A viral toolbox for conditional and transneuronal gene expression in zebrafish

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Abstract:

Zebrafish are an important model in systems neuroscience but a key limitation is the lack of viral tools to interrogate the structure and function of neuronal circuitry. We developed methods for efficient gene transfer and retrograde tracing by herpes simplex viruses (HSV1), for conditional HSV1-mediated gene expression using the Gal4/UAS system, and for transneuronal tracing by rabies viruses. This toolbox creates new opportunities to visualize and manipulate specific sets of neurons through the combination of genetic and viral approaches in zebrafish.

Introduction

The zebrafish is an important vertebrate model in systems neuroscience because its small, optically accessible brain provides unique opportunities to study neuronal circuits and behavior^{1,2}. Key methods established in zebrafish include large-scale imaging of neuronal population activity, behavioral approaches including virtual realities, and genetic manipulations¹⁻⁸, but efficient methods for viral gene transfer are still lacking. Viral vectors are important experimental tools in mammals because they enable the visualization and manipulation of defined neurons with high spatial, temporal and molecular specificity, and because they can bypass the need to generate transgenic animals^{9,10}. Moreover, engineered viral tools that cross synapses allow for the visualization and physiological analysis of synaptically connected neuronal cohorts¹¹⁻¹³. Our goal was to establish similar methods for specific viral gene transfer and transneuronal tracing in zebrafish.

Results

Previous studies demonstrated that adeno-associated viruses (AAVs), which are widely used in mammals and other amniotes, fail to infect neurons in the zebrafish brain¹⁴. Infection of zebrafish neurons by other viruses has been reported but efficiency was usually low and viral vectors for conditional gene expression in transgenic fish have not been described. To improve upon these points, we first focused on herpes simplex virus 1 (HSV1), a DNA virus that can infect zebrafish neurons both locally and retrogradely via projecting axons without obvious signs of cytotoxicity¹⁵. We first explored whether HSV1-mediated gene transfer can be further improved by optimizing the temperature regime. Zebrafish are usually kept at 26 – 28.5 °C but the temperature range of natural habitats is broad (up to >38 °C) and temperature tolerance in the laboratory extends up to ~41 °C^{16,17}. We therefore tested whether viral gene expression is more efficient at temperatures similar to those of mammalian hosts (37 °C).

We injected an amplicon type of HSV1 with an insert encoding the red fluorescent protein DsRed under a non-specific promoter for long-term expression (HSV1[*LTCMV*:DsRed]) unilaterally into the olfactory bulb (OB) of adult zebrafish expressing green fluorescent protein (GFP) under the *vglut1* promoter (Tg[*vglut1*:GFP]). Fish were then maintained at 26 °C or 36 °C for 6 days after injection (Fig. 1a). Consistent with previous results¹⁵, reporter expression (DsRed) was observed in the injected OB (Fig. S1a). Moreover, retrogradely labeled neurons were present in telencephalic areas that project to the OB, most notably the posterior zone of the dorsal telencephalon (Dp), the homolog of mammalian olfactory cortex (Fig. S1a). After incubation at 36 °C, substantially more neurons expressed DsRed (Fig. 1b). In addition, retrograde labeling was detected in a small but distinct population of neurons in the dorsal telencephalon that was not seen at 26 °C (Fig. 1b, c). DsRed expression at 36 °C was first detected ~12 hours after injection and stable for at least 10 days (Fig. S1b). Moreover, increasing the temperature from 26 °C to 36 °C three days after the injection failed to enhance expression (Fig. 1a, c). These results show that adjusting the temperature to that of mammalian hosts can substantially increase the efficiency of HSV1-mediated viral gene transfer in zebrafish.

Viral vectors are often combined with transgenic lines to target specific cell types using two-component expression systems (e.g., injection of a Cre-dependent viral construct into Cre-expressing mice). In zebrafish, the most widely used two-component expression system is the Gal4/UAS system. We therefore explored the possibility to combine viral delivery of UAS-dependent expression constructs with Gal4-expressing driver lines. We first created a Gateway expression vector¹⁸ to simplify the construction step of HSV1 for UAS-dependent expression (Fig. 1d). We then generated HSV1[*UAS*:TVA-mCherry] to express a fusion of the transmembrane protein TVA and the red fluorescent protein mCherry under UAS control. This virus did not drive expression when injected into the brain of wt zebrafish (n = 3; not shown).

