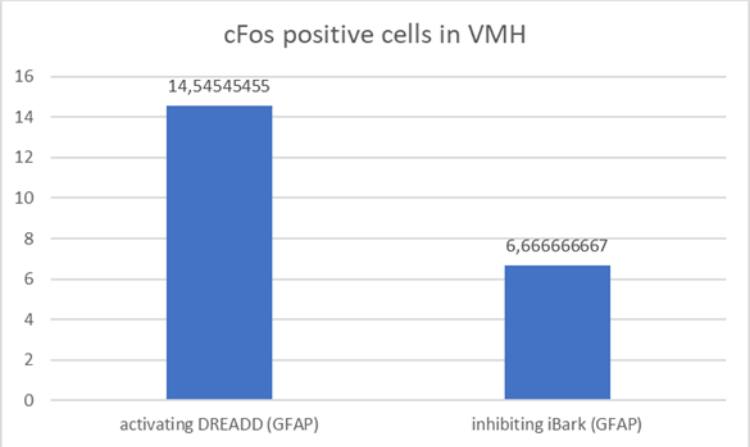

Figure 3.15: amount of cFos- positive cells after injection with activating DREADD compared to injection with inhibitory iBARK construct
After injection with virus v97-5 carrying the activating DREADD, high levels of cFos- positive cells were found in the VMH. This number significantly decreased when animas were instead injected with a virus v784-9 carrying an inhibitory iBark sequence.

In addition, I compared the uninjected and injected VMH of the same animal and found that there is a reduction in cFos positive cells in the infected VMH compared to uninfected VMH (Figure 3.16). This once again suggests that there is a basal cFos activity in the VMH which is not related to the virus

injection, but can be significantly increased with either an activating DREADD or, as in this case, reduced using the inhibitory iBark system.

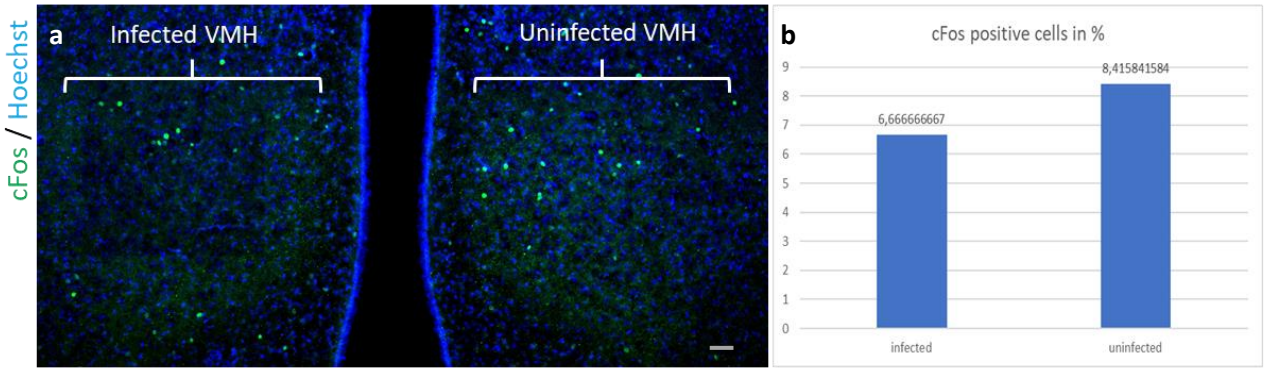

Figure 3.16: cFos- positive cells in injected VMH compared to uninjected VMH of the same animal
After injection with virus v748-9 carrying the inhibitory iBark sequence, the already low level of basal cFos activity was reduced
a, injected and uninjected VMH
b, quantification of cFos- positive cells found in injected/ uninjected VMH

Interestingly, I found no activation in either the median eminence or the ependymal layer, as it was observed before with the activating construct. These findings are in line with our expectations and the research results of Nagai et al., suggesting that astroglial GPCR signalling can in fact be attenuated in order to reduce the activity of astrocytes.

