Targeting Tanycytes: Balance between Efficiency and Specificity

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Abstract

Tanycytes are peculiar ependymoglia cells lining the bottom and the lateral wall of the third ventricle. For a decade, the utilization of molecular genetic approaches allowed us to make important discoveries about their diverse physiological functions. Here, I review the current methods used to target tanycytes, focusing on their specificity, their efficiency, their limitations, as well as their potential future improvements.

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Introduction

Since their characterization by Horstmann [1], tanycytes are now considered as key cells in the regulation of numerous physiological functions, ranging from reproduction to energy balance [2–4]. Elongated unipolar ependymoglia cells lining the bottom and the lateral wall of the third ventricle, they send a long radial process to connect blood-brain barrier capillaries in the mediobasal hy-

pothalamus and the fenestrated capillaries of the hypothalamo-hypophysial portal system in the median eminence [5–7]. While still controversial, tanycytes would also contact other neural cells – including neurons – located in the arcuate nucleus, the ventromedial nucleus, and the dorsomedial nucleus [8, 9]. This strategic position at a “3-way exchange interface” between the blood, the cerebrospinal fluid and the brain parenchyma is complemented by peculiar properties [2] such as blood/brain traffic controllers [10–14], metabolic sensors [15, 16], neuronal modulators [8, 17, 18], and neural stem/progenitor cells [19–23], making them a promising cell population to understand neuroendocrine control of physiological functions and to potentially treat their related diseases.

Tanycytes have consequently begun to attract considerable attention, driving different groups to develop and continuously improve state-of-the-art investigative techniques to study them. Among them, molecular genetic approaches theoretically allow us to study tanycyte functions in different contexts including development, adult physiology, and diseases, by making possible the manipulation of any tanycyte gene, in any tanycyte type, at a defined time point. However, these approaches prompted us to beg crucial questions to ensure the relevance of our work. What is the best development period to target tanycytes? What is the best balance between efficiency and

specificity? This mini-review aims to describe the current methods used to selectively target tanycytes and study their function. It will also discuss the specificity, the efficiency, and the limitations of these methods as well as new possibilities for their improvements.

A Question of Time: When to Target Tanycytes?

The first critical point to efficiently target a cell population is the time window: indeed, the timeline of gene expression as well as cell “story” (e.g., its birth, its connectivity, its lifespan) have to be taken into consideration. These concerns are particularly true for tanycytes. Remaining radial glial cells in the adult brain, tanycytes share numerous properties with these progenitor cells [24, 25]. During brain development, radial glial cells give birth to neuronal populations and certain glia lineages [25]. Their cell bodies lie in the embryonic ventricular zone, and they send a long basal process contacting the pial surface: these processes form a scaffold for the migration of newly generated neurons up to their final destinations [25]. Along the third ventricle, tanycytes are born around E19 in rat brain, but some of them continue to be generated and to mature during the first and the second postnatal week [26]. Interestingly, tanycytes maintain the expression of numerous radial glial cell markers such as *Nestin*, *Glast*, or *Rax* [2, 4, 24]. Most importantly, they also maintain their capacity not only to proliferate in the postnatal brain to renew part of the tanycyte population but also to generate other neural cells including glial cells and neurons that will be involved in the same regulatory systems as tanycytes [20–23]. According to previous reports, this postnatal neuro- and gliogenesis is robust perinatally and may be observed as late as P60/65 [21, 22]. Therefore, targeting tanycyte genes during development or in the postnatal brain in order to study their physiological role in adult mice would consequently lead to a leaky expression in the “wrong” cell types. The basic Cre-loxP recombination by crossing cell-specific Cre-expressing mice with Cre-dependent transgenic mice has consequently been banished by several groups to avoid leaks during embryonic development. In contrast, models where tanycytes are targeted after birth have been preferred to be able to choose the time window appropriate to what they want to analyze (i.e., in pups and young animals (up to P60) to examine the impact of tanycyte populations on hypothalamus development, or in adult mice (after 8 weeks) to study the physiological role of tanycytes on different neuroendocrine functions).

A Question of Specificity: How to Target Tanycytes?