We injected HSV1[*UAS*:TVA-mCherry] into the cerebellum of Tg[*gad1b*:GFP; *gad1b*:Gal4] fish¹⁹, which express Gal4 and GFP under the control of the *gad1b* promoter, a marker of GABAergic neurons. Consistent with the distribution of GABAergic neurons in the cerebellum, GFP was expressed in Purkinje neurons and in a sparse neuronal population in the granular layer, presumably Golgi cells. mCherry was co-expressed with GFP in a subset of Purkinje neurons and putative Golgi cells (97.7 ± 1.2 % of mCherry-positive neurons were also GFP-positive; mean ± s.d.; n = 4 fish; Fig. 1e and f). No expression was detected in the dense population of GFP-negative granule cells in the granular layer. Co-expression of mCherry and GFP was also observed when the injection of HSV1[*UAS*:TVA-mCherry] was directed at a cluster of GABAergic neurons near Dp in Tg[*gad1b*:GFP; *gad1b*:Gal4] fish (94.8 ± 4.5 % of mCherry+ neurons coexpressed GFP; mean ± s.d.; n = 5 fish; Fig. 1g, Fig. S2). Interestingly, mCherry-expressing

neurites arborized extensively in the lateral pallium but spared a region of Dp immediately adjacent to the *gad1b*-positive cluster. These observations indicate that the dorsal telencephalon contains specific long-range projections of GABAergic neurons (Fig. S2a).

In additional experiments we first injected HSV1[*LTCMV*:Gal4] into the OB of wt zebrafish and subsequently injected HSV1[*UAS*:GFP] into the dorsal telencephalon. Expression of GFP was observed specifically in OB-projecting neurons of the dorsal telencephalon without expression elsewhere (Fig. 1g). To assess the efficiency of this intersectional targeting approach we compared the number of labeled neurons using either the Gal4/UAS system or the one-component approach (injection of HSV1[*LTCMV*:DsRed] into OB). Both approaches yielded similar numbers of labeled neurons in the dorsal telencephalon (Fig. 1g). We therefore conclude that HSV1 can be combined with the Gal4/UAS system in intersectional approaches with high efficiency and low leakiness.

We next explored strategies for co-expression of multiple transgenes in the same neurons. When red and green reporter constructs were co-packaged into the same virus (HSV1[*UAS*:TVA-mCherry & *UAS*:GFP]), injection into the cerebellum of Tg[*gad1b*:Gal4] resulted in a high rate of co-expression, as expected (89.5 ± 8.7 % co-expression; mean $\pm$ s.d.; $n = 3$ fish; Fig. S3a, c). Interestingly, co-injection of the same responder constructs packaged into separate viruses (HSV1[*UAS*:TVA-mCherry] and HSV1[*UAS*:GFP]) produced a similar rate of co-expression, even when overall expression was sparse (84.3 ± 6.0 % co-expression; mean $\pm$ s.d.; $n = 3$ fish; Fig. S3b, c). These results indicate that co-injection of viruses can be used to co-express multiple transgenes in the same neurons.

In rodents, modified rabies viruses have become important tools to analyze connectivity and structure-function relationships in neuronal circuits. These vectors can infect specific “starter” neurons and are transmitted to presynaptic neurons across one synapse. Specific infection is achieved by expressing the receptor protein TVA in the starter neuron and pseudotyping the virus with the envelope protein EnvA¹¹. To limit retrograde transfer to one synapse, an essential glycoprotein (G) is deleted from the viral genome and supplied in *trans* only in the starter neurons. In zebrafish, the G-deleted rabies virus (RVΔG) has been reported to infect neurons¹⁴ and to cross synapses when complemented with G but the efficiency of retrograde synaptic transfer appeared very low²⁰.

To enhance trans-neuronal tracing efficiency we first explored the effect of temperature. When we injected EnvA-coated RVΔG expressing GFP (EnvA-RVΔG-GFP) into the telencephalon of wt fish at 36°C, no GFP expression was observed (not shown). We then injected EnvA-RVΔG-GFP into the telencephalon of Tg[*gad1b*:Gal4; *UAS*:TVA-mCherry] fish that express TVA-mCherry in GABAergic neurons. After 6 days, efficient and selective infection of neurons expressing TVA-mCherry was

observed at 36°C but not at 26°C, nor when the temperature was raised from 26°C to 36°C three days post injection (Fig. S4). Hence, infection of zebrafish neurons by EnvA-RVΔG-GFP required TVA and was substantially more efficient when the temperature was close to the body temperature of the natural host.