Simultaneous inhibition and activation of astroglia

We next investigated the effect of combining an AAV construct carrying an activating DREADD with a construct encoding the inhibitory iBark. For this, I mixed the GFAP- controlled viruses v97-5 (activating) and v784-9 (inhibiting) in a 1:1 ratio and injected 400nl unilateral into the VMH of wildtype Bac- Glast-CreER mice. Previous results showed an increase in cFos positive cells in the injected VMH compared to the uninjected VMH with the activating v97-5 virus, suggesting that cell activity could in fact be DREADD induced. With the inhibiting virus v784-9 I observed that almost no cells in the targeted region stained positive for cFos. In fact, even less cells were active than in the uninjected VMH of both animals. Therefore, a mixture of both activating and inactivating construct should at the same time increase and decrease the number of active cells. In fact, I found that animals injected with the 1:1 mix showed less cell activation (10,8%) in the target region compared to animals injected with the activating DREADD (14,5%) and more activation when compared to the animals injected with the iBark construct (6,6%, Figure 3.17).

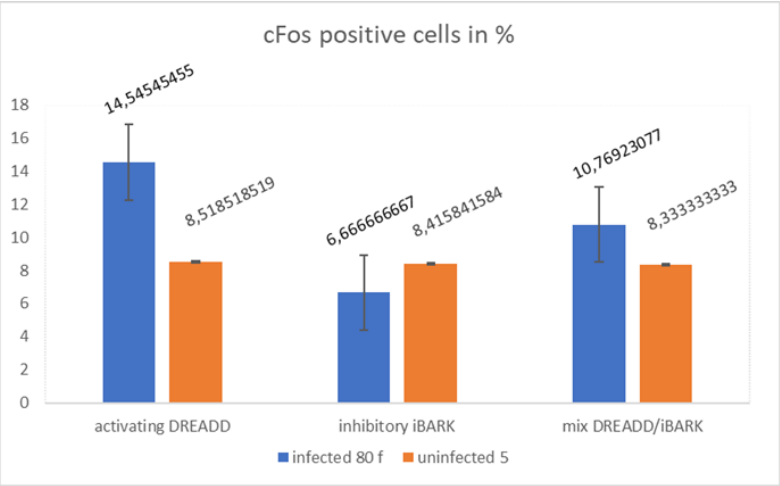

Figure 3.17: amount of cFos- positive cells found in the VMH after injection of activating DREADD, inhibitory iBARK and 1:1 mixture of DREADD/iBARK

Animals that were infected with a mixture of both activating DREADD and inhibitory iBARK showed reduced cFos activity compared to the activating DREADD alone. In line with that, cFos activity was increased in comparison to animals that were injected with the inhibitory iBARK construct.

Interestingly, I still found cFos positive cells in the ependymal layer just like with the activating GFAP construct, but the number of active cells was significantly reduced (Figure 3.18). This again demonstrates that the GFAP DREADD can be in fact used to activate astroglia in the VMH, whereas with the iBARK system the exact opposite is possible, i. e. the inhibition of astroglia.

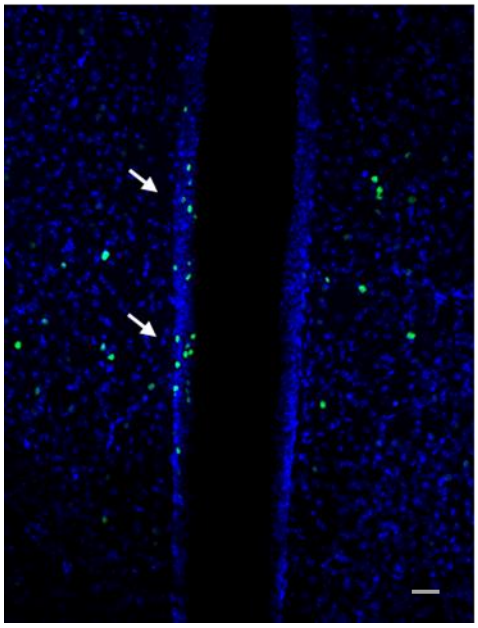

Figure 3.18: cFos positive cells in the ependymal layer after injection with mixture of virus v97-5 and v748-9

When compared to animals injected with the virus v98-9 carrying an activating DREADD, fewer cFos positive cells were found in the ependymal layer of animals injected with a mixture of activating DREADD and inhibitory iBark.