In the literature, current methods to target specific cell populations *in vivo* are based on marker gene expression, location, anatomical projection pattern, synaptic connectivity, as well as combinations of them [27]. In tanycyte research, authors mainly chose to target tanycytes using their markers and/or their advantageous location (Table 1).

CreERT2 Models

CreERT2 models are inducible systems that allow the temporal control of gene expression [28]. CreERT2 recombinase is a fusion protein containing the Cre recombinase and a mutant form of the human estrogen receptor binding the synthetic estrogen receptor ligands (e.g., tamoxifen) [28]. When CreERT2 mice are bred with mice containing a loxP-flanked sequence of interest, tamoxifen injections will induce the nuclear translocation of CreERT2 recombinase and consequently the Cre-mediated recombination at the floxed sites. Different mouse models harboring a CreERT2 transgene driven by a tanycyte-specific promoter (i.e., *Nestin* [21–23], *Fgf10* [21], or *Glast* [14, 20, 21]) have been used to temporally control DNA recombination in tanycytes. In some studies, these models have been bred with reporter mice encoding fluorescent proteins (e.g., YFP or tdTomato kept silent by upstream STOP^{LoxP/LoxP} sites) to lineage-trace tanycytes: their expressions were then induced in tanycytes by tamoxifen injections at different time points in order to monitor their division and the differentiation pattern of their progeny [20–23]. Besides lineage tracing, CreERT2 mouse lines such as *Rax*-CreERT2 mouse line [29] have also been used to study tanycyte functions [30, 31]. Using tdTomato reporter mice (Ai9) and tamoxifen injection at different developmental ages (from E8.5 to P53), Pak et al. [29] first described the brain distribution of *Rax* expression and confirmed that *Rax* could be used as tanycyte marker. Indeed, in postnatal brains, tdTomato expression was mainly observed in tanycytes but not in more dorsally located ependymal cells of the third ventricle [29]. Tanycytes are more efficiently labeled at P7 or P28 than at P50, as well as by series of injections rather than a single injection [29]. However, they also observed a robust and selective tdTomato expression after tamoxifen injections in radial Müller glia and the non-pigmented zone of the ciliary body in the neuroretina, in fibrous pituicytes in the neurohypophysis, and in some cells in the granule layer of the flocculus of the cerebellum [29]. This *Rax*-CreERT2 mouse line was then crossed with mice expressing Cre-dependent *Eno2-lsl*-

Table 1. Summary of available genetic tools to target tanycytes and their specificity

Mouse model	Tools	Administration			Targeted cells in adult mice	Purpose	Application	Reference
		mode	dose	site				
<i>Genetic approach</i> <i>Rax</i> -CreERT2 mouse line (crossed with Cre-dependent mice)	Tamoxifen	i.p.	Multiple	–	Tanycytes + Müller cells + pituicytes	Tanycyte ablation, LepR deletion	Physiological tests	[30, 31]
<i>Glast</i> -CreERT2 mouse line (crossed with Cre-dependent mice)	Tamoxifen	i.p.	Multiple	–	Tanycytes + parenchymal cells (astrocytes)	Gene silencing in tanycytes	Calcium signaling	[14]
		i.p.	Multiple	–		Induction of GFP, or B-Gal	Lineage tracing	[20]
<i>Fgf10</i> -CreERT2 (crossed with Cre-dependent mice)	Tamoxifen	i.p.	Single	–	β-tanycytes	Induction of YFP, B-Gal or tdTomato	Lineage tracing	[21]
<i>Nestin</i> -CreERT2 (crossed with Cre-dependent mice)	Tamoxifen	Gavage	Simple	–	Neuroprogenitor cells (tanycytes)	Induction of YFP	Lineage tracing	[22]
<i>Anatomical approach</i> Ai9 Cre reporter mice	TAT-Cre	i.c.v.	Single	3v	3v Ependyma	Tdtomato expression in tanycytes	Cell sorting	[10, 41]
Cre-dependent mice	TAT-Cre	i.c.v.	Single	3v	3v Ependyma	Gene silencing in tanycytes	Physiology + morphology	[41]
Ai14 Cre reporter mice	rAAV1/2-CAG-Cre	i.c.v.	Single	Lateral	Ependyma	Tdtomato expression in tanycytes	Morphology	[14]
WT mice	AAV-CAG-GCaMP6s	i.c.v.	Single	Lateral	Ependyma	Expression of GCaMP6s in tanycytes	Calcium signaling	[14]
WT rat	Ad-shRNA	i.c.v.	Singlet	3v	Ependyma	Gene silencing in tanycytes	Physiological tests	[18, 44, 45]
<i>Genetic + anatomical approaches</i>								
<i>Glast</i> -CreERT2 mouse line	rAAV1/2-CAG-flex (hM3D-mCherry)	i.c.v.	Single	Lateral	Tanycytes	Expression of hM3D in tanycytes	Tanycyte stimulation	[14]
	Tamoxifen	i.p.	Multiple	i.p.				
Cre-dependent mice	rAAV1/2-Dio2-iCre-GFP	i.c.v.	Single	Lateral	Tanycytes	Gene silencing in tanycytes	Physiological tests	[14]
WT mice and Tas1r2 null mice	AdV-pTSHR-GCaMP3	i.c.v.	Single	Lateral	Tanycytes	Expression of GCaMP3 in tanycytes	Calcium signaling	[49]
Cre-dependent mice	AAV1-Rax-Cre-GFP	i.c.v.	Single	3v	Tanycytes	Gene silencing in tanycytes	Physiology + tanycyte functions	[46]