Efficient trans-neuronal spread of the rabies virus depends on the expression level of the glycoprotein in starter cells^{21,22}. We therefore took two steps to enhance glycoprotein expression. First, we optimized codon usage for zebrafish. Second, we expressed TVA and the codon-optimized glycoprotein (zoSADG) using HSV1 because viral vectors typically reach higher expression levels than transgenics¹⁵. In rodents, starter neurons expressing high levels of G often disappear in parallel with the emergence of transneuronal expression, presumably because long-term expression of G is toxic^{23,24}. We therefore determined the time course of transgene expression under different experimental conditions. We first focused on the cerebellum where GABAergic Purkinje neurons receive local synaptic input from different types of cerebellar neurons and long-range input from neurons in the contralateral inferior olive (climbing fibers; Fig. 2a). We injected a mixture of HSV1[*UAS:TVA-mCherry*] and EnvA-RVΔG-GFP w into the cerebellum of Tg[*gad1b:Gal4*] fish and examined expression for up to 10 days. As observed before (Fig. 1e), mCherry expression was localized to Purkinje neurons and putative Golgi cells. Neurons expressing GFP were concentrated around the injection site and usually co-expressed mCherry (Fig 2b, d). Rarely, GFP expression was observed in mCherry-negative neurons (Fig. 2d), which may indicate TVA-independent viral entry. However, as no GFP expression was observed when EnvA-RVΔG-GFP was injected into wt animals, it appears more likely that GFP+/mCherry- neurons initially expressed TVA but subsequently lost expression due to the slight decline of expression driven by the viral promoter (Fig. S1b). Co-expression of mCherry and GFP was stable for at least 10 days after injection and neurons expressing GFP only remained very rare (Fig. 2d). These results confirm the specificity of EnvA-RVΔG-GFP infection and show that expression of TVA-mCherry or the infection of rabies virus alone are not toxic in the absence of glycoprotein. No expression of GFP was observed in the inferior olive, consistent with the expectation that the RVΔG does not spread in the absence of glycoprotein.

To examine whether the delivery of glycoprotein to starter cells can drive trans-neuronal spread, we injected a mixture of HSV1[*UAS:TVA-mCherry*], HSV1[*UAS:zoSADG*] and EnvA-RVΔG-GFP into the cerebellum of Tg[*gad1b:Gal4*] fish. Unlike in the absence of glycoprotein, the number of mCherry-expressing Purkinje neurons and putative Golgi cells now declined over the incubation period of 10 days. The number of neurons expressing GFP, in contrast, was low initially but increased steeply between 6 and 10 days after injection. In the cerebellum, GFP was expressed in Purkinje cells and throughout the granular layer (Fig. 2c). The number and spatial distribution of GFP-expressing neurons in the granular layer was clearly different from mCherry expression in the absence of glycoprotein (Tg[*gad1b:Gal4*] fish

injected with HSV1[*UAS:TVA-mCherry*]; Fig. 1e, 2b), indicating that GFP was expressed in granule cells. Moreover, GFP-positive neurons were found in the contralateral inferior olive (Fig. 2c). We therefore conclude that *trans*-complementation with zoSADG in starter cells promoted transneuronal spread of the rabies virus.

Transneuronal viral transfer for synaptic inputs to Purkinje neurons from the inferior olive was quantified by a convergence index established previously in rodents^{22,25}. This index is defined as the number of transneuronally labeled neurons (here: GFP+/mCherry- neurons in the inferior olive) normalized to the number of starter cells (here: GFP+/mCherry+ Purkinje neurons) in each individual. The clear spatial separation of starter neurons from transneuronally labeled neurons minimizes potential counting errors. The convergence index was 1.00 ± 0.95 (mean $\pm$ s.d.; $n = 4$ fish; Fig. 2e), which is comparable to values reported in mammals²².

