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 manipulations[1–8], 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 cohorts[11–13]. 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 brain[14]. 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 cytotoxicity[15]. 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 ºC but the temperature range of natural habitats is broad (up to >38 ºC) and temperature tolerance in the laboratory extends up to ~41 ºC[16,17]. We therefore tested whether viral gene expression is more efficient at temperatures similar to those of mammalian hosts (37 ºC).

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 ºC or 36 ºC for 6 days after injection (Fig. 1a). Consistent with previous results[15], 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 ºC, 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 ºC (Fig. 1b, c). DsRed expression at 36 ºC was first detected ~12 hours after injection and stable for at least 10 days (Fig. S1b). Moreover, increasing the temperature from 26 ºC to 36 ºC 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 vector[18] to simplify the construction step of HSV1 for UAS-dependent expression (Fig. 1d). We then generated HSV1[*UAS*:TVA-mCherry] to expresses 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] fish[19], 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

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

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

76 We next explored strategies for co-expression of multiple transgenes in the same neurons. When red and
77 green reporter constructs were co-packaged into the same virus (HSV1[*UAS*:TVA-mCherry &
78 *UAS*:GFP]), injection into the cerebellum of Tg[*gad1b*:Gal4] resulted in a high rate of co-expression, as
79 expected (89.5 ± 8.7 % co-expression; mean ± s.d.; n = 3 fish; Fig. S3a, c). Interestingly, co-injection of
80 the same responder constructs packaged into separate viruses (HSV1[*UAS*:TVA-mCherry] and
81 HSV1[*UAS*:GFP]) produced a similar rate of co-expression, even when overall expression was sparse
82 (84.3 ± 6.0 % co-expression; mean ± s.d.; n = 3 fish; Fig. S3b, c). These results indicate that co-injection
83 of viruses can be used to co-express multiple transgenes in the same neurons.

84 In rodents, modified rabies viruses have become important tools to analyze connectivity and structure-
85 function relationships in neuronal circuits. These vectors can infect specific "starter" neurons and are
86 transmitted to presynaptic neurons across one synapse. Specific infection is achieved by expressing the
87 receptor protein TVA in the starter neuron and pseudotyping the virus with the envelope protein EnvA[11].
88 To limit retrograde transfer to one synapse, an essential glycoprotein (G) is deleted from the viral genome
89 and supplied in *trans* only in the starter neurons. In zebrafish, the G-deleted rabies virus (RVΔG) has
90 been reported to infect neurons[14] and to cross synapses when complemented with G but the efficiency of
91 retrograde synaptic transfer appeared very low[20].

92 To enhance trans-neuronal tracing efficiency we first explored the effect of temperature. When we
93 injected EnvA-coated RVΔG expressing GFP (EnvA-RVΔG-GFP) into the telencephalon of wt fish at
94 36ºC, no GFP expression was observed (not shown). We then injected EnvA-RVΔG-GFP into the
95 telencephalon of Tg[*gad1b*:Gal4; *UAS*:TVA-mCherry] fish that express TVA-mCherry in GABAergic
96 neurons. After 6 days, efficient and selective infection of neurons expressing TVA-mCherry was

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

100 Efficient trans-neuronal spread of the rabies virus depends on the expression level of the glycoprotein in
101 starter cells[21,22]. We therefore took two steps to enhance glycoprotein expression. First, we optimized
102 codon usage for zebrafish. Second, we expressed TVA and the codon-optimized glycoprotein (zoSADG)
103 using HSV1 because viral vectors typically reach higher expression levels than transgenics[15]. In rodents,
104 starter neurons expressing high levels of G often disappear in parallel with the emergence of
105 transneuronal expression, presumably because long-term expression of G is toxic[23,24]. We therefore
106 determined the time course of transgene expression under different experimental conditions. We first
107 focused on the cerebellum where GABAergic Purkinje neurons receive local synaptic input from different
108 types of cerebellar neurons and long-range input from neurons in the contralateral inferior olive (climbing
109 fibers; Fig. 2a). We injected a mixture of HSV1[*UAS*:TVA-mCherry] and EnvA-RVΔG-GFP w into the
110 cerebellum of Tg[*gad1b*:Gal4] fish and examined expression for up to 10 days. As observed before
111 (Fig. 1e), mCherry expression was localized to Purkinje neurons and putative Golgi cells. Neurons
112 expressing GFP were concentrated around the injection site and usually co-expressed mCherry (Fig 2b,
113 d). Rarely, GFP expression was observed in mCherry-negative neurons (Fig. 2d), which may indicate
114 TVA-independent viral entry. However, as no GFP expression was observed when EnvA-RVΔG-GFP
115 was injected into wt animals, it appears more likely that GFP+/mCherry- neurons initially expressed TVA
116 but subsequently lost expression due to the slight decline of expression driven by the viral promoter
117 (Fig. S1b). Co-expression of mCherry and GFP was stable for at least 10 days after injection and neurons
118 expressing GFP only remained very rare (Fig. 2d). These results confirm the specificity of EnvA-RVΔG-
119 GFP infection and show that expression of TVA-mCherry or the infection of rabies virus alone are not
120 toxic in the absence of glycoprotein. No expression of GFP was observed in the inferior olive, consistent
121 with the expectation that the RVΔG does not spread in the absence of glycoprotein.