Discussion

Compared to other brain regions, the hypothalamus does not consist of strictly separated functional and anatomical units. Because of its high cellular heterogeneity and complex neuronal circuitry, revealing the interplay that lies behind metabolic and physiological processes maintained by the hypothalamus has always been a highly demanding task for researchers (Saper & Lowell, 2014). Nevertheless, understanding the regulatory mechanisms that are responsible for the maintenance of the bodies internal milieu could contribute crucially to the diagnosis and treatment of some of the most prevailing metabolic diseases, including obesity, trauma- associated behaviour, growth defects, failure to reproduce or even aggression behaviour and insomnia/ narcolepsy (Romanov et al., 2019). Significant progress in this field was facilitated by the development of molecular and genetic techniques like single-cell RNA sequencing and spatial transcriptomics. Those methods allow the better understanding of cellular codes together with the identification of specific molecular blueprints in hypothalamic neurons at high resolution (Romanov et al., 2019). However, what is missing here is the link between the specific cell types to the functional circuitry that finetunes the physiological output. In order to understand the detailed mechanisms that lie behind the various physiological and metabolic outcomes maintained by the hypothalamus, it is necessary to take a step back from the bigger picture and rather start by unravelling the very basic elements. In contemporary neuroscience, the current approach is to genetically dissect individual neuronal circuits to understand first their function and then put them in in context with the behavioural or physiological outcome. This is mainly done by manipulating and controlling genetic elements involved in the regulation of transcription or translation in order to generate different cell types. With this approach, transgenes can be expressed in neuronal subpopulations within the highly heterogeneous cell populations throughout the whole CNS, allowing the targeted and specific manipulation of those populations. The challenging part of this operation is the exact delivery to the desired cell population across the CNS and the PNS. Viral vectors have proven to be a flexible and fast method to face this challenge, most commonly used are nonpathogenic adeno- associated viruses (AAVs) (Bedbrook et al., 2018). This tool has shown to be very efficient when it comes to targeting cells of neuronal identity. In the hypothalamus, AAV technology was successfully used for the delivery of Tracers, optogenetic and chemogenetic tools, calcium sensors and the delivery of shRNA for gene silencing. This altogether revealed a connection between the hypothalamic A14 locus, the suprachiasmatic nucleus and the lateral septum which is apparently involved in the diurnal regulation of locomotion behaviour in the mouse (Korchynska et al., 2021). Those results were the foundation of some of the experiments that are part of this thesis, i. e. the AAV- based activation of somatostatin cells in the lateral septum. My results also show that AAV-based approaches can be very efficiently used for targeting of cells of neuronal identity. However, in the past years it became more and more obvious that when it comes to fully understanding the

complex interplay in different regions of the brain, neurons are by far not the only cell group that should be of interest. In fact, astrocytes, who make up 20-40% of the cells in the CNS, are also crucially involved in physiological responses and the development of pathological states. They are also important supporters to ensure proper neuronal circuit functions (Khakh & Sofroniew, 2015). In order to understand their various roles, AAV- based genetic manipulation is a promising approach. Nevertheless, there are some complications in using this approach for astrocyte manipulation: limited specificity and high off- target expression in neurons are a big challenge. In my work, I also experienced a low transgene expression rate, especially with cre- dependent approaches. As a consequence, I tested different strategies, changed virus serotypes and promoters, all with the goal to develop an efficient model to specifically target and manipulate astrocytes in the hypothalamus. The longterm goal after the successful establishment of this model was to use it for behavioural experiments, which would have been very promising to reveal how much astrocytes can influence neuronal circuits that modulate physiological and behavioural responses. However, I unfortunately was not able to further conduct this research, as all my experiments had to be discontinued due to legal issues. Nevertheless, I still got some interesting results which can hopefully be included in further research into the topic of AAV- based manipulation of neuronal cells and astrocytes.