To corroborate these results we also examined transneuronal tracing from starter neurons in telencephalic area Dp. In this area, both glutamatergic principal neurons and GABAergic interneurons receive long-range input from mitral cells in the OB (Fig. 2f). In addition, Dp receives input from other telencephalic areas that is not well characterized. When a mixture of HSV1[*UAS:TVA-mCherry*] and EnvA-RVΔG-GFP was injected into Tg[*gad1b:Gal4*] fish (Fig. 2g), GFP expression was largely restricted to a cluster of neurons that co-expressed mCherry (Fig. 2g). This cluster was located near a prominent furrow that separates an anterior from a posterior compartment of Dp, consistent with the known location of GABAergic neurons in Dp¹⁹. When HSV1[*UAS:zoSADG*] was added to the injected virus mixture, mCherry expression became rare while a large number of neurons in different telencephalic areas expressed GFP (Fig. 2h, i). Moreover, GFP was expressed in the olfactory bulb by neurons with the characteristic morphology of mitral cells, but not by other cells (Fig. 2h). Similar observations were made when the infection was targeted to glutamatergic starter neurons by injecting the cocktail of viruses into Dp of Tg[*vglut1:Gal4*] fish (Fig. S6. Note that *vglut1* is expressed in excitatory neurons in the telencephalon but not in the OB (Fig. S5), implying that labeling of OB neurons cannot be explained by direct infection. Convergence indices for projections of mitral cells to GABAergic or glutamatergic starter neurons in Dp were 0.70 ± 0.21 and 1.31 ± 1.20 (mean $\pm$ s.d.; $n = 5$ and $n = 4$ fish, respectively; Fig. 2j). These results confirm that EnvA-RVΔG selectively infects TVA-expressing target neurons and undergoes monosynaptic retrograde transneuronal transfer when complemented with zoSADG.

Conclusions

In summary, we found that viral infection and transgene expression in zebrafish can be significantly enhanced by adjusting the temperature to that of the natural viral host. HSV1 vectors can be combined with the Gal4/UAS system for intersectional gene expression without obvious leakiness. Retrograde HSV1-mediated delivery of Gal4 and subsequent HSV1-mediated delivery of a UAS responder can be used to target projection neurons. Conceivably, viral delivery of UAS responders may also be useful to overcome the slow loss of expression observed in some Gal4/UAS lines over generations because it bypasses epigenetic silencing of the highly repetitive UAS elements^{26,27}. HSV1-based viral tools thus provide novel opportunities to target narrowly defined types of neurons in zebrafish by combining the genetic specificity of promoters and the Gal4/UAS system with the spatial and temporal precision of injections. Such approaches can immediately capitalize on the broad spectrum of Gal4 driver lines that has been created using defined promoters and random insertion strategies^{28–30}. The development of a viral toolbox for conditional gene expression thus enables experimental approaches in zebrafish that have previously been restricted mainly to rodents.

We further established procedures for retrograde monosynaptic tracing using rabies viruses that greatly enhanced the efficiency of transneuronal transfer compared to a previous study in zebrafish²⁰. Temperature adjustments were important but unlikely to be the only factor underlying the enhanced transneuronal spread because the temperature difference to the previous study was only 1.5 °C²⁰. We therefore assume that increased glycoprotein expression by codon optimization and viral gene delivery also contributed significantly. Moreover, efficiency may be affected by the relative timing of glycoprotein expression and rabies virus infection. Convergence ratios were similar to those observed in rodents despite the fact that zebrafish neurons are smaller and probably receive fewer synaptic inputs. These results suggest that the efficiency of transneuronal tracing was in the same range as in rodents although quantitative comparisons are difficult. In the future, further improvements may be achieved by modifications that are currently explored in rodents such as the design of optimized glycoproteins²² or trans-complementation with a second viral gene³¹. The apparent long-term toxicity of the glycoprotein may be circumvented by transient expression using a negative feedback system³². Hence, rabies-based transneuronal tracers are promising tools for the structural and functional dissection of neuronal circuitry in zebrafish.

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Author contributions

C.S. developed the methodology, designed and performed experiments, analyzed data and wrote the manuscript. R.L.N. produced HSV1. H.K.O. produced rabies virus. E.A.B. helped molecular biology to establish HSV1[*UAS:GFP*], M.T. helped to establish rabies virus tracing in zebrafish. S.H. created transgenic fish. G.B.K. supervised the project. R.W.F. supervised the project, and wrote the manuscript.

Competing interests

The authors declare no competing interests.

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All HSV1 viruses were produced by Gene Delivery Technology Core in the Massachusetts General Hospital (<https://researchcores.partners.org/mvvc/about>). Rabies virus (EnvA-RVΔG-GFP) was produced by FMI viral core.

