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Research article

Failure of AAV retrograde tracer transduction in hypothalamic projections to the periaqueductal gray matter

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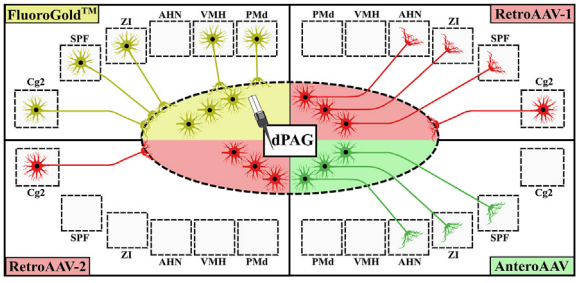
HIGHLIGHTS

- The use of AAVs to examine neural circuits is growing and well accepted, but some limitations should be considered.
- We used two commercially available retrograde AAV, which failed to infect hypothalamic neurons.
- One of two commercially available retrograde AAV tested also showed significant anterograde labeling.

GRAPHICAL ABSTRACT



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ABSTRACT

Connectomics is an important field of neuroscience that examines how neurons are connected and form functional circuits that underly the brain's functions. Conventional tracers based on dye have led to great advances in mapping these connections, and now, neurotropic viruses are contributing to connectomics. In this work, two retrograde adeno-associated virus failed to transduce in projections from hypothalamic neurons to periaqueductal gray matter (PAG) but worked well in cortical connections to PAG. One of this virus also marked a substantial amount of PAG efferent projections, therefore working as an anterograde tracer. We also used hydroxystilbamidine (FluoroGold™) as a gold standard in retrograde tracing for comparison with the projections shown by the retrograde virus. As determined in past works, FluoroGold™ shows connections from the hypothalamus and cortex to the PAG. Also, an anterograde AAV was compared with one of the retrograde AAV, which showed a similar pattern of axonal projections and terminal fields. Hence, although neurotropic viruses are revolutionizing connectomics and other areas, their mechanism, neurotropism, and cell invasion need to be addressed. Their use is a great challenge and requires further studies to clarify their interaction with the nervous system's cells.

1. Introduction

The structural and functional units of the nervous system were defined long ago as "nerve cells" by the neuron doctrine, which was mainly achieved using techniques such as Golgi's method (Swanson, 2000; Guillery, 2005). This doctrine states that these functional units (now known as neurons) communicate at sites of cell-to-cell contact with a space between them, which were defined later as synapses (Guillery, 2005). Neuroscience has since confirmed most of this doctrine, showing that neurons connect to each other through neurites and synapses in a great number of functional circuits (Sun et al., 2019). The field of connectomics was born with the objective of mapping these circuits across

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the brain in different species, which are thought to underlie functions such as cognition, emotion, memory, sensation, and movement (Lanciego and Wouterlood, 2011; Saleeba et al., 2019; Sun et al., 2019).

As stated by Saleeba et al. (2019), conventional tracers can be stained by immunohistochemical, intrinsic fluorescence, or conjugation processing and visualized by light microscopy for use as retrograde or anterograde tracers. Starting around 1970, conventional tracers led to a revolution in neuroanatomy and an avalanche of data on unknown neural connections (Swanson, 2000). Basically, retrograde tracers deposited in the terminals of neurons are transported back to cell bodies (Lanciego and Wouterlood, 2011; Saleeba et al., 2019). One example of this class of tracer is hydroxystilbamidine (FluoroGold™), which is considered as a "gold-standard" tracer (Lanciego and Wouterlood, 2011; Saleeba et al., 2019) or "robust synthetic retrograde tracer" (Tervo et al., 2016). FluoroGold™ is taken up into nerve terminals through endocytosis and transported retrogradely to cell bodies (Saleeba et al., 2019).