122 To examine whether the delivery of glycoprotein to starter cells can drive trans-neuronal spread, we
123 injected a mixture of HSV1[*UAS*:TVA-mCherry], HSV1[*UAS*:zoSADG] and EnvA-RVΔG-GFP into the
124 cerebellum of Tg[*gad1b*:Gal4] fish. Unlike in the absence of glycoprotein, the number of mCherry-
125 expressing Purkinje neurons and putative Golgi cells now declined over the incubation period of 10 days.
126 The number of neurons expressing GFP, in contrast, was low initially but increased steeply between 6 and
127 10 days after injection. In the cerebellum, GFP was expressed in Purkinje cells and throughout the
128 granular layer (Fig. 2c). The number and spatial distribution of GFP-expressing neurons in the granular
129 layer was clearly different from mCherry expression in the absence of glycoprotein (Tg[*gad1b*:Gal4] fish

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

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

141 To corroborate these results we also examined transneuronal tracing from starter neurons in telencephalic
142 area Dp. In this area, both glutamatergic principal neurons and GABAergic interneurons receive long-
143 range input from mitral cells in the OB (Fig. 2f). In addition, Dp receives input from other telencephalic
144 areas that is not well characterized. When a mixture of HSV1[*UAS*:TVA-mCherry] and EnvA-RVΔG-
145 GFP was injected into Tg[*gad1b*:Gal4] fish (Fig. 2g), GFP expression was largely restricted to a cluster of
146 neurons that co-expressed mCherry (Fig. 2g). This cluster was located near a prominent furrow that
147 separates an anterior from a posterior compartment of Dp, consistent with the known location of
148 GABAergic neurons in Dp[19]. When HSV1[*UAS*:zoSADG] was added to the injected virus mixture,
149 mCherry expression became rare while a large number of neurons in different telencephalic areas
150 expressed GFP (Fig. 2h, i). Moreover, GFP was expressed in the olfactory bulb by neurons with the
151 characteristic morphology of mitral cells, but not by other cells (Fig. 2h). Similar observations were made
152 when the infection was targeted to glutamatergic starter neurons by injecting the cocktail of viruses into
153 Dp of Tg[*vglut1*:Gal4] fish (Fig. S6. Note that *vglut1* is expressed in excitatory neurons in the
154 telencephalon but not in the OB (Fig. S5), implying that labeling of OB neurons cannot be explained by
155 direct infection. Convergence indices for projections of mitral cells to GABAergic or glutamatergic starter
156 neurons in Dp were 0.70 ± 0.21 and 1.31 ± 1.20 (mean ± s.d.; n = 5 and n = 4 fish, respectively; Fig. 2j).
157 These results confirm that EnvA-RVΔG selectively infects TVA-expressing target neurons and undergoes
158 monosynaptic retrograde transneuronal transfer when complemented with zoSADG.

159

160 **Conclusions**

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

174 We further established procedures for retrograde monosynaptic tracing using rabies viruses that greatly
175 enhanced the efficiency of transneuronal transfer compared to a previous study in zebrafish[20].
176 Temperature adjustments were important but unlikely to be the only factor underlying the enhanced
177 transneuronal spread because the temperature difference to the previous study was only 1.5 ºC[20]. We
178 therefore assume that increased glycoprotein expression by codon optimization and viral gene delivery
179 also contributed significantly. Moreover, efficiency may be affected by the relative timing of glycoprotein
180 expression and rabies virus infection. Convergence ratios were similar to those observed in rodents
181 despite the fact that zebrafish neurons are smaller and probably receive fewer synaptic inputs. These
182 results suggest that the efficiency of transneuronal tracing was in the same range as in rodents although
183 quantitative comparisons are difficult. In the future, further improvements may be achieved by
184 modifications that are currently explored in rodents such as the design of optimized glycoproteins[22] or
185 trans-complementation with a second viral gene[31]. The apparent long-term toxicity of the glycoprotein
186 may be circumvented by transient expression using a negative feedback system[32]. Hence, rabies-based
187 transneuronal tracers are promising tools for the structural and functional dissection of neuronal circuitry
188 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.

Methods

Animals

Experiments were performed in adult (5 – 15 month old) zebrafish (*Danio rerio*) of both sexes. Fish were bred under standard laboratory conditions (26 – 27°C, 13 h/11 h light/dark cycle). All experiments were approved by the Veterinary Department of the Canton Basel-Stadt (Switzerland).

The following transgenic fish lines were used: Tg[*gad1b*:GFP][28], Tg[*gad1b*:Gal4][19], Tg [*vglut2a*:loxP-DsRed-loxP-GFP][28], Tg[*vglut1*:GFP] (created in this study), Tg[*vglut1*:Gal4] (created in this study), and Tg[*UAS*:TVA-mCherry] (created in this study). Note that Tg[*gad1b*:GFP] and Tg[*gad1b*:Gal4] were created using the same BAC (zC24M22), that likely produce the same expression pattern under *gad1b*.

Transgenic fish, DNA constructs and virus production

Tg[*UAS*:TVA-mCherry] fish were created using standard procedures based on the Tol2 transposon.[33] TVA-mCherry was amplified by PCR from a plasmid (gift from Dr. Uchida, Addgene plasmid #38044 ; http://n2t.net/addgene:38044 ; RRID:Addgene_38044,[34]) and inserted into a 5xUAS vector[29]. Tg[*vglut1*:GFP] fish were established using the CRISPR/Cas9 method[35]. Insertion of a construct containing the hsp70 promoter was targeted at a site upstream of the *vglut1* gene using the target sequence gagagagactcgggcgcgcg. The same procedure and target sequence was used to generate Tg[*vglut1:*loxP-mCherry-loxP-Gal4]. This line was then crossed to Tg[*hspa8*:Cre-mCherry-NLS] (ZFIN ID: ZDB-ALT-201210-1), which expresses Cre ubiquitously, to generate Tg[*vglut1:*Gal4].