Virus injection

Virus injection was performed as described¹⁵ with minor modifications. Fish were anesthetized in 0.03% tricaine methanesulfonate (MS-222) and placed under a dissection microscope. MS-222 (0.01%) was continuously delivered into the mouth through a small cannula. A small craniotomy was made over the dorsal telencephalon near the midline, over the OBs, or over the cerebellum. If multiple viruses were injected in the same region, virus suspensions were mixed. Phenol red (0.05 %) was added to the suspension to visualize the injection. Micropipettes pulled from borosilicate glass were inserted vertically through the craniotomy. The depth of injections was approximately 100 μm in the dorsal telencephalon, 50 μm in the OB, and 100-150 μm in the cerebellum, 300-500 μm in Dp. The injected volume was 50 nl – 100 nl. After surgery, fish were kept in standard holding tanks at 35 °C – 37 °C unless noted otherwise.

Clearing of brain samples

We adapted the original Cubic protocol³⁶ to small samples such as adult zebrafish brains. After fixation with 4% paraformaldehyde overnight, samples were soaked with reagent 1A (10%w/v Triton, 5wt% NNNN-tetrakis (2HP) ethylenediamine and 10%w/v urea) for 2.5 h at room temperature and for 6 h at 37 °C with mild shaking and multiple solution exchanges. Subsequently, samples were washed in PBS overnight at room temperature. On the next day, samples were treated with reagent 2 (25% w/v urea, 50% w/v sucrose and 10% w/v triethanolamine) for refractive index matching, mounted in glass bottom dishes, and covered by 16 x 16 mm cover glasses to avoid drift. Images were acquired using a Zeiss 10x water-immersion objective lens (N.A. = 0.45) on an upright Zeiss LSM 700 confocal microscope.

Immunohistochemistry

For rabies virus tracing of the cerebellum circuit, GFP and mCherry signals were detected by immunocytochemistry. Brain samples were fixed in 4% paraformaldehyde overnight and sectioned (100 μm) on a Leica VT1000 vibratome. Primary antibodies were anti-GFP (ThermoFisher, A10262, 1:200) and anti-RFP (5F8, chromotek, 1:200). Secondary antibodies were conjugated to Alexa Fluor 488 or 594 (Invitrogen, 1:200).

Neuron counts

For whole brain imaging (Fig1c, 1g and S1b), z stacks (7-10 μm steps) were acquired using an upright Zeiss LSM 700 confocal microscope with a 10x objective (water, N.A. = 0.45, pixel size 1.25 μm). GFP or DsRed-expressing neurons in the dorsal telencephalon were counted manually.

Z stacks from individual brain slices (1 – 2 μm steps, Fig1f, 2d, 2i, 2j, S1 and S3c) were acquired using an upright Zeiss LSM 700 confocal microscope with a 20x objective (air, N.A. = 0.8, pixel size 0.625 μm). For non-rabies injections, a single slice, containing the largest number of labelled neurons, was chosen

366 from each brain sample for cell counting. For rabies injections, all slices were used for cell counting.
 367 Cells expressing GFP and/or mCherry in specific regions (cerebellum, telencephalon, olfactory bulb)
 368 were counted manually.

369

370 **Data availability**

371 The data that support the findings of this study are available from the corresponding authors upon
 372 reasonable request.

373 **Code availability**

374 All code used in this study is available from the corresponding authors upon reasonable request.

Figure Legends

Fig 1. HSV1-mediated gene delivery in adult zebrafish and the Gal4/UAS system

(a) Procedure for testing temperature-dependence of HSV1-mediated gene expression.

(b) Maximum intensity projection after injection of HSV1[*LTCMV*:DsRed] into one olfactory bulb (magenta arrow) of a Tg[*vglut1*:GFP] fish and incubation at 36°C. White arrowhead indicates the OB-projecting area in the dorsal telencephalon used for quantification in (c) and (g). *vglut1*:GFP expression served as a morphological marker.

(c) Mean number of labeled neurons in the dorsal telencephalon after injection of HSV1[*LTCMV*:DsRed] into the ipsilateral olfactory bulb and incubation at different temperatures. In this and similar plots, black dots represent data from individual fish, box plot indicates median and 25% and 75% percentiles, circles and error bars indicate mean and s.d. over individual fish. n: number of fish.

(d) Construction of the UAS vector for HSV packaging. Genes of interest (GOI) are inserted downstream of the 5xUAS sequences by recombination cloning using the Gateway system. The transcriptional blocker minimizes leaky expression³⁷.

(e) Injection of HSV1[*UAS*:TVA-mCherry] into the cerebellum of Tg[*gad1b*:Gal4; *gad1b*:GFP] double transgenic fish. Note co-localization of mCherry and GFP in Purkinje cells (arrowheads) and putative Golgi cells (arrows). ML: molecular layer; PL: Purkinje layer; GL: granular layer.