Neurotropic viruses, such as adeno-associated viruses (AAVs), are currently leading to great advancements in neuroscience, not only as tracers in connectomics, but also in imaging neuronal activity, pharmacogenetics, and optogenetics (Sun et al., 2019). Used as tracers, neurotropic viruses have advantages compared to conventional tracers in terms of specificity since conventional tracers tag most of the neurons that they have contact with. Once cell types are defined by particular gene expression and other factors, specific cell populations can be targeted differently by neurotropic viruses and transgenic approaches with animal models. This enables the manipulation of specific neuronal populations defined by chemical identity or particular gene expression, which provides further understanding of circuits throughout the brain (Luo et al., 2018).

The hypothalamus is a necessary structure for survival and coordinates neuroendocrine, autonomic, and behavioral responses. The hypothalamic medial nuclei column is involved in defensive behaviors and is composed of the medial preoptic nucleus, anterior hypothalamic nucleus (AHN), descending division of the paraventricular nucleus, ventromedial nucleus (VMH), adjacent tuberal nucleus, dorsal (PMd), and the ventral pre-mammillary nucleus and mammillary body (Swanson, 2000).

The periaqueductal gray matter (PAG) is a mesencephalic structure that is subdivided in four columns known as dorsomedial (dmPAG), dorsolateral (dlPAG), lateral (lPAG) and ventrolateral (Bandler et al., 2000). Dorsal PAG (dPAG) stands as the union of dorsomedial and dorsolateral columns. Both receive inputs from different areas across encephalon, as medial prefrontal cortex (Franklin et al., 2017), zona incerta (ZI) (Chou et al., 2018), VMH (Canteras et al., 1994; Lindberg et al., 2013; Wang et al. 2015, 2019; Carvalho et al., 2020) and PMd (Canteras and Swanson, 1992; Motta et al., 2009, Wang et al., 2021a,b). The PAG is involved in a wide array of functions, including defensive behaviors (LeDoux, 2012; Motta et al., 2017). Defensive behaviors are processed in different structures and circuits when executed in response to a conspecific aggressor or a predator. When faced by a predator, rat's defensive behavior is coordinated by the dorsomedial portion of VMH and ventrolateral portion of PMd. Those subdivisions have anterograde projections to dlPAG and dmPAG activating both columns when faced by a predator. On the other hand, while threatened by a conspecific, ventrolateral portion of VMH and dorsomedial portion of PMd activates dmPAG and lPAG (Motta et al., 2009). Therefore, dmPAG and dlPAG receive dense projections from both VMH and PMd, known to be related to defensive behaviors (Canteras and Swanson, 1992; Canteras et al., 1994, 2015; Motta et al., 2009; Gross and Canteras 2012; Silva et al., 2013; Lindberg et al., 2013; Wang et al., 2015, 2019, 2021a,b; Carvalho et al., 2020).

The role of hypothalamic projections to dPAG in defensive behaviors against predators and conspecifics (Motta et al., 2009, 2017; Gross and Canteras 2012) is considerably studied. However, further detailing of such circuits, mainly regarding neural populations morphology and physiology, would greatly benefit from the application of properly functioning neurotropic viruses. Taking this into account, the present work examines the ability of AAV retrograde tracers to transduce known afferent connections to dPAG. It also examines whether retrograde AAV tracers also work as anterograde tracers.

2. Experimental procedures

2.1. Animals

Experiments were done using adult male C57BL/6 (n = 30) and Vglut^{CRE} mice (n = 8) at approximately 70–140 days of age at the time of surgery. Food and water were available ad libitum to the animals in light-controlled (07:00 AM to 07:00 PM) and temperature-controlled (24–26 °C) rooms. The animal housing and all experimental procedures were conducted according to the institutional guidelines of the National Council for Animal Experimentation Control and Animal Ethics Committee of the Institute of Biomedical Sciences (CEUA# 9782090821).