For HSV1 production, constructs containing *LTCMV*:DsRed and *LTCMV*:Gal4 were created at the Gene Delivery Technology Core of the Massachusets General Hospital. Constructs containing *UAS*:GFP, *UAS*:TVA-mCherry, and *UAS*:zoSADG were created using the Gateway construct described in this paper (Fig. 1d). The transcriptional blocker (Fig. 1d) and zoSADG (Fig. 2) were synthesized with the following sequences:

Transcriptional blocker:
caataaaatatctttattttcattacatctgtgtgttggtttttgtgtgaatcgatagtactaacatacgctctccatcaaaacaaaacgaaacaaaacaaactag
caaaataggctgtccccagtgcaagtgcaggtgccagaacatttctct

zoSADG:
atggtgcctcaggctctgctgtttgtgcctctgctggtgttccctctgtgcttcggaaaattccctatctacacaatcccggataaactgggaccttggagccc
tatcgacatccaccacctgagctgtcctaacaacctggtggtggaggatgagggatgcactaacctgagcggattctcctacatggagctgaaagtggga
tacatcctggctatcaaggtgaacggattcacctgtacaggagtggtgactgaggctgagacatacacaaacttcgtgggatacgtgacaaccacattca
aaaggaaacacttcagacctacacctgatgcttgtagagctgcctacaactggaagatggctggtgaccctagatatgaggagtccctgcacaaccctta
ccctgactacagatggctgcgtacagtgaagacaacaaaagagagcctcgttatcatcagcccttccgtggccgatctcgatccatatgacagaagcctg
cactctagagtgttcccaagcggaaagtgctctggcgtggcagtgtcttccacttactgctcaaccaaccacgactacaccatctggatgccagagaacc
caagactgggaatgtcttgtgacatcttcactaactctagaggaaaaagagcttctaaaggatctgagacctgcggattcgtggatgagagaggactgtac
aagtctctgaagggagcttgtaaactgaagctgtgtggagtgctgggactgagactgatggacggaacctgggtcagcatgcagacaagcaacgagac
caagtggtgtcctccagacaaactggtgaacctgcacgactttagaagcgatgagattgagcaccttgtggtggaggagctggtgagaaaaagagagg
agtgtctggacgctctggagagcatcatgacaacaaaaagcgtgtctttcagaagactgagccacctgagaaaactggtgcctggattcggaaaggctta
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ggaagatgtcacccacacgtgaacggagtgttctttaacgggatcattttgggtcccgacggcaatgtgctcatcccggaaatgcagagcagcctgctcc
agcaacacatggagttgctcgagagtagtgtgataccccttagtccatccactcgcagatccttccacagtgttcaaggatggtgacgaggctgaggacttt

328 gtagaggttcatctccctgatgtgcacaaccaggtgtctggagtggatctgggactgccaaactggggaaagtacgtgctgctgtctgctggagctctgac
329 cgctctgatgctgatcatcttcctgatgacatgttgtagaagagtgaacagatctgagcctacacagcacaacctgagaggaactggaagagaggtgagc
330 gtgacacctcagagcggaaagatcatctctagctgggagtcacataagtctggaggtgaaactagactgtga.

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

334 **Virus injection**

335 Virus injection was performed as described[15] with minor modifications. Fish were anesthetized in 0.03%
336 tricaine methanesulfonate (MS-222) and placed under a dissection microscope. MS-222 (0.01%) was
337 continuously delivered into the mouth through a small cannula. A small craniotomy was made over the
338 dorsal telencephalon near the midline, over the OBs, or over the cerebellum. If multiple viruses were
339 injected in the same region, virus suspensions were mixed. Phenol red (0.05 %) was added to the
340 suspension to visualize the injection. Micropipettes pulled from borosilicate glass were inserted vertically
341 through the craniotomy. The depth of injections was approximately 100 μm in the dorsal telencephalon,
342 50 μm in the OB, and 100-150 μm in the cerebellum, 300-500 μm in Dp. The injected volume was 50 nl
343 – 100 nl. After surgery, fish were kept in standard holding tanks at 35 °C – 37 °C unless noted otherwise.

344 **Clearing of brain samples**

345 We adapted the original Cubic protocol[36] to small samples such as adult zebrafish brains. After fixation
346 with 4% paraformaldehyde overnight, samples were soaked with reagent 1A (10%w/v Triton, 5wt%
347 NNNN-tetrakis (2HP) ethylenediamine and 10%w/v urea) for 2.5 h at room temperature and for 6 h at
348 37 °C with mild shaking and multiple solution exchanges. Subsequently, samples were washed in PBS
349 overnight at room temperature. On the next day, samples were treated with reagent 2 (25% w/v urea, 50%
350 w/v sucrose and 10% w/v triethanolamine) for refractive index matching, mounted in glass bottom dishes,
351 and covered by 16 x 16 mm cover glasses to avoid drift. Images were acquired using a Zeiss 10x water-
352 immersion objective lens (N.A. = 0.45) on an upright Zeiss LSM 700 confocal microscope.

353 **Immunohistochemistry**

354 For rabies virus tracing of the cerebellum circuit, GFP and mCherry signals were detected by
355 immunocytochemistry. Brain samples were fixed in 4% paraformaldehyde overnight and sectioned
356 (100 μm) on a Leica VT1000 vibratome. Primary antibodies were anti-GFP (Thermofisher, A10262,
357 1:200) and anti-RFP (5F8, chromotek, 1:200). Secondary antibodies were conjugated to Alexa Fluor 488
358 or 594 (Invitrogen, 1:200).

359 **Neuron counts**

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

363 Z stacks from individual brain slices (1 – 2 um steps, Fig1f, 2d, 2i, 2j, S1 and S3c) were acquired using an
364 upright Zeiss LSM 700 confocal microscope with a 20x objective (air, N.A. = 0.8, pixel size 0.625 um).
365 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.

375 **Figure Legends**

376

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

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

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

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

387 **(d)** Construction of the UAS vector for HSV packaging. Genes of interest (GOI) are inserted downstream
388 of the 5xUAS sequences by recombination cloning using the Gateway system. The transcriptional blocker
389 minimizes leaky expression[37].

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

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

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

401

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

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

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

411 **(c)** Same as in (b) but the glycoprotein (zoSADG) supplied to starter neurons in *trans* by co-injection of
412 HSV1[*UAS*:zoSADG]. Note expression of GFP in putative granule cells and in neurons of the inferior
413 olive, indicating transneuronal spread.