(f) Fraction of mCherry-positive neurons that co-expressed GFP after injection of HSV1[*UAS*:TVA-mCherry] into the cerebellum or Dp of Tg[*gad1b*:Gal4; *gad1b*:GFP] double transgenic fish. n: number of fish.

(g) Injection of HSV1[*LTCMV*:Gal4] into the olfactory bulb and HSV1[*UAS*:GFP] into the dorsal telencephalon of wt fish. Note selective expression of GFP in OB-projecting neurons (arrowhead; dashed outline). Number of neurons labeled in the dorsal telencephalon by a single injection of HSV1[*LTCMV*:DsRed] (a) into the OB (one-way), or by two injections using the two-component Gal4/UAS system (g). n: number of fish.

Fig 2. Transneuronal tracing using pseudotyped rabies virus in adult zebrafish

(a) Schematic of the cerebellar circuit. Glutamatergic neurons are shown in red colors, GABAergic neurons in blue colors. Purkinje cells receive extra-cerebellar input exclusively from the inferior olive.

(b) Co-injection of EnvA-RVΔG-GFP and HSV1[*UAS*:TVA-mCherry] into the cerebellum of Tg[*gad1b*:Gal4] fish in the absence of glycoprotein. Left: schematic. Center: expression of TVA-mCherry (magenta) and GFP (green) in the cerebellum. Regions in the Purkinje and granular layers (dashed rectangles) are enlarged. Right: expression of GFP in the hindbrain. Region covering the inferior olive (dashed rectangle) is enlarged. Expression of GFP was restricted to putative starter neurons. No expression was detected in the inferior olive. ML: molecular layer; PL: Purkinje layer; GL: granular layer.

- (c) Same as in (b) but the glycoprotein (zoSADG) supplied to starter neurons in *trans* by co-injection of HSV1[*UAS:zoSADG*]. Note expression of GFP in putative granule cells and in neurons of the inferior olive, indicating transneuronal spread.
- (d) Number of neurons that expressed GFP and mCherry (putative starter neurons) or GFP alone (putative presynaptic neurons) at different time points after injection of EnvA-RVΔG and HSV1[*UAS:TVA-mCherry*] into the cerebellum of Tg[*gad1b:Gal4*] fish. Left: without glycoprotein; right: with trans-complementation of zoSADG in starter neurons. Note that labeling of putative presynaptic neurons emerged between 6 and 10 days post injection only when zoSADG was trans-complemented in starter neurons. See the method for details. In all plots, black dots represent data from individual fish, box plot indicates median and the 25% and 75% percentiles, circles and error bars indicate mean and s.d. over individual fish. n: number of fish.
- (e) Convergence index for the projection from the inferior olive to the cerebellum at different time points. The convergence index is the numerical ratio of transneuronally labeled neurons (GFP+/mCherry- neurons in the inferior olive) and putative starter cells in the cerebellum (GFP+/mCherry+ Purkinje neurons). n: number of fish.
- (f) Schematic of the circuitry in telencephalic area Dp. Glutamatergic neurons are shown in red colors, GABAergic neurons in blue colors. Long-range projections from mitral cells in the olfactory bulb terminate on glutamatergic neurons and on GABAergic interneurons in Dp. Additional long-range projections originate in other telencephalic areas.
- (g) Co-injection of EnvA-RVΔG and HSV1[*UAS:TVA-mCherry*] into Dp of Tg[*gad1b:Gal4*] fish in the absence of glycoprotein. Coronal section through the injected telencephalic hemisphere at the level of Dp. Area outlined by dashed rectangle is enlarged. Co-expression of GFP (green) and mCherry (magenta) indicates starter cells.
- (h) Same as in (g) but with trans-complementation of zoSADG in starter neurons by co-injection of HSV1[*UAS:zoSADG*]. Left: coronal section through the injected telencephalic hemisphere. Right: coronal section through the ipsilateral olfactory bulb. Expression of GFP only (green) indicates transneuronally labeled neurons.
- (i) Number of neurons in the telencephalon that expressed GFP and mCherry (putative starter neurons) or GFP alone (putative presynaptic neurons) after injection of EnvA-RVΔG and HSV1[*UAS:TVA-mCherry*] into Dp with (+) or without (-) trans-complementation with zoSADG in starter neurons (HSV1[*UAS:zoSADG*]). Left: injection into Tg[*gad1b:Gal4*] fish; right: injection into Tg[*vglut1:Gal4*] fish (right). Expression was analyzed 10 days post injection. n: number of fish.
- (j) Convergence index for the projection of transneuronally labeled neurons in the OB to Dp when EnvA-RVΔG was targeted to GABAergic neurons (viral injections into Tg[*gad1b:Gal4*] fish) or to glutamatergic neurons (injections into Tg[*vglut1:Gal4*] fish) in Dp. Expression was analyzed at 10 days post injection. n: number of fish.