2.2. Retrograde and anterograde tracing experiments with FluoroGold™ or AAVs

To deposit FluoroGold™ (FG, n = 18, 9 of them were included in the analyses), C57BL/6 animals were anesthetized with Isoforine (Isoflurano, Cristália, 2.5–1.5%), and unilateral iontophoretic deposits of a 2% solution of FluoroGold™ (Fluorochrome Inc., Colo, USA) were placed stereotaxically into the dPAG (AP = −3.95 mm, ML = 0 mm, DV = −2.35 mm). Deposits were made over 5 min through a glass micropipette (tip diameter: 24 μm) by applying a +3-μA current pulsed at 7-second intervals with a constant current source (Midgard Electronics, Wood Dale, Ill, USA, model CS3). In the case of AAV injections, AAV5-hSyn-hChR2(H134R)-EYFP-WPRE-PA (anteroAAV, n = 8, C57BL/6), AAV Retro -EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA (retroAAV-1, n = 8, 5 of them were included in the analysis, Vglut^{CRE}) and AAV Retro-Ef1a-mCherry-IRES-Cre (retroAAV-2, n = 4, C57BL/6) (Addgene) were injected with a microsyringe (Hamilton 0.5 μL) placed stereotaxically into the dPAG (AP = -3.95 mm, ML = 0 mm, DV = -2.35 mm) using an automatic pump (Harvard Apparatus, pump11 elite), volume (7–15 nl). The mice used to describe retrograde AAV (VGLUT-DM-V32-7) and anterograde AAV (WT-DM-V5-7) had infusion volumes of 7 and 15 nl, respectively.

2.3. Tissue processing and cell counting

After survival times of 7 days for FluoroGold™ deposits and 30 days or more for AAV injections, the animals were deeply anesthetized with Isoforine and perfused transcardially with saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. After the perfusion, the encephalon was removed from the skull and left overnight in a solution of 20% sucrose in 0.1 M phosphate buffer at 4 °C. The brains were then frozen, four series of 40-μm-thick sections were cut on a sliding microtome in the transverse (frontal) plane, and sections were collected from the caudal brainstem through the rostral tip of the prefrontal cortex. One complete series was processed for each animal.

Immunohistochemistry analysis was performed for one series of animals that received FluoroGold™ (FG group) deposits using an antiserum directed against FluoroGold™ (Chemicon International, Calif, USA) at a dilution of 1:10,000. The antigen-antibody complex was localized with a variation of the avidin-biotin complex system (ABC) and a commercially available kit (ABC Elite Kit, Vector laboratories, Calif, USA). The sections were mounted on gelatin-coated slides, dehydrated, and covered with DPX. Sections were examined under a bright-field microscope. For visualization of viral retrograde and anterograde projections, sections were mounted on slides and visualized using a fluorescence microscope (Nikon Eclipse 80i). Figures were prepared using Inkscape (1.0.1). For neuroanatomical and Bregma referencing, The Mouse Brain atlas was used (Franklin and Paxinos 2007).

Retrogradely labeled cells were quantified in PMd, VMH and Cg2 for animals in retroAAV-1, retroAAV-2 and FG groups. ImageJ software (1.52a) were used to count neurons and calculate area, resulting in neuronal density by mm2. Data processing, visualization and statistical analyses were done using anaconda platform (conda 4.12.0) for python programming language (3.8.8) and libraries: pandas (1.2.4), numpy (1.20.1) matplotlib (3.3.4) and scipy (1.6.2). Some groups did not show normal distribution and groups were analyzed using Mann-Whitney test ($\alpha = 0.05$).

3. Results

3.1. retroAAV-1 and retroAAV-2 do not transduce efferent connections from hypothalamus to dPGA, but transduces from Cg2 to dPAG

Eight cases of FG, 5 cases of retroAAV-1 and 4 cases of retroAAV-2 were used to obtain the density of retrograde cells labelled after dPAG injections. Figure 1 shows a comparison between a FG case and a retroAAV-1 case as retrograde tracers deposited (1A) or injected (1E) in the dPAG (Bregma: −3.88 mm), and shows structures that contain retrograde labeled cells in PMd (Bregma: −2.54 mm), VMH (Bregma: −1.7 mm) and Cingulate Cortex Area 2 (Cg2, Bregma: 0.5mm). Fluoro-Gold™ deposits in the case WT-DM-FG-15 (FG group) showed a representative amount of retrogradely labeled cells in PMd (1B and 2B), VMH (1C and 2A), and Cg2 (1D). However, VGLUT-DM-V32-7 case (group retroAAV-1) showed few retrogradely labeled cells in PMd (1F) and VMH (1G) or none at all. Cg2 showed a considerable number of cells (1H). The comparison shows that FluoroGold™ retrogradely labeled neurons in VMH, PMd, and Cg2. retroAAV-1 failed to transduce neurons in VMH and PMd, but it succeeded in Cg2. Greater magnification of VMH and PMd retrogradely labeled neurons by FluoroGold™ is shown in Figure 2.