414 **(d)** Number of neurons that expressed GFP and mCherry (putative starter neurons) or GFP alone (putative
415 presynaptic neurons) at different time points after injection of EnvA-RVΔG and HSV1[*UAS*:TVA-
416 mCherry] into the cerebellum of Tg[*gad1b*:Gal4] fish. Left: without glycoprotein; right: with trans-
417 complementation of zoSADG in starter neurons. Note that labeling of putative presynaptic neurons
418 emerged between 6 and 10 days post injection only when zoSADG was trans-complemented in starter
419 neurons. See the method for details. In all plots, black dots represent data from individual fish, box plot
420 indicates median and the 25% and 75% percentiles, circles and error bars indicate mean and s.d. over
421 individual fish. n: number of fish.

422 **(e)** Convergence index for the projection from the inferior olive to the cerebellum at different time points.
423 The convergence index is the numerical ratio of transneuronally labeled neurons (GFP+/mCherry-
424 neurons in the inferior olive) and putative starter cells in the cerebellum (GFP+/mCherry+ Purkinje
425 neurons). n: number of fish.

426 **(f)** Schematic of the circuitry in telencephalic area Dp. Glutamatergic neurons are shown in red colors,
427 GABAergic neurons in blue colors. Long-range projections from mitral cells in the olfactory bulb
428 terminate on glutamatergic neurons and on GABAergic interneurons in Dp. Additional long-range
429 projections originate in other telencephalic areas.

430 **(g)** Co-injection of EnvA-RVΔG and HSV1[*UAS*:TVA-mCherry] into Dp of Tg[*gad1b*:Gal4] fish in the
431 absence of glycoprotein. Coronal section through the injected telencephalic hemisphere at the level of Dp.
432 Area outlined by dashed rectangle is enlarged. Co-expression of GFP (green) and mCherry (magenta)
433 indicates starter cells.

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

438 **(i)** Number of neurons in the telencephalon that expressed GFP and mCherry (putative starter neurons) or
439 GFP alone (putative presynaptic neurons) after injection of EnvA-RVΔG and HSV1[*UAS*:TVA-mCherry]
440 into Dp with (+) or without (-) trans-complementation with zoSADG in starter neurons
441 (HSV1[*UAS*:zoSADG]). Left: injection into Tg[*gad1b*:Gal4] fish; right: injection into Tg[*vglut1*:Gal4]
442 fish (right). Expression was analyzed 10 days post injection. n: number of fish.

443 **(j)** Convergence index for the projection of transneuronally labeled neurons in the OB to Dp when EnvA-
444 RVΔG was targeted to GABAergic neurons (viral injections into Tg[*gad1b*:Gal4] fish) or to
445 glutamatergic neurons (injections into Tg[*vglut1*:Gal4] fish) in Dp. Expression was analyzed at 10 days
446 post injection. n: number of fish.