DTA in order to ablate tanycytes after tamoxifen injection [30]. After 3-week tamoxifen treatment in 6-week-old mice, they observed a complete ablation of tanycytes of the arcuate nucleus and median eminence associated to metabolic defects (e.g., increase in visceral fat distribution, insulin insensitivity) [30]. *Glast*-CreERT2 mouse line has also been used to study the role of thyrotropin-releasing hormone signaling in tanycytes by deleting *Gna11* [14].

The greatest advantage of this approach is the simplicity to implement it. Indeed, CreERT2 mouse lines can be easily crossed with any Cre-dependent mouse line, and tamoxifen can be administrated by gavage or by intraperitoneal injection at diverse time points. The absence of surgery also makes it a good tool to study physiological effects induced by the deletion of endogenous genes or the expression of exogenous/ectopic genes in tanycytes. However, tamoxifen treatment may create different is-

sues. First, tamoxifen acts through the estrogen receptor: tanycytes expressing ER- α and β receptors and responding to estrogen [2, 32]; chronic administration of tamoxifen may modulate tanycyte functions. Most importantly, tamoxifen may have an impact on tanycyte neurogenesis per se, given that the levels of estrogens may affect neurogenesis [33] and, consequently, lead to numerous off-targets in the brain parenchyma over time. Moreover, tamoxifen could also act on other cell populations and organs [34–36], impacting mice physiology. Finally, tanycyte-specific promoters have to be carefully chosen in order to specifically target tanycyte population or tanycyte subgroups without targeting the neighboring parenchymal cells. Unfortunately, we still do not have the necessary background knowledge about tanycyte markers: so far, the known markers are shared with other cell populations, in particular stem/progenitor cells (i.e., *Nestin*, *Rax*) or other glial cells (i.e., *Glast*), leading to potential off-targets. In particular, *Glast* is an astrocytic marker and *Glast*-CreERT2 mice are usually used to induce a “glia-specific” knockout [37, 38]. Moreover, the distribution of *Glast* expression in tanycytes is also controversial [24]. While some authors reported α -tanycyte-specific *Glast* expression using *Glast*-CreERT2 reporter line [21, 39], its expression has been observed in both α - and β -tanycytes by others [14, 20, 40].

TAT-Cre Model

Taking advantage of their unique position in the brain, tools for genetic manipulations have also been infused in the ventricular system in order to target tanycytes. By this way, we developed an approach using TAT-Cre [10, 41]. TAT-Cre is a recombinant cell-penetrating protein containing Cre recombinase to catalyze the site-specific recombination event between 2 loxP DNA sites, TAT (Trans-Activator of Transcription) sequence which is a cell-penetrating peptide, and a nuclear localization sequence. Its cellular uptake is therefore enhanced compared to Cre-recombinase [42]. Infusion of TAT-Cre in the ventricular system of Cre reporter line mice (i.e., Ai9 mice) showed the DNA recombination in ependymal cells, including both tanycytes and common cuboidal ependymal cells as well as the choroid plexus [10]. Interestingly, this recombinant protein does not cross the ependymal layer and does not target neural cells present in the parenchyma. However, it is worth noticing that few neurons – likely cerebrospinal fluid-contacting neurons – may sometimes be observed. We first used this approach in order to isolate tanycytes by fluorescence-activated cells sorting and to study tanycyte gene expression dynamics according to the physiological