Density of retrogradely labeled cells were assessed in PMd, VMH and Cg2 in FG group compared to retroAAVs group (combining retroAAV-1 and retroAAV-2). Mann-Whitney test showed greater efficiency for retrograde labeling of Flurogold™ compared to retroAAVs in PMd [Figure 3; $U = 0$, $p = 0.00008$] and VMH [Figure 3; $U = 0$; $p = 0.0002$].

3.2. retroAAV-1 shows similar pattern of anterograde labeling compared to anteroAAV

Five cases of retroAAV-1 group were compared to 8 cases of anteroAAV in terms of efferent projections after dPAG injections. Figure 4 shows representative cases of retroAAV-1 and anteroAAV in comparison as anterograde tracers retroAAV-1 and anteroAAV injections (4A and 4E) show representative amounts of anterograde projections close to or within the subparafascicular thalamic nucleus (4B, 4F, Bregma: −2.3 mm), in ZI, Subl, DM, and a region close to those structures (4C, 4G,

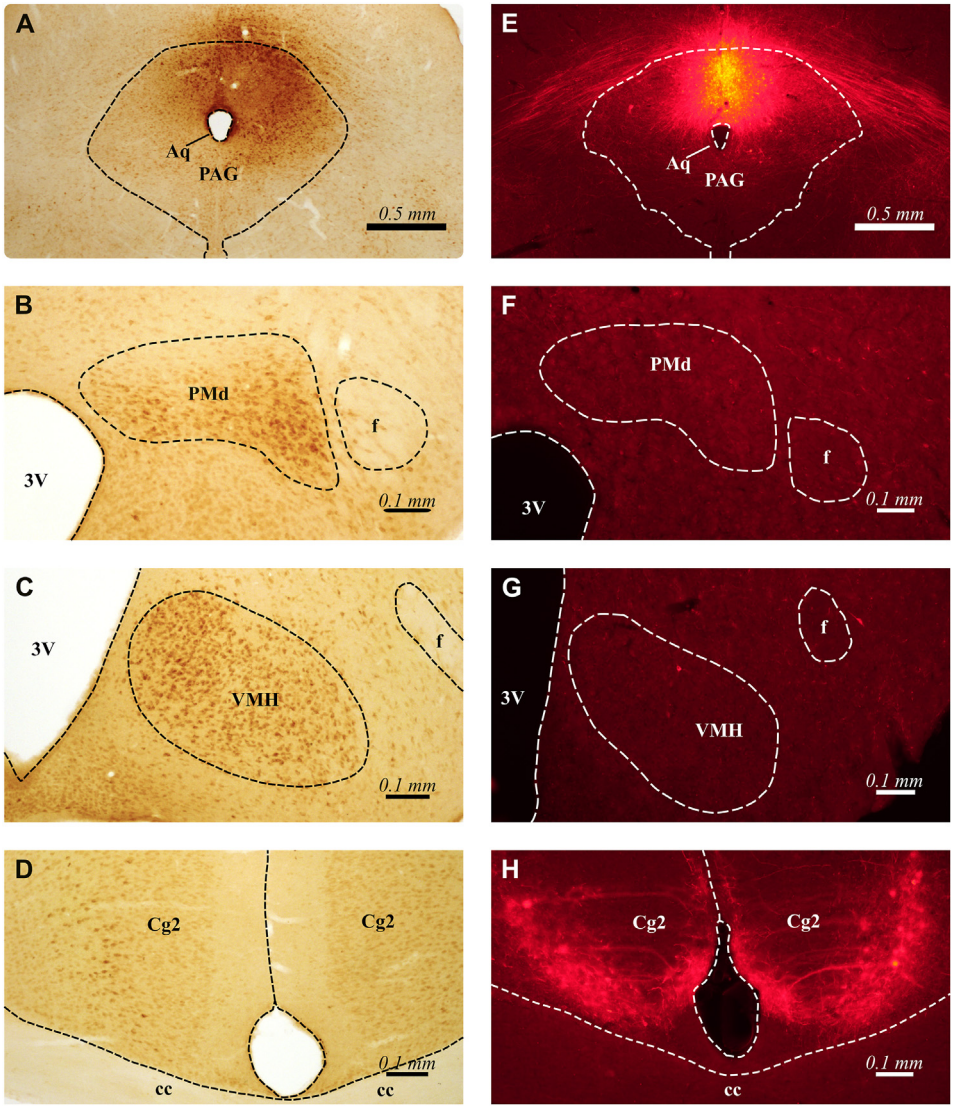

Figure 1. Photomicrograph of frontal sections of retrogradely FluoroGold-positive cells (A, B, C, D) and retroAAV-1 retrogradely positive cells (E, F, G, H), after dPAG injection (A, E, Bregma: −3.88 mm). Retrogradely stained cells are shown in PMd (B, F, Bregma: −2.54 mm), VMH (C, G, Bregma: −1.7 mm) and Cg2 (D, H, Bregma: 0.5 mm). Abbreviations: Aq (aqueduct), PAG (peri-aqueductal gray matter), 3V (3rd Ventricle), PMd (premammilary nucleus dorsal part), f (fornix), VMH (ventromedial hypothalamic nucleus), Cg2 (cingulate Cortex Area 2), cc (corpus callosum).

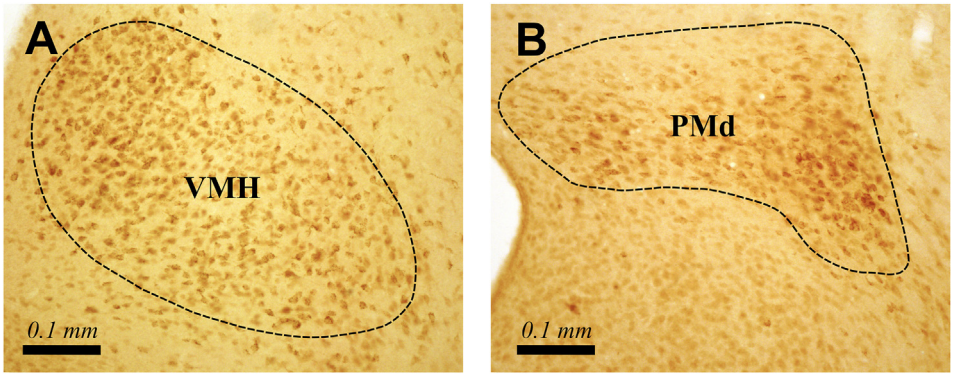

Figure 2. Photomicrograph of frontal sections of retrogradely labeled FluoroGold-positive cells deposited in dPAG. Retrogradely stained cells are shown in VMH (A, Bregma: −1.7 mm) and PMd (B, Bregma: −2.54 mm).