Dopaminergic A14 neurons are involved in locomotion control as well as feeding behaviour

In the recent work my colleagues have identified that A14 DA neurons innervate both intrahypothalamic (ARC, VMH and other regions (not shown)), and extrahypothalamic cell targets, specifically lateral septum (LS) (Korchynska et al., 2022). Circuit mapping combined with pharmacological analysis identified lateral septal neurons expressing both D1 and D2 dopamine receptors as preferred synaptic targets of extrahypothalamic afferents originating in the PeVN (Korchynska et al., 2022). My colleagues also found that in vivo chemogenetic activation of A14 DA cells stimulated locomotion mediated by LS neurons (Besnard et al., 2019) and can be disrupted by the antagonistic inhibition of the lateral septal cellular cohort. Moreover, chemogenetic inhibition of dopamine output from the A14 cells normalized amphetamine-induced hyperlocomotion, particularly during sedentary periods. Cumulatively, those findings identify a hypothalamic locus for the diurnal control of locomotion and pinpoint a midbrain-independent cellular target of psychostimulants. We additionally found out that chemogenetic stimulation of A14 DA cells in with CNO injection suppresses eating. Altogether, our preliminary data suggest the multiple behavioural effects on complex feeding behaviour with the overall decrease in food consumption. Importantly, the observed drop down of food consumption upon chemogenetic activation of A14 cells rather supports the idea of direct regulation of food consumption by A14 neurons and cannot be explained by the indirect effect of increased physical activity because of the opposite anticipated effect, that is the rise of energy needs.

A14 neurons in the PeVN can be successfully activated using flp- dependent DREADDs delivered by AAVs

I showed that dopaminergic cells of the A14 group which is located in the periventricular nucleus can be successfully activated using an AAV virus encoding a flp- dependent DREADD, as several TH- positive cells additionally showed colocalization of mCherry and cFos. This finding can be used for further investigation on the circuit function of those A14 neurons, as it gives rise to the possibility of using a combination of both cre- and flp- dependent DREADDs in order to allow bidirectional manipulation. Before, it was only possible to be specific from one side, whereas now the cell groups of interest can be targeted more precisely, e.g. SSt- cells in the LS together with the dopaminergic A14 group in the PeVN. Additionally, my results show that not only neurons of the A14 group can be targeted with this flp- dependent approach, but also dopaminergic A12 neurons, which are located in the arcuate nucleus, can be successfully infected and activated. Regarding the experimental procedure, I also made some useful discoveries: As the PeVN is closely located to the third ventricle, the risk is that, if the injection procedure is not accurate enough, the virus accidentally gets injected into the ventricle, which would reduce or even completely prevent the infection of the targeted cell group. Keeping this in mind, it makes sense to adjust the injection coordinates a bit more in the lateral direction, as because of the virus spread the cells of interest can still be reached. Another thing to consider is that infection and activation of target cells seemed to be more efficient in animals that were homozygous for the flp- transgene than in heterozygous one. We therefor tried to shift the animal breeding so that more homozygous animals would be available. Nevertheless, mating of two homozygous animals hardly generated useable offspring, as there were either only few animals per litter or the animals did not survive the experiments due to weak health or other medical issues like skin conditions.

Cre- dependent chemogenetic activation of Sst- neurons in the lateral septum leads to increased locomotion

Previous experiments showed that DREADD- induced activation of dopaminergic A14 neurons leads to increased animal mobility, whereas the inhibition of those neurons results in decreased locomotion. Further, locomotion behaviour seems to be regulated by a circuit involving not only the dopaminergic A14 locus in the PeVn, but also the suprachiasmatic nucleus and the lateral septum (Korchynska et al., 2022). Therefore, the next logic step was to test whether manipulating somatostatin neurons in the LS, which are the projection targets of neurons of the A14 locus, also leads to changes in locomotion behaviour. I showed that chemogenetic activation of Sst- neurons in the LS using a cre- dependent AAV- virus indeed leads to increased animal movement. I additionally found that those Sst- neurons have several other projection targets, including the median preoptic area and the lateral preoptic area. It is known that the lateral septum displays huge heterogeneity, as it can be subdivided into several

regions that receive input mainly from subregions of the hippocampus and generate output to various different brain regions that include areas of the hypothalamus like the lateral hypothalamic area, lateral preoptic area, medial hypothalamic area and medial preoptic area (Rizzi- Wise & Wang, 2021). It is also known that somatostatin- positive neurons of the dorsal lateral Septum (dLS) seem to be involved in the mediation of stress responses, as they receive innervation from the locus coeruleus which is the primary source of norepinephrine, a neurotransmitter that is highly connected to stress responses. Their known projection targets also include areas that are known to mediate stress- related functions (An et al., 2022). Stress can be induced as a response that environmental threats that in turn require defensive reactions like fight/ flight or also freezing behaviour. In fact, somatostatin- neurons seem to regulate mobility in a context- dependent manner (Besnard et al., 2019). Altogether, my findings are in line with the fact that the lateral septum with its different subregions serves as an important relay station between various different brain regions that are involved in the contextual regulation of behavioural and physiological responses.