state (i.e., during the estrous cycle [41], during a metabolic challenge [10]). For that, tdTomato reporter mice were infused with TAT-Cre in the third ventricle; 2 days later, the mediobasal hypothalamus were microdissected in order to only keep the ependyma formed by tanycytes, dissociated to obtain a single cell suspension and processed for fluorescence-activated cells sorting [10]. The high expression of tanycyte markers (i.e., DARPP32) confirmed that our sample was enriched in tanycytes [10, 41]. Labeling tanycyte populations may also be critical for different applications: it enables the visualization of their morphology, as well as the validation of gene expression. Besides tanycyte labeling, we also used TAT-Cre in Cre-dependent mice to delete gene expression in tanycytes (i.e., *Itgb1*) in order to analyze mice fertility [41].

The greatest advantage of TAT-Cre is the efficiency and rapidity to modulate tanycyte function. Indeed, it is not necessary to cross mice line: TAT-Cre is directly infused in any Cre-dependent mice at the desired time point. The recombination is very efficient and quick (<24 h after injection). This is consequently ideal for labeling, neuroanatomy, and cell sorting (associated with mediobasal hypothalamus microdissections to specifically collect tanycytes). The main cons are the use of surgery: 1 week of recovery with anti-inflammatory treatment is therefore necessary before starting physiological studies. The specificity is also limited given that TAT-Cre will target any ependymal cells along the ventricle, as well as the choroid plexus. However, some specificity may be added by adjusting the conditions of infusion. Indeed, as reported by others [43], we observed a lack of widespread Cre activity: TAT-Cre induces the DNA recombination close to the site of injection. Therefore, the flow rate, the site, and the volume of injection may influence the proportion of targeted tanycytes versus ependymal cells. For instance, an injection in the left ventricle will mainly target the left ventricle, but not the right one. The third ventricle will be partially targeted, whereas the fourth one will not. An injection in the third ventricle will target the third ventricle, including the entire tanycyte population, but not the lateral ventricles, the fourth ventricle, or the choroid plexus. This TAT-Cre property might be due to its quick cellular uptake or to molecules present in the cerebrospinal fluid interfering with its activity. However, further studies are needed to fully understand this property. Finally, another potential limitation is related to its peptidic nature: TAT-Cre quickly acts and is then quickly cleared. The state of chromatin structure at the moment of the injection could therefore impact TAT-Cre efficiency as it has to find target pair of loxP sites and complete

Fig. 1. Combining genetic and location approaches to specifically target tanycytes or tanycyte subgroups. Polarized ependymoglial cells lining the basal part of the third ventricle (3V), tanycytes are classified in 4 subtypes: $\beta 2$ (orange), $\beta 1$ (brown), $\alpha 2$ (green), and $\alpha 1$ (blue). Recent studies, in particular single-cell RNAseq [57, 58], allowed the characterization of markers for the global population as well as for each subgroup. Some of them are suggested here. The combination of tools targeting only the ependymal layer (e.g., adenoviruses, AAV1/2) and tanycyte marker promoter (e.g., *Rax* [46], *Dio2* [14], *Tshr* [49]) will allow us to specifically study tanycyte populations and to make significant advances in the understanding of their functions. AAV, adeno-associated virus; ARH, arcuate nucleus; VMH, ventromedial nucleus; DMH, dorsomedial nucleus.

the reaction in a short delay. Further knowledge about tanycyte chromatin structure according to the physiological state could help to limit this issue.