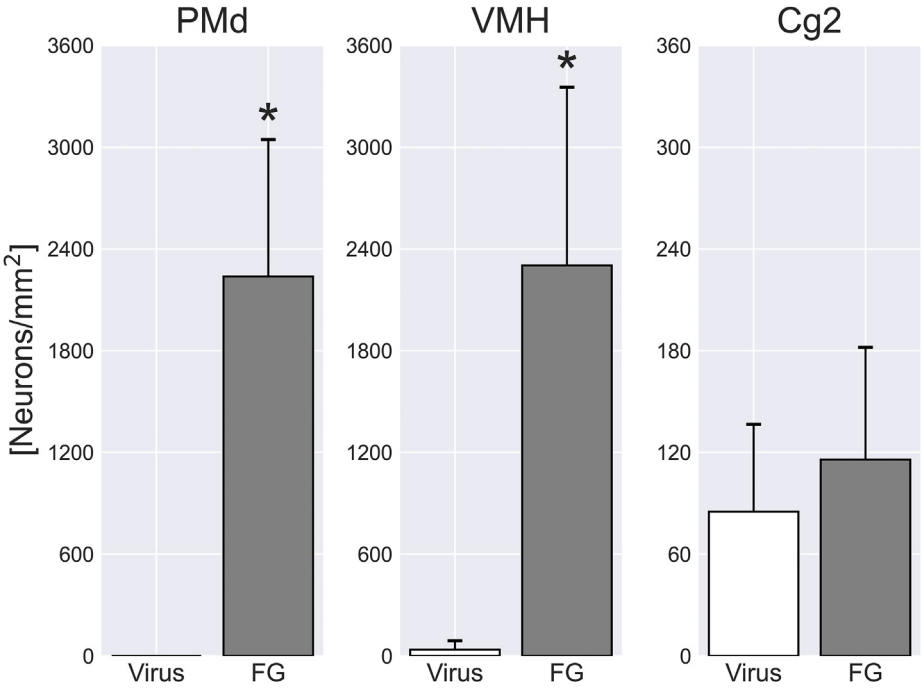

Figure 3. Neuronal density of retrogradely labeled cells after dPAG injection of retroAAVs (retroAAV-1 and retroAAV-2) or Fluorogold™ (FG). Data expressed as mean ± SD in neurons/mm^2. Mann-Whitney test. * = $p < 0.05$ compared to the Virus group (retro-AAV1 and retro-AAV2) in the same structure. Virus (n = 9) and FG (n = 9).

Bregma: −1.46 mm), and in ZI, AHN, and hypothalamic area (4D, 4H, Bregma: −1.22 mm). As a result, retroAAV-1 shows a similar pattern to an anterograde neurotropic viral tracer. It is important to highlight that Figures 1 and 4 show the same animal (VGLUT-DM-V32-7) for retroAAV1 group but different brain areas. The retroAAV-2 group did not show patterns of anterograde projections.

4. Discussion

In neuroscience, the last decade has been marked by many diverse uses of neurotropic viruses. Such tools have been used to trace, manipulate, and note neurons and their physiology, expanding the boundaries of knowledge in neuroscience. However, the use of neurotropic viruses needs attention and may have limitations, particularly when used to describe neuronal projections.

The results presented here indicate the presence of efferent connections from VMH, PMd, and Cg2 to the dPAG in mice. These projections were previously described using techniques with *Phaseolus vulgaris* leucoagglutinin, a conventional anterograde tracer in rats (Canteras and Swanson, 1992; Canteras et al., 1994; Motta et al., 2009) or using anterograde neurotropic tracers from hypothalamic nuclei to dPAG (Silva et al., 2013; Lindberg et al., 2013; Wang et al. 2015, 2019, 2021a,b; Carvalho et al., 2020). Nevertheless, mice injected with retroAAV-1 or retroAAV-2 in dPAG did not present positive cells in VMH and PMd, but they showed positive cells in Cg2. RetroAAV-1 was also able to show a significant amount of efferent dPAG projections, behaving not only as a retrograde tracer but also as an anterograde tracer.

It is important to highlight that injection sites may vary slightly between cases. Sometimes centralized in dmPAG or dlPAG and never restricted to one solely column. Both dmPAG and dlPAG receive dense inputs from PMd and VMH. Differences in the injection site would not explain the difference in our results, especially taking into account that this variation of injection site also exists in the FG group. When analyzing anterograde patterns, differences may exist between dmPAG and dlPAG, but results are focused on their common anterograde projections.

