# Lentiviral Expression of GAD67 and CCK Promoter-Driven Opsins to Target Interneurons in Vitro and in Vivo

**Short Title: Targeting Opsin Vectors to Interneurons**

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## Abstract

### Background

The ability to manipulate the activity of interneurons with optogenetic tools offers the possibility to interfere with diseases caused by altered neuronal inhibition and synchrony, including epilepsy and schizophrenia. To develop vectors for therapeutic approaches, targeting optogenetic constructs to interneurons is therefore a key requirement. We asked if the interneuron-specific promoters glutamic acid decarboxylase (GAD)67 and cholecystokinin (CCK) allowed targeted lentiviral delivery of opsins to interneurons as a whole, or specifically CCK-positive(+) interneurons.

### Methods

We generated lentiviral (LV) plasmids encoding channelrhodopsin (ChR2) and halorhodopsin (NpHR) tagged with fluorophores and driven by GAD67 or CCK promoters. Adeno-associated virus (AAV) and LV vectors carrying opsins driven by pyramidal cell promoters were used as controls. We transduced neuronal cultures and rodent brain in vivo, immunostained specimens 6-8 weeks following in vivo injection, 7-14 days after in vitro transduction, and evaluated volume and specificity of expression by confocal microscopy.

### Results

In vitro 90% (19/21) of LV-CCK-NpHR2.0-EYFP expressing neurons were CCK+. In vivo LV-GAD67-ChR2-mCherry was expressed in 2.6%(5/193), LV-GAD67-NpHR2.0-EYFP in ~15%(43/279), LV-CCK-NpHR2.0-EYFP in 47%(9/19) of hippocampal GABA+ interneurons. GAD67 vectors expressed in larger volumes than CCK-driven constructs, AAV vector controls achieved the largest expression volumes.

### Conclusions

LV-CCK-NpHR2.0-EYFP may be useful to target CCK+ interneurons in culture. GAD67/CCK-driven lentiviral constructs are expressed in vivo, but expression is not specific for interneurons. Overall, expression levels are low compared to opsins driven by pyramidal cell promoters. Better understanding of GAD67 and CCK promoter structure or alternative techniques are required to reliably target opsins to interneurons using viral vectors.

## 1. Introduction

Optogenetic techniques [1,2] combine optical and genetic tools, and allow targeted optical control of firing in neurons through expression of opsins on neuronal cell membranes. Excessive or altered neuronal synchrony underlies common nervous system diseases, such as epilepsy and schizophrenia [3]. As interneurons are responsible for timing and synchronizing pyramidal cell activity [4], and are known to prevent seizure spread [5], targeting opsins to these inhibitory cells is key to influence interneuron function for therapeutic purposes. Augmenting interneuron activity or altering their firing patterns could be used as treatment tool, to interfere with epileptiform activity [6], or to re-synchronize oscillating neuronal ensembles in schizophrenia and Parkinson's disease, but also to dissect the roles of different subclasses of interneurons in various diseases of the nervous system [6,7].

Targeting interneurons in vitro and in vivo can be achieved by either using opsin-carrying neurotropic viral vectors, or transgenic models. In both cases, a cell-type specific promoter drives opsin expression. The advantage of using viral vectors is that they transduce a limited volume of tissue, which allows precise targeting of very specific areas of interest. A further advantage of viral vectors includes their potential translational applications, such as their use in human patients. To date, transcription of virally delivered genes in the nervous system has been achieved by either using strong but non-specific neuronal promoters (including synapsin-1 [8], elongation factor 1a (EF-1a) [9], neuron-specific enolase[10]), or by using weaker but more specific promoters such as calcium calmodulin-binding kinase 2a (Camk2a [11], specific for excitatory neurons) and glutamic acid decarboxylase 1 (Gad1, equivalent to GAD67 in mouse and specific for GABAergic interneurons as a whole [12]). Other cell-type specific promoters used are prepro-hypocretin (Hcrt, specific for hypocretin neurons [13]), glial fibrillary acidic protein (GFAP, specific for astrocytes [14]), tyrosine hydroxylase (for targeting catecholaminergic neurons [15]) and neurofilament [16]. An alternative technique to achieve expression of virally delivered genes in the nervous system consists of adding enhancing cassettes or intronic sequences to the transgene of interest [17, 18].

To understand how manipulation of inhibitory interneuron excitability alters pyramidal cell activity [19] and influences epileptic networks [20], we set out to investigate if targeted viral delivery of the two major opsins channelrhodopsin-2 (ChR2) and halorhodopsin (NpHR) to interneurons was feasible in vivo and in vitro.