Viral Model

Using a similar approach, different laboratories developed viral constructs such as adenoviruses [18, 44, 45] and recombinant adeno-associated virus (rAAV) [14, 46] to target tanycytes. As for CreERT2 mouse lines or TAT-Cre, these vectors have been first validated using fluorescent protein such as GFP [18, 19, 44, 45] or tdTomato [14]. While adenoviruses efficiently target the ependymal layer in every ventricle without diffusing in the parenchyma [18, 45], rAAV serotype has to be carefully chosen: indeed, some of them are more prone to target neurons such as AAV2 or AAV8, whereas others will target glial cells such as AAV4 or AAVDJ8 [47, 48]. Moreover, some of them (i.e., AAV1 and 2) are known to hardly diffuse into the parenchyma. For tanycytes research, rAAV1/2 have been successfully used [14]: indeed, once infused in the ventricular system, this virus efficiently targets the ependymal layer of the ventricular system – including tanycytes – without diffusing in the parenchyma as shown by the expression of the reporter tdTomato [14]. So far, viruses have been mainly used to silence genes in tanycytes in order to determine their functions from the cellular level to physiology in the intact organism. In this perspective, Garcia-Robles laboratory developed adenoviruses

expressing different shRNA to silence gene expression in rat tanycytes (i.e., *Glut2*, *Glucokinase*, *Mct1*), in order to study their glucose-sensing properties and their impact on mice physiology [18, 44, 45]. Alternatively, viruses have also been used to express proteins in tanycytes, such as the calcium sensor GCaMP6s in order to evaluate on slices the increase in $(Ca^{2+})_i$ in tanycytes in response to stimuli [14].

The greatest advantage of viruses is their efficiency: indeed, a single injection in the lateral ventricle is sufficient to target the ependymal layer in every ventricle. The expression of fluorescent reporters through viral vectors is consequently ideal for labeling, neuroanatomy, and cell sorting (associated with microdissection). The main cons are similar to those of TAT-Cre. This approach requires a surgery, and the specificity is limited as viruses will target any ependymal cells in every ventricle. To deal with such issues and to further enhance selectivity, genetic (i.e., using tanycyte markers) and location approaches (i.e., using viruses targeting the ventricular wall) may be combined (Table 1; Fig. 1). In this perspective, 3 different constructs have been built using the promoter of *Dio2* [14], *Tshr* [49], and *Rax* [46] genes. Introduced in AAV or adenoviral vectors that incorporate the ependymal layer without targeting parenchymal cells, these promoters only drive an expression of the protein of interest (i.e., Cre recombinase [14, 46] or *Gcamp3* [49]) in the tanycyte population. Alternatively, Müller-Fielitz et al. [14] com-

bined Cre-dependent viruses and CreERT2 mouse lines to specifically target tanycytes. They injected rAAV1/2-CAG-flex(hM3D-mCherry) vector into the lateral ventricle of *Glast*-CreERT2 mice: by injecting tamoxifen, Cre recombinase was specifically activated in tanycytes and glial parenchymal cells. However, rAAV1/2 vectors get trapped in the ventricle wall, and hM3D-mCherry expression was only observed in hypothalamic tanycytes [14]. This approach allowed them to chemogenetically activate Gq/11 signaling in tanycytes and study its impact on tanycyte morphology [14].

Future Improvements and Applications

So far, molecular genetic approaches in tanycyte research have been restricted to the expression of Cre recombinase, shRNA, fluorescent reporters, DREADD receptors, or calcium sensors in tanycytes [10, 14, 44, 49]. However, already available tools are becoming more versatile and sophisticated and could therefore give us new information about tanycyte biology. While engineered DREADD receptors have already been expressed in tanycytes to analyze tanycyte function following their activation [14], this approach as well as optogenetics (i.e., expression of light-sensitive ion channels in tanycytes) could allow us to study the impact of tanycyte activation on neighboring neural cell functions as well as on physiology [50]. Brainbow multicolor labeling strategies could also be useful to decipher tanycyte lineage as well as connectivity [51]. Finally, the induction of transgene expression (e.g., ectopic expression of genes or mutated genes) could allow us to study their role in tanycytes in physiological and pathological conditions.