Cre- independent targeting allows AAV- mediated activation and inhibition of astrocytes in the VMH and the ependymal layer

My results show that targeting astrocytes in the VMH using a cre- dependent approach only produces a relatively small number of successfully activated cells of interest. In fact, with this approach I infected mostly cells of neuronal identity. Switching to a virus that delivers the transgene in a cre- independent manner drastically improved the results, as this led to more astrocyte- specific infection. Additionally, I discovered that the EF-1α promoter is more efficient for transgene expression in astrocytes than the previously used human synapsin promoter. Lastly, changing the virus serotype from AAV-8 to AAV-9/2 also improved the outcome. Therefor, it can be said that out of all 3 viruses (cre- dependent 44361-AAV8 and cre- independent v98-9 and v97-5), the most efficient one was the cre- independent virus v97-5. Nevertheless, with this virus I was still not able to limit infection to astrocytes only: due to the huge spread and high infection rate, cells with neuronal identity were still infected. Also, the mCherry-signal that marks infected cells was so strong that it was difficult to distinguish between individual cells. Thus, this also lad to complication regarding the analysis of colocalization with other markers like cFos, GFAP or NeuN, which is of crucial importance in order to monitor the state of cell activity and the cellular identity, respectively. However, this problem could eventually be solved by reducing the injection volume. Also, changing again either the promoter or the serotype could generate more specific results. Interestingly, I observed that cells in the ependymal layer next to the third ventricle at the level of the DMH and the Arc showed a rather specific activation pattern that seems to be linked to the virus injection. This suggest that this approach can be used not only for targeting astrocytes in the VMH or DMH, but also tanycytes and other glial cells which are a part of an astroependymal group.

Tanycytes were found to be involved in the regulation of important metabolic processes, as they transport metabolites such as leptin, ghrelin and also glucose between the third ventricle and the general circulation or the parenchymal area (Balland et al., 2014; Collden et al., 2014). They also regulate the secretion of neuropeptides into the hypothalamus- pituitary portal circulation and generate the active form of thyroid hormones (Prevot et al., 2018). My research implies that those cells can be chemogenetically activated using AAV- based transgene delivery. This could facilitate further research into the topic of metabolic processes that involve the transport of molecules over the blood- brain barrier or between the CSF and the general circulation. I also found that astrocytes in the VMH can not only be chemogenetically activated, but also inhibited using an ibark- construct to actively disrupt astrocytic Gq- GPCR Ca2+ signalling (Nagai et al., 2021). When animals were injected with the inhibiting virus v748-9, the number of activated cells was significantly reduced in the target region (VMH) and also in the ependymal layer and the arcuate nucleus. Nevertheless, I had to face the same challenge as with the activating virus v97-5: The number of infected cells in total was way to high and not only limited to astrocytes. However, this problem can eventually be solved as previously described. Lastly, I injected a mixture of both activating and inactivating viruses. The results were as expected: Less cells were active than with the activating virus v97-5 alone, but still more than with only the inhibiting virus v748-9. I observed this in all three regions (VMH, ependymal layer and Arc). Altogether, I found that manipulating astroglia is more efficient when a cre- independent approach is used. This could maybe even facilitate further research, i.e. when it comes to combining two viruses, one cre-dependent and one cre- independent, in order to investigate neurons and astrocytes at the same time, respectively. However, there is still work that has to be done: Immunohistochemical analysis of brain slices is only the first step when it comes to monitoring the outcome of successful activation or inactivation of target cells. The main goal is to determine whether the manipulation of specific cell groups in the animal brain has behavioural or physiological consequences. My experimental outlook therefore is to slightly adjust the experimental strategies as described above and then, as a next step I plan to do behavioural experiments. This includes basic settings like home cage monitoring but additionally also the investigation of higher cognitive tasks like aggression behaviour or stress response.

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