447

448

449

450 **Supplementary Figure Legends**

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

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

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

459

460 **Fig S2. Targeting of GABAergic neurons in the telencephalon**

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

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

467

468 **Fig S3. Co-packaging versus co-injection**

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

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

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

482

483 **Fig S4. Temperature-dependence of infection by rabies virus**

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

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

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

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

492

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

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

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

501

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

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

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

512

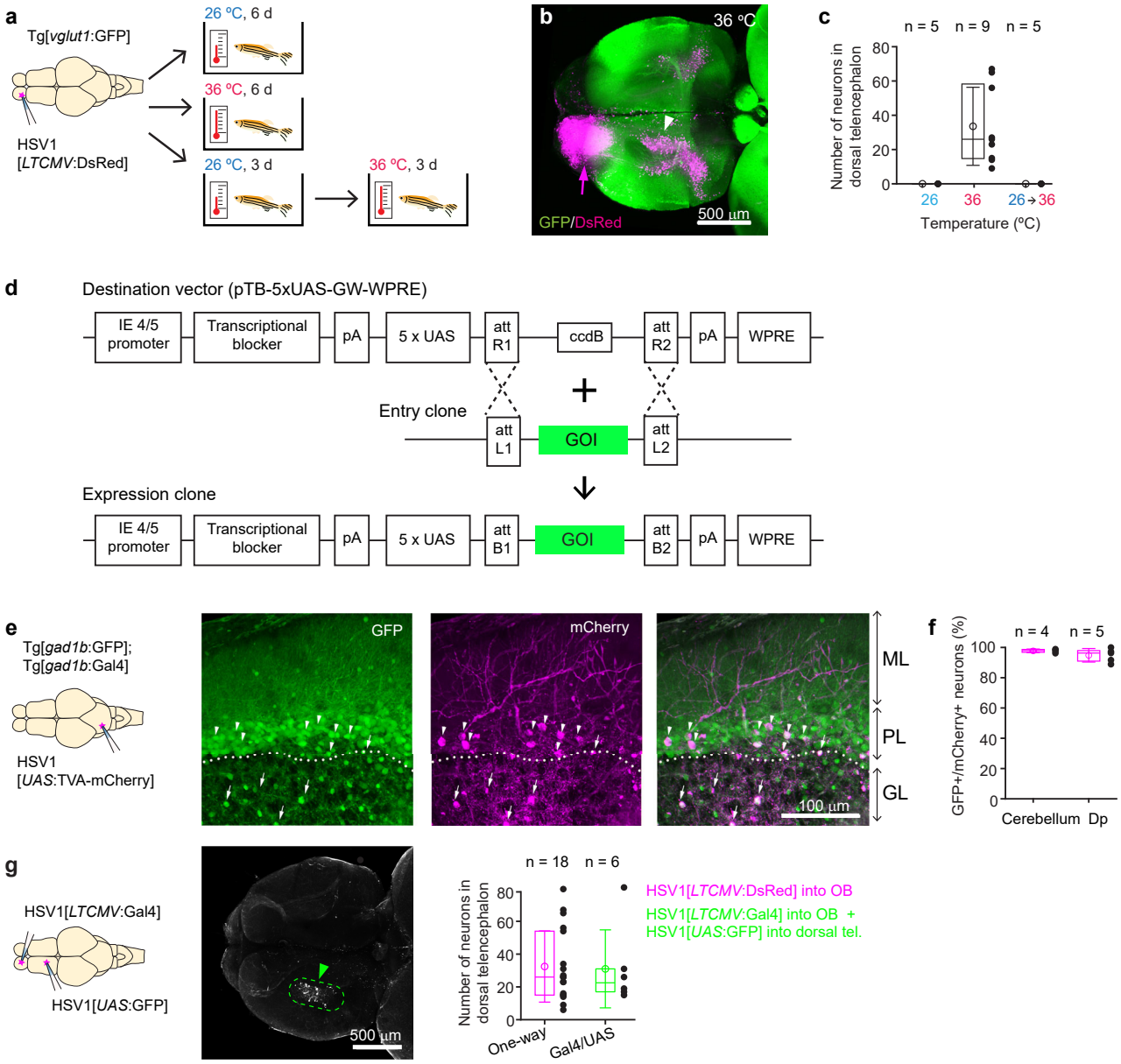

Fig 1. HSV1-mediated gene delivery in adult zebrafish and the Gal4/UAS system

(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 36ºC. 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 expression[37].
(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.

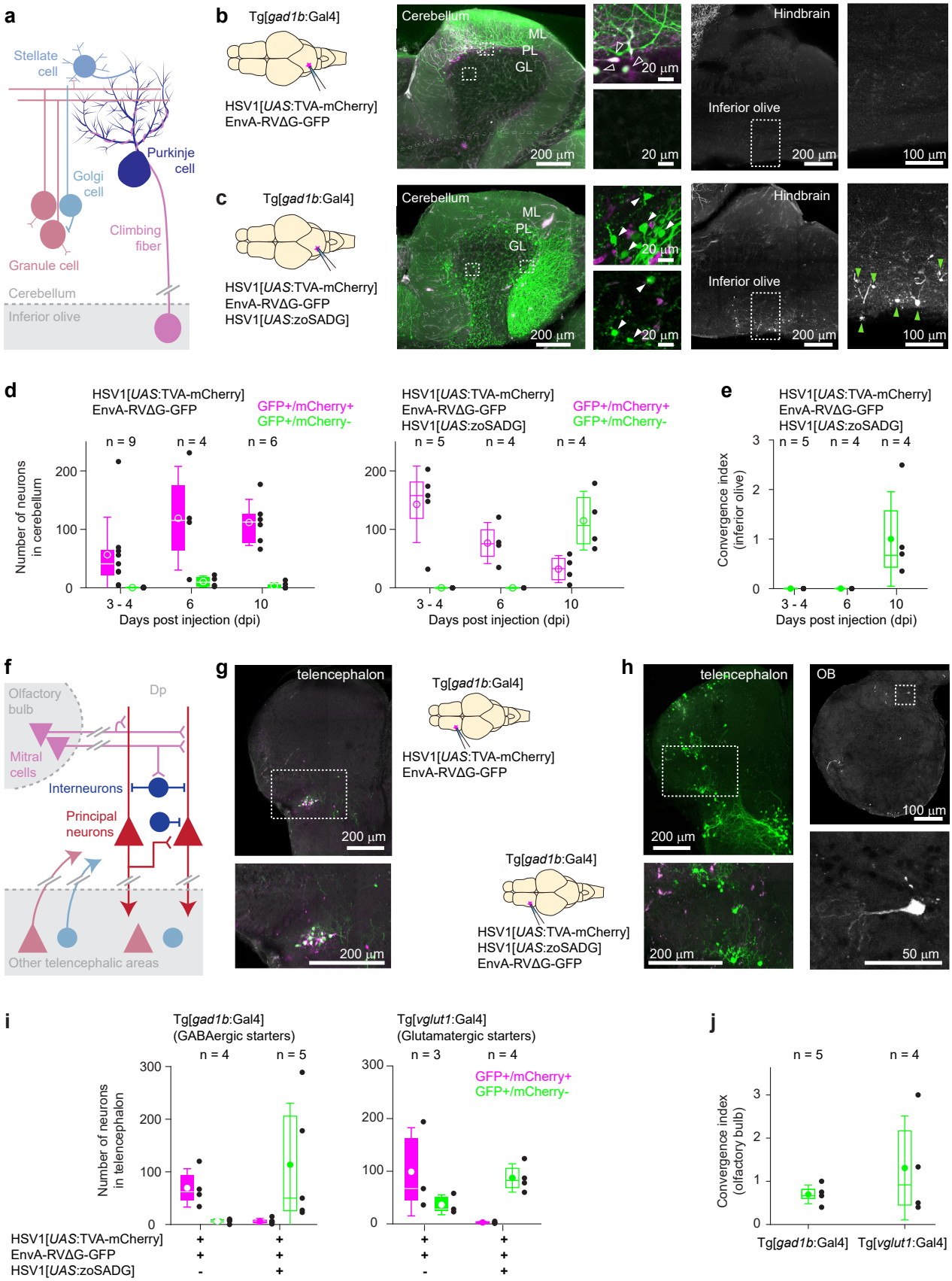
a
Stellate cell
Purkinje cell
Golgi cell
Climbing fiber
Granule cell
Cerebellum
Inferior olive
b
Tg[gad1b:Gal4]
HSV1[UAS:TVA-mCherry]
EnvA-RVΔG-GFP
Cerebellum
ML
PL
GL
200 µm
20 µm
Hindbrain
Inferior olive
100 µm
c
Tg[gad1b:Gal4]
HSV1[UAS:TVA-mCherry]
EnvA-RVΔG-GFP
HSV1[UAS:zoSADG]
d
HSV1[UAS:TVA-mCherry]
EnvA-RVΔG-GFP
GFP+/mCherry+
GFP+/mCherry-
n = 9
n = 4
n = 6
Number of neurons in cerebellum
Days post injection (dpi)
HSV1[UAS:TVA-mCherry]
EnvA-RVΔG-GFP
HSV1[UAS:zoSADG]
n = 5
n = 4
n = 4
e
HSV1[UAS:TVA-mCherry]
EnvA-RVΔG-GFP
HSV1[UAS:zoSADG]
Convergence index (inferior olive)
Days post injection (dpi)
f
Olfactory bulb
Dp
Mitral cells
Interneurons
Principal neurons
Other telencephalic areas
g
telencephalon
Tg[gad1b:Gal4]
HSV1[UAS:TVA-mCherry]
EnvA-RVΔG-GFP
200 µm
Tg[gad1b:Gal4]
HSV1[UAS:TVA-mCherry]
HSV1[UAS:zoSADG]
EnvA-RVΔG-GFP
h
telencephalon
OB
200 µm
100 µm
50 µm
i
Tg[gad1b:Gal4]
(GABAergic starters)
n = 4
n = 5
Number of neurons in telencephalon
Tg[vglut1:Gal4]
(Glutamatergic starters)
n = 3
n = 4
GFP+/mCherry+
GFP+/mCherry-
HSV1[UAS:TVA-mCherry]
EnvA-RVΔG-GFP
HSV1[UAS:zoSADG]
j
n = 5
n = 4
Convergence index (olfactory bulb)
Tg[gad1b:Gal4]
Tg[vglut1:Gal4]