Very few mammalian promoters have been characterized sufficiently enough to allow viral expression of transgenes specific to interneurons: these include fragments of the GAD67 promoter to target γ-amino butyric acid (GABA)ergic interneurons as a whole [21, 22, 12], and cholecystokinin (CCK) [23, 24], to target a subset of CCK-expressing interneurons. In both cases

these promoter sequences span over 3 kb. Whilst successful expression of fluorophores in interneurons has been reported using a GAD67 promoter-carrying adeno-associated virus (AAV) vectors [21, 22, 12], the remaining AAV payload is too small to also carry an opsin gene. Targeted expression of opsins to interneurons has so far only been achieved by injection of vector carrying a floxed-stop opsin construct into Cre-mice lines expressing Cre-recombinase under a promoter specific for interneurons. Examples include Cre-driver lines under the promoters parvalbumin (PV) and somatostation (SST), specific for subsets of GABAergic interneurons [25, 26, 27, 6], and glutamic acid decarboxylase 2 (Gad2), specific for GABAergic interneurons as a whole [6].

We asked if a lentivirus (LV) vectors (LVV) incorporating the GAD67 or CCK-promoter could reliably target opsins to interneurons in wild-type tissue in vitro and in vivo. Targeting opsins to interneurons as a whole, or to CCK-positive interneurons, using lentiviral vectors has not been previously described. We chose to employ lentiviral constructs as they have a large transgene capacity of 8-10 kb, allowing them to carry the genes for promoter, opsin and fluorophore. A further advantage considering future clinical applications is the lack of immune response to LVVs and the possibility of large scale production of clinical grade vectors.

We were able to demonstrate that our opsin carrying lentiviral constructs driven by the interneuron-specific promoters GAD67 and CCK are expressed in neuronal cultures and rat motor cortex and hippocampus. In cultures enriched in interneurons, such as those from the ganglionic eminence, LV-CCK-NpHR2.0-EYFP may be useful to target CCK+ interneurons. Expression levels in vivo, however, were low and expression was not specific for interneurons. Our results suggest that, in their current form, the two promoters may not contain all regulatory sequences necessary to target opsins to interneurons in cortex and hippocampus [21], or may indicate that LVVs are less suitable than other viral vectors to target inhibitory neurons [28].

## 2. Materials and Methods

**Plasmid Design and Construction**

Lentiviral plasmids used to target excitatory neurons contained NpHR2.0–EYFP (enhanced yellow fluorescent protein, EYFP) and hChR2(H134R)-mCherry under the excitatory neuron specific promoter Camk2a (gift of K.Deisseroth, Stanford University [11]). They were used as backbones to generate the constructs carrying the GAD67 (gift of S. Kasparov [22]) and CCK promoters (gift of K. Ressler [23, 24]). The vectors have a truncated HIV 3'LTR, contain the HIV-1 central polypurine tract (cPPT) [29] and the Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE) [30] to improve transduction efficiency. To construct the vectors pLenti-CCK-NpHR–EYFP and pLenti-CCK-ChR2-mCherry, an XbaI site was inserted into pLenti-CCKpro-GFP (green

fluorescent protein, GFP) by mutagenizing it with the following primers: forward 5'-acaaaaacaaattacaaaaattcaaaattttatctagatttcccagggaagatgaagaa-3' and reverse 5'-ttcttcatcttccctgggaaatctagataaaattttgaatttttgtaatttgtttttgt-3'. The ~3 kb CCK promoter sequence was subsequently removed (XbaI/BamHI fragment) and ligated into pLenti-Camk2a-NpHR2.0–EYFP and pLenti-Camk2a-ChR2-mCherry cut with the restriction enzymes XbaI/BamHI. Plasmids were purified using conventional kits (Qiagen) and all sequences were confirmed by restriction enzyme digestion and sequencing. GAD67 subcloning was outsourced to Entechelon GmBH, Regensburg, Germany. The adenoviral vector (AVV) shuttle carrying 3.7 kb of GAD67 promoter sequences was mutagenized and a PacI site inserted 5'-upstream of the PmlI site. The Camk2a promoter was subsequently removed from pLenti-Camk2a-NpHR2.0–EYFP and pLenti-Camk2a-ChR2-mCherry via restriction digest with PacI/PmlI and the GAD67 promoter cloned in.