Supplementary Figure Legends

Fig S1. Temperature-dependence and time course of HSV1-mediated gene expression

(a) Injection of HSV1[*LTCMV*:DsRed] into the OB (arrow) and incubation at 26°C (maximum projection of confocal stack). Boxed areas (OB and Dp) are enlarged below. Note that number of DsRed-expressing neurons is low compared to the expression after incubation at 36 °C (Fig. 1).

(b) Time course of DsRed expression in the dorsal telencephalon after injection of HSV1[*LTCMV*:DsRed] into the ipsilateral OB. Fish were kept at 36 °C for different durations. Black dots represent data from individual fish, box plot indicates median and 25% and 75% percentiles, circles and error bars indicate mean and s.d. over individual fish. n: number of fish.

Fig S2. Targeting of GABAergic neurons in the telencephalon

(a) Coronal section through the telencephalon at the level of Dp after injection of HSV1[*UAS*:TVA-mCherry] Tg[*gad1b*:Gal4]; Tg[*gad1b*:GFP] double transgenic fish. The injection was targeted to a volume around Dp. mCherry was expressed predominantly in a cluster of GFP-positive neurons associated with Dp. Note long-range projections of mCherry-expressing neurons to multiple telencephalic areas.

(b) Enlargements of boxed region in (a).

Fig S3. Co-packaging versus co-injection

(a) Expression of GFP and mCherry after injection of HSV1[*UAS*:GFP & *UAS*:TVA-mCherry] into the cerebellum of Tg[*gad1b*:Gal4] fish. In this virus, two expression constructs, *UAS*:GFP and *UAS*:TVA-mCherry, are packaged into the same virus particles. Expression is observed in Purkinje neurons and in putative Golgi cells. Note high rate of co-expression of GFP and mCherry. ML: molecular layer; PL: Purkinje layer; GL: granular layer.

(b) Expression of GFP and mCherry in the Purkinje layer after co-injection of two independent viruses (HSV1[*UAS*:GFP] and HSV1[*UAS*:TVA-mCherry]) into the cerebellum of Tg[*gad1b*:Gal4] fish. Note that the rate of co-expression was high even though GFP and mCherry were delivered by separate viruses. Note also that the overall expression was sparse, implying that co-expression was unlikely to occur by chance.

(c) Percentage of GFP and mCherry-expressing neurons among all fluorescent neurons. Filled circles represent data from individual fish, box plot indicates median and the 25% and 75% percentiles, and open circles indicate mean over individual fish. n: number of fish.

Fig S4. Temperature-dependence of infection by rabies virus

(a) Experimental scheme: Rabies virus (EnvA-RVΔG-GFP) was injected into the telencephalon of transgenic fish expressing TVA-mCherry in GABAergic neurons (Tg[*gad1b*:Gal4; *UAS*:TVA-mCherry]).

(b) Expression of TVA-mCherry and GFP when fish were kept at 26 °C for 6 days after injection. Note almost complete absence of GFP expression.

(c) Expression of TVA-mCherry and GFP when fish were kept at 36 °C for 6 days after injection. Note strong GFP expression.

(d) Expression of TVA-mCherry and GFP six days after injection when the housing temperature was increased from 26 °C to 36 °C 3 days after injection. GFP expression was weak and sparse.

Fig S5. Expression pattern of *vglut1* and *vglut2* in olfactory bulb and Dp

(a) Coronal cross sections through the OB and anterior telencephalon from Tg[*vglut2a*:RFP; *vglut1*:GFP] double transgenic fish. Note that *vglut2a* (magenta) is expressed by axons of olfactory sensory neurons innervating glomeruli in the OB and by a subset of mitral cells, while expression of *vglut1* (green) in the OB is weak or absent. Dotted lines outline OBs.