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.

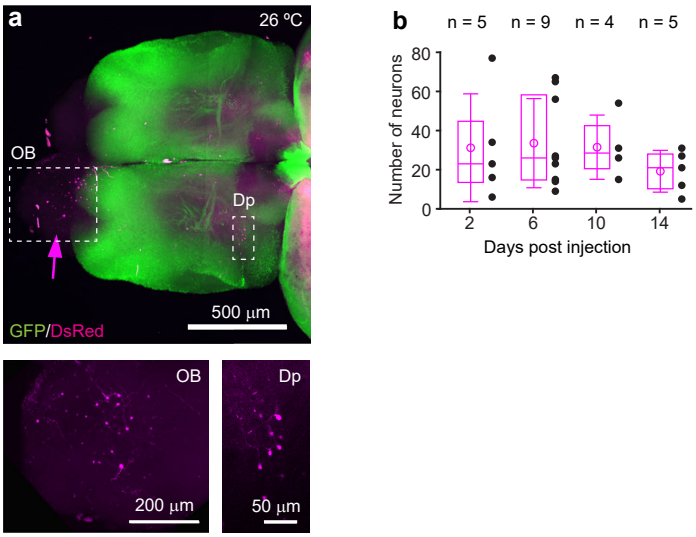

Fig S1. Temperature-dependence and time course of HSV1-mediated gene expression

(a) Injection of HSV1[*LTCMV*:DsRed] into the OB (arrow) and incubation at 26ºC (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 ºC (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 ºC 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.