Some hypotheses can explain why hypothalamic neural connections to the dPAG fail to be transduced and why cortical neural connections to the dPAG work well. The main reason could be the technique used to

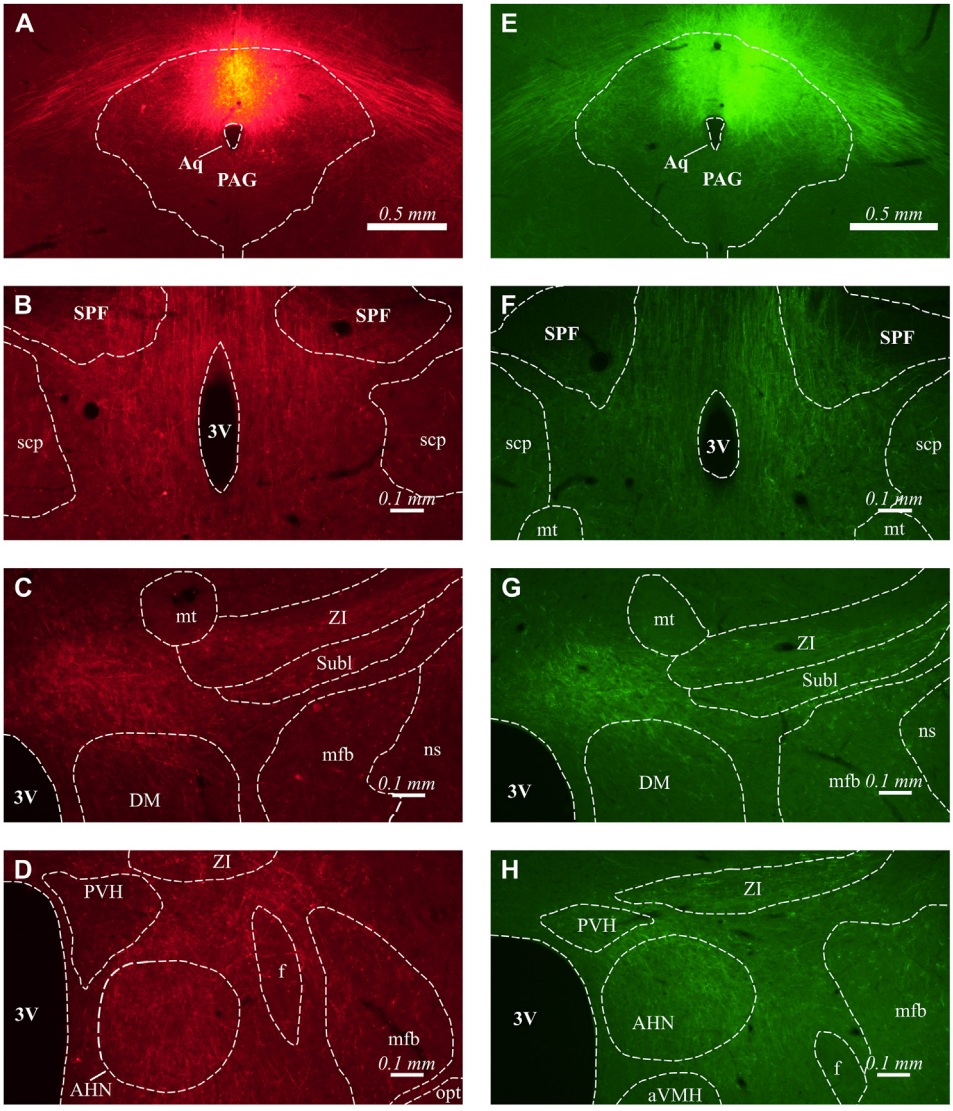

Figure 4. Photomicrograph of frontal sections with terminal fields or axonal projections from retroAAV1 positive cells (A, B, C, D) and from anteroAAV-positive cells (E, F, G, H) both injected in dPAG (A, E). Anterograde projections are shown in a region close to SPF (B, F), close to ZI and DM (C, G), and in AHN (D,H). Abbreviations: Aq (aqueduct), PAG (periaqueductal gray matter), 3V (3rd ventricle), SPF (subparafascicular thalamic nucleus), scp (superior cerebellar peduncle), mt (mammillothalamic tract), ZI (zona incerta), DM (dorsomedial hypothalamic nucleus), Subl (subincertal nucleus), mfb (medial forebrain bundle), f (fornix), ns (nigrostriatal tract), PVH (paraventricular hypothalamic nucleus), AHN (anterior hypothalamic area), opt (optic tract).

design the AAV. A common technique applied for the development of new viral vectors is directed evolution (Kotterman and Schaffer, 2014; Tervo et al., 2016). In this technique, libraries of AAV variants are generated by random processes that increase diversification, and these variants are subjected to selection processes to accumulate beneficial mutations and improve a desired function (Kotterman and Schaffer, 2014).