(b) More posterior coronal cross sections through the telencephalon of the same fish at the level of Dp. Note that expression of *vglut2a* and *vglut1* in the telencephalon are largely complementary. Neurons in Dp express primarily *vglut1*. Dotted areas indicate the dorsal lateral telencephalic area (Dl) and Dp.

Fig S6. Transneuronal tracing using pseudotyped rabies virus from *vglut1*+ neurons in Dp in adult zebrafish

(a) Co-injection of EnvA-RVΔG and HSV1[*UAS*:TVA-mCherry] into Dp of Tg[*vglut1*:Gal4] fish in the absence of glycoprotein. Coronal section through the injected telencephalic hemisphere at the level of Dp. Area outlined by dashed rectangle is enlarged. Co-expression of GFP (green) and mCherry (magenta) indicates starter cells.

(b) Same as in (a) but with trans-complementation of zoSADG in starter neurons by co-injection of HSV1[*UAS*:zoSADG]. Left: coronal section through the injected telencephalic hemisphere. Right: coronal section through the ipsilateral olfactory bulb. Expression of GFP only (green) indicates transneuronally labeled neurons.

Fig 1. HSV1-mediated gene delivery in adult zebrafish and the Gal4/UAS system

(a) Procedure for testing temperature-dependence of HSV1-mediated gene expression.

(b) Maximum intensity projection after injection of HSV1[LTCMV:DsRed] into one olfactory bulb (magenta arrow) of a *Tg[vglut1:GFP]* fish and incubation at 36°C. White arrowhead indicates the OB-projecting area in the dorsal telencephalon used for quantification in (c) and (g). *vglut1:GFP* expression served as a morphological marker.

(c) Mean number of labeled neurons in the dorsal telencephalon after injection of HSV1[LTCMV:DsRed] into the ipsilateral olfactory bulb and incubation at different temperatures. In this and similar plots, black dots represent data from individual fish, box plot indicates median and 25% and 75% percentiles, circles and error bars indicate mean and s.d. over individual fish. n: number of fish.

(d) Construction of the UAS vector for HSV packaging. Genes of interest (GOI) are inserted downstream of the 5xUAS sequences by recombination cloning using the Gateway system. The transcriptional blocker minimizes leaky expression³⁷.

(e) Injection of HSV1[UAS:TVA-mCherry] into the cerebellum of *Tg[gad1b:Gal4; gad1b:GFP]* double transgenic fish. Note co-localization of mCherry and GFP in Purkinje cells (arrowheads) and putative Golgi cells (arrows). ML: molecular layer; PL: Purkinje layer; GL: granular layer.

(f) Fraction of mCherry-positive neurons that co-expressed GFP after injection of HSV1[UAS:TVA-mCherry] into the cerebellum or Dp of *Tg[gad1b:Gal4; gad1b:GFP]* double transgenic fish. n: number of fish.

(g) Injection of HSV1[LTCMV:Gal4] into the olfactory bulb and HSV1[UAS:GFP] into the dorsal telencephalon of wt fish. Note selective expression of GFP in OB-projecting neurons (arrowhead; dashed outline). Number of neurons labeled in the dorsal telencephalon by a single injection of HSV1[LTCMV:DsRed] (a) into the OB (one-way), or by two injections using the two-component Gal4/UAS system (g). n: number of fish.

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- (a)** Schematic of the cerebellar circuit. Glutamatergic neurons are shown in red colors, GABAergic neurons in blue colors. Purkinje cells receive extra-cerebellar input exclusively from the inferior olive.
- (b)** Co-injection of EnvA-RVΔG-GFP and HSV1[UAS:TVA-mCherry] into the cerebellum of Tg[*gad1b*:Gal4] fish in the absence of glycoprotein. Left: schematic. Center: expression of TVA-mCherry (magenta) and GFP (green) in the cerebellum. Regions in the Purkinje and granular layers (dashed rectangles) are enlarged. Right: expression of GFP in the hindbrain. Region covering the inferior olive (dashed rectangle) is enlarged. Expression of GFP was restricted to putative starter neurons. No expression was detected in the inferior olive. ML: molecular layer; PL: Purkinje layer; GL: granular layer.
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(b) Expression of GFP and mCherry in the Purkinje layer after co-injection of two independent viruses (HSV1[*UAS:GFP*] and HSV1[*UAS:TVA-mCherry*]) into the cerebellum of Tg[*gad1b:Gal4*] fish. Note that the rate of co-expression was high even though GFP and mCherry were delivered by separate viruses. Note also that the overall expression was sparse, implying that co-expression was unlikely to occur by chance.

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