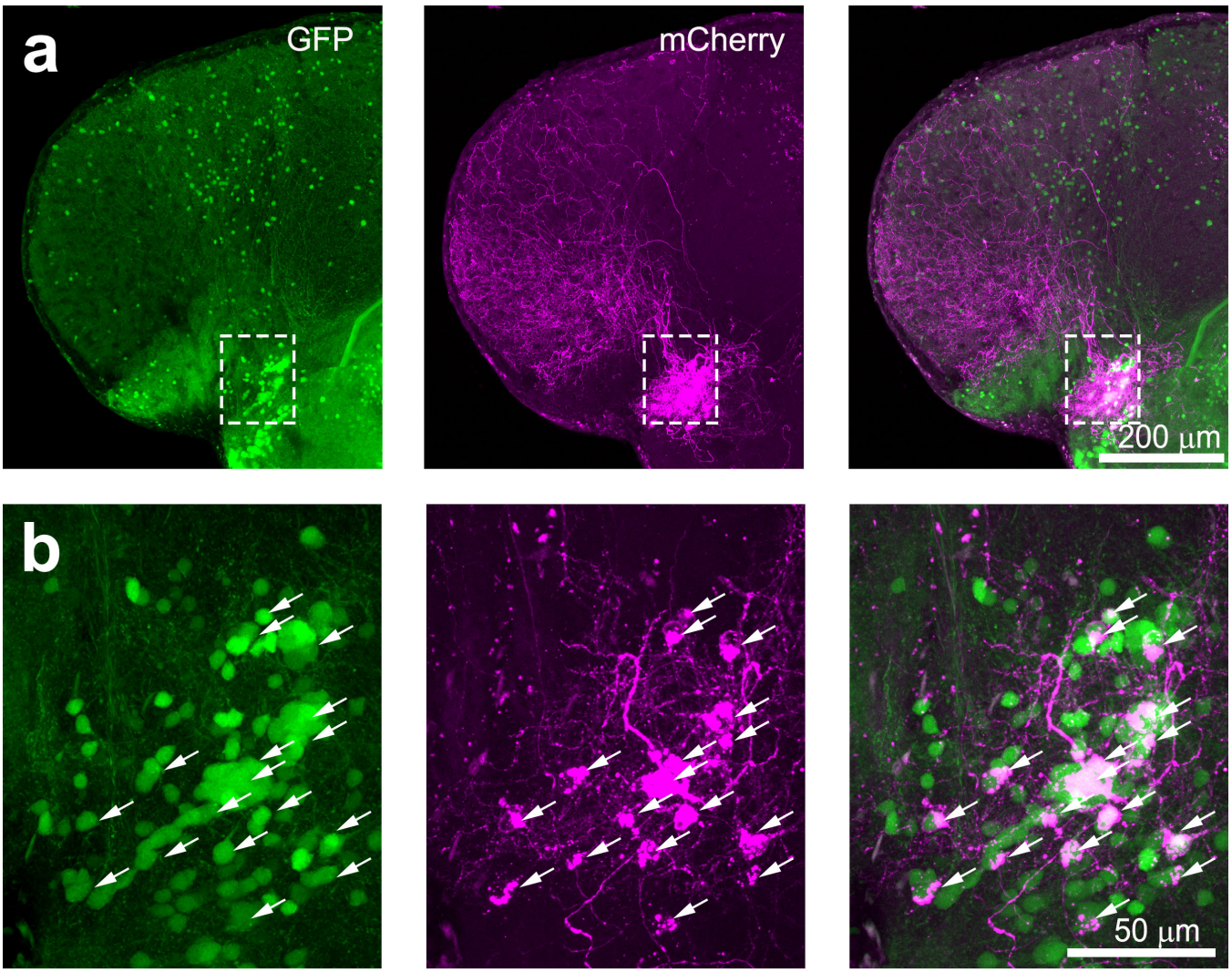

Fig S2. Targeting of GABAergic neurons in the telencephalon

(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).

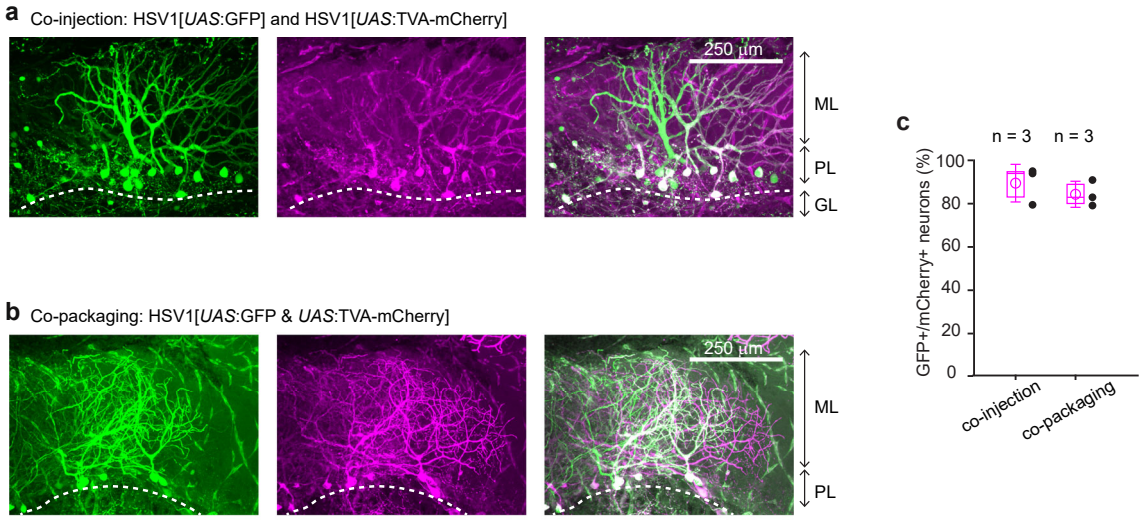

Fig S3. Co-packaging versus co-injection.

(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.