However, some issues persist on tanycyte research and have to be addressed if we want to efficiently use these new approaches. The 3 main concerns are the specificity, the level of gene expression, and the timing. As described above, the best way to specifically target tanycytes is to combine genetic (i.e., tanycyte markers) and location (i.e., the use of viruses targeting the ependyma) approaches (Fig. 1). This first requires good tanycyte markers to target either the entire population or most interestingly each tanycyte subgroup. Research has therefore to further focus on tanycyte gene expression profiling as well as their “connectivity” with other neural cells. Indeed, the complete characterization of the different tanycyte subgroups is needed. While this classification currently defines 4 subgroups with specific markers for each of them, recent advances clearly showed that >4 subgroups may be

defined (see review [4]). In addition, according to their location along the ventricle wall, tanycytes likely interact with different neural cell types and different neuronal populations: this variable “connectivity” certainly has an impact on their transcriptomic profiles resulting in different subgroups with different functions. The identification of new tanycyte subgroup-specific markers will allow the development of new genetic tools (i.e., relevant AAV-Cre or CreERT2 mouse lines) for delineating these different subgroups, as well as achieving their specific manipulation in order to comprehensively dissect their diverse functions. The second aspect to take into consideration is the level of transgene expression. Indeed, transgene overexpression or expression in the bad time windows would put the mice in non-physiological conditions and biased our studies. Therefore, the expression system and the promoter should be chosen with the desired expression levels in mind. As tanycyte gene expression varies according to the physiological conditions as well as the photoperiod for seasonal animals [4, 15, 52], a temporal control of gene expression through inducible promoter such as Tet-Off and Tet-On expression systems could be useful to express the transgene at the good time point [53]. In these systems, the transgene of interest is under the control of a tetracycline-inducible promoter (tetracycline response element [TRE]) and its expression is therefore dependent on the activation of an inducible transcriptional activator (i.e., tTA for the Tet-off and rtTA for the Tet-on system) by tetracycline [53]. tTA requires the absence of tetracycline to bind to TRE, whereas rtTA requires the presence of tetracycline to bind to TRE. In mice, as tetracycline may take time to be eliminated, rtTA (i.e., Tet-on system) is preferred to turn on a transgene. For tanycyte research, the co-expression of rtTA under the promoter of tanycyte marker and of the transgene of interest under TRE promoter could allow us to control the time window of its expression.

Recently, CRISPR/Cas9 systems have revolutionized genome engineering approaches. These systems rely on the Cas9 protein to cleave the genomic DNA and the guide RNA strand to target the genomic site. When the double-stranded DNA is broken, the endogenous cellular repair mechanisms will induce either an incorporation of random nucleotides to the break site, leading to gene disruption or knock-out, or the insertion of new engineered genomic sequences, leading to mutation or insertion of a new gene (see reviews [54]). More than a tool to efficiently edit any genes within organisms, CRISPR systems are highly adaptable leading to various applications ranging from transient activation of endogenous genes to epige-

netic modifications. These approaches are slowly translated for in vivo research through different delivery methods including viruses [55], nanocarriers [56], or physical methods [54]. Tanycytes being in a strategic position to receive these carriers, this approach would be ideal for tanycyte research and could offer numerous advantages. By expressing Cas9 under tanycyte marker promoter, the expression of the gene of interest could be induced or deleted in tanycytes, and the impact on tanycyte function and mice physiology studied. Most interestingly, tanycytes being associated to pathological conditions such as obesity [11], rare pathogenic mutations, or single-nucleotide polymorphism associated to diseases by genome-wide association study may also be studied in tanycytes by achieving the desired modification of the gene sequence.

Conclusion

For a decade, the adaptation of different genetic approaches to tanycyte research allowed us to largely improve our knowledge about this cell population. However,

expanding our toolbox to fine-tune tanycyte functions is required to fully understand their role in the regulation of different physiological functions. For this purpose, recent technical advances in gene expression profiling, chromatin accessibility profiling, as well as high-efficiency genomic editing could allow the development of novel efficient genetic tools for tanycyte research.

In addition to improving our knowledge about their biology, these approaches represent a hope for the development of potential therapeutic strategy. Given their unique neuroanatomical position in relationship with the median eminence of the hypothalamus (i.e., a region outside the blood-brain barrier), tanycytes are prime targets in the brain for pharmacological treatments. Their genetic manipulation could therefore offer a valuable tool for modulating their neuroendocrine functions in order to tackle any related diseases.

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