In the context of engineering new AAVs to study of connectomics and functionality of the encephalon, directed evolution is done using described libraries of AAVs that are subjected to diverse processes to increase diversity. These AAV variants are injected in some structures of the encephalon, and after the transduction period of the AAV, another encephalon structure that sends projections to the injected structure is collected to extract the AAVs for the selection of a new AAV retrograde tracer (Tervo et al., 2016). This process involves an effect of randomness and selection of successful cases without a deep evaluation on how these selected AAVs work differently from the past ones.

In nervous systems, neural populations have considerable differences in cell type. These include the cells' body location, dendritic morphology, axonal projection, physiological characteristics, developmental history, gene expression pattern, and function (Luo et al., 2018). Selection of neurotropic viruses through directed evolution based on a given neural population does not guarantee efficiency to all other neural populations among the great diversity of cell types in the brain. For example, Tervo et al. (2016) used only two independent populations of projections for the selection of new AAV variants in directed evolution from the substantia nigra pars reticulata and hindbrain neurons to the cerebellar cortex. They tested the new AAV in areas ranging from the basal pontine nuclei to layer V in the cortex. Albeit testing the efficiency of the new AAV variant in different structures, Tervo et al. (2016) observed that some projections presented weak or very weak efficiency.

In the same direction, Sun et al. (2019) explored differences in neurotropism of retro adeno-associated virus (rAAV) and glycoprotein-deleted rabies virus (RV-ΔG). They showed that injections of retrograde tracer in the lateral hypothalamic area (LHA) and medial pre-optic area preferentially label neurons in the cerebral cortex for rAAV and preferentially label neurons in the basal ganglia and hypothalamus for RV-ΔG. In the case of rAAV, the connections of LHA to the paraventricular nucleus of the hypothalamus and of LHA to the nucleus accumbens completely fail to tag retrograde neurons. Another hypothesis was explained by Nathanson et al. (2009), who found that in cortical neurons, a difference in dilution of the same viral vector (AAV) changes the bias of transduction in glutamatergic or gabaergic neurons. This shows a possible preference of transduction for gabaergic instead of glutamatergic neurons in the same structure for the used AAV.

The present results also showed that the commercially available retroAAV-1 that was used may work as an anterograde tracer. The retroAAV-1 tracer showed axonal projections in the thalamus and hypothalamus and terminal fields in the ZI and AHN between other

structures and areas. The same pattern was also observed using a commercially available anterograde AAV. One possible explanation for the retroAAV-1 tracer working as an anterograde tracer could be that AAV2 is naturally transported anterogradely (Salegio et al., 2013).

The rAAV-2 developed by Tervo et al. (2016) was artificially selected through directed evolution to perform retrograde transport, but it is still an AAV2. Therefore, one explanation is that the new variant developed still has the anterograde-transporting feature plus a new ability to infect terminal axons and to be transported retrogradely. Thus, it may work as an anterograde and retrograde tracer at the same time, as observed with retroAAV-1. This dubious functioning may create many misunderstandings and malfunctioning not only when using it as a tracer, but also when using it with functional techniques. In addition, dubious transport could generate second-order anterograde projections from a retrograde transduced structure from the injection site.

5. Conclusion

Neurotropic viruses used as genetic delivery systems are currently revolutionizing connectomics and brain physiology. In the case of AAVs, however, their mechanism, cell invasion, and neurotropism still remain poorly understood (Salegio et al., 2013; Sun et al., 2019). Future studies should provide greater detail about this important tool in neuroscience. When using neurotropic viruses for neural tracing, it is important to have a positive control, such as conventional tracers.

Declarations

Author contribution statement

Bortoloci, J.G.T: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Motta, S.C: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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