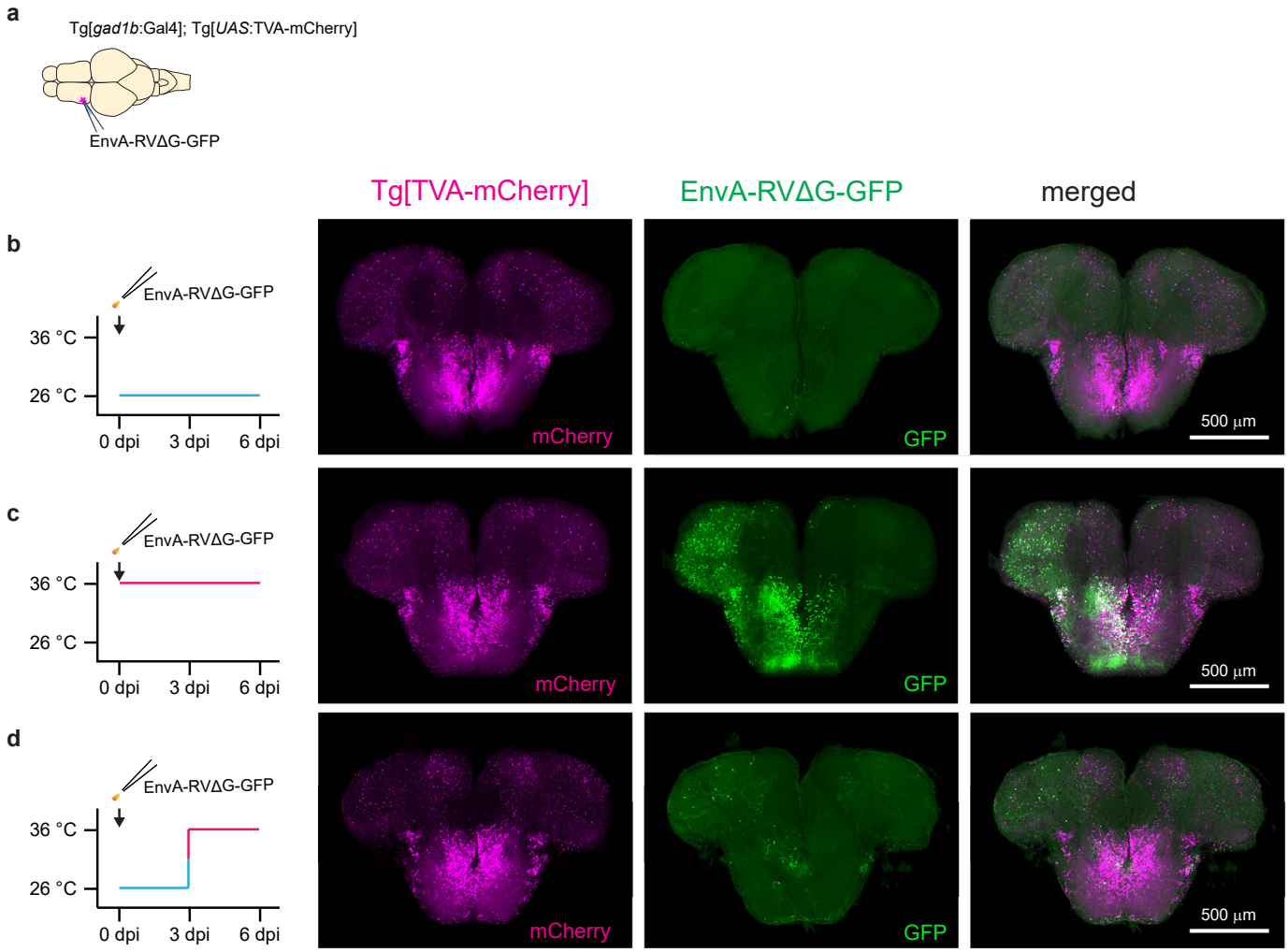

Fig S4. Temperature-dependence of infection by rabies virus

(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 ºC 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 ºC 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 ºC to 36 ºC 3 days after injection. GFP expression was weak and sparse.

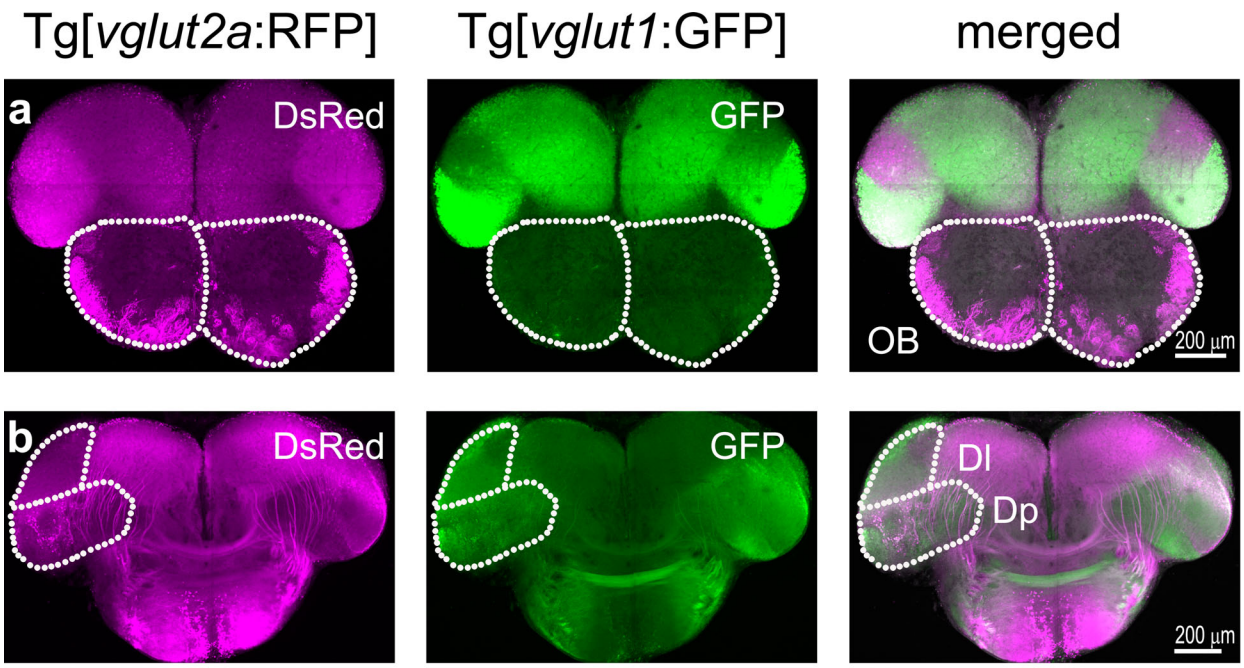

Fig S5. Expression pattern of vglut1 and vglut2 in olfactory bulb and Dp

(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.

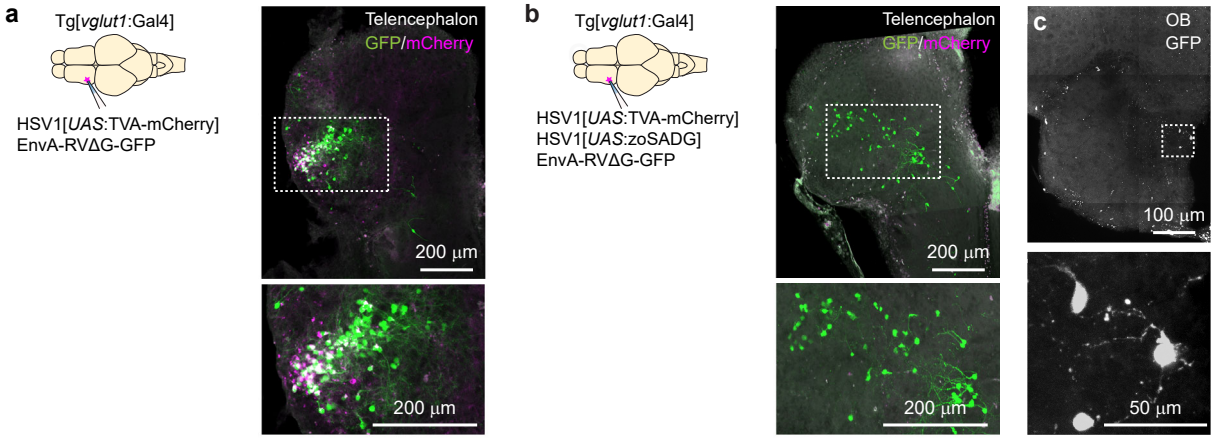

Fig S6. Transneuronal tracing using pseudotyped rabies virus from vglut1+ neurons in Dp in adult zebrafish

(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.