**Viral Production and Titration**

VSVg pseudotyped lentiviral vectors were generated according to local protocols: 293FT cells (Invitrogen) were seeded 24 hours prior to transfection in order to reach 90% confluence the following day. Cells were co-transfected with the transfer vector, p8.91 (gag/pol expressor) and pMD.G (VSVg expressor) in Fugene 6 (Roche), Optimem and sterile Tris-Ethylenediaminetetraacetic acid buffer (both Invitrogen), pH 8. LVV-containing supernatant was harvested approximately 24, 48 and 72 hours after transfection. LVV was concentrated by ultracentrifugation at 20,000 rpm for 2 hours at 4 °C in a swing-out rotor ultracentrifuge (Beckman Coulter). After centrifugation, the vector was re-suspended, aliquoted and stored long term at -80 °C. The concentrated viral titer was determined by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) to be between $10^8$ and $10^9$ copies/ml.
pAAV5-hSyn-eNpHR3.0-2A-ChR2-EYFP (AAV-eNPAC [31] serotype 5, purchased from UNC vector core, titres $1x10^{12}$ vector genome(vg)/ml), carrying a strong pan-neuronal promoter, and pLenti-Camk2a-NpHR2.0–EYFP/pLenti-Camk2a-ChR2-mCherry, carrying a promoter specific for excitatory neurons, were used as a control vectors for in vivo experiments.

**Viral Transduction in Vitro**

Neuronal cultures from the medial ganglionic eminence (embryonal day 14) were a gift of W. Andrews (J. Parnavelas laboratory, Department of Cell and Developmental Biology, UCL). Day 7 neuronal cultures were transduced with serial dilutions of the LVV. Images were taken using a Leica Fluorescence microscope. Cultures were incubated at 37 °C for 7–14 days before fixation in 4 % paraformaldehyde for subsequent immunofluorescence.

**Stereotactic Surgery and Viral Transduction in Vivo for Targeting Opsins to Interneurons**

Male postnatal day 20 (p20) Sprague-Dawley rats were used for in vivo viral vector injections. The correct coordinates for the hippocampal injection site were established through calibration

experiments by injection of Trypan Blue into the target area. All animals were housed on a 12-hour light/dark cycle in a temperature- and humidity-controlled environment with free access to food and water. All efforts were made to minimize animal suffering and to reduce the number of animals used. Animal experiments were conducted in accordance with the Animals (Scientific Procedures) Act 1986.

For stereotactic injections each rat was anaesthetized with isoflurane (Forane; Abbot). Animals were placed in a stereotactic frame (Kopf instruments). Surgery was performed under a Leica S6E microscope. LVV was injected directly with a Hamilton syringe (5 µl Hamilton Syringe, 900 Series, liquid tight with reinforced plunger; 33 G blunt needle; injection rate 200 nl/min with a microinjector unit). Injection volume was of 1 µl to cortex at the following stereotaxic coordinates AP -1.5 , L -1.5, V -1.45, and 1.5 µl to dorsal CA1/3 at AP -2.8 , L +3.6, V -2.9 and ventral CA3: AP -4.1, L +4.45, V +5.35. Before lightening anaesthesia, rats were injected with 0.05 mg/kg subcutaneous (s.c.) buprenorphine analgesia and 1–2 ml s.c. 0.9% saline and the animal allowed to recover.

**Tissue Processing**

Animals were sacrificed by an overdose of pentobarbitone up to 6–8 weeks following viral vector injection. For immunohistochemistry, rats were perfused transcardially with ice-cold artificial cerebrospinal fluid, decapitated and the brains removed and post-fixed for 24 h in 4% paraformaldehyde (PFA; adjusted to pH 7.4 with 1 M HCl) at 4°C. They were then transferred to a solution containing 30% sucrose in 1x phosphate buffered saline (PBS) and left at 4°C until they had sunk. Using a Leica freezing vibratome, 30 µm coronal sections were cut through the targeted brain regions.

**Immunohistochemistry**

Immunohistochemical staining was conducted on free floating sections. Sections were washed in PBS and permeabilized in 0.2% Triton X-100 for 10 minutes and then blocked with DAKO-blocking medium (dako) for 1 hour on a shaker. Series of sections were incubated for 2 days in the primary antibodies against γ-amino butyric acid (GABA, 1:100-1:200, Thermo Scientific, PA1-18027), cholecystokinin-8 (CCK-8, 1:100-1:200, Thermo Scientific, PA1-18016), Camk2a (1:100-1:200, Thermo Scientific, #MA1-048), green-fluorescent protein (GFP, 1:3000, abcam, ab13970 or aves 1:800), dsRed and mCherry (1:200, Living Colors®; DsRed or mCherry Monoclonal Antibody, catalogue no. 632392 and 632543). Following washing in PBS (3 x 15 min), the sections were incubated in secondary antibodies (1:1000, all abcam and labelled with Alexa Fluor (AF) 488, AF 568 or Tetramethylrhodamine (kindly gifted by A. Cariboni and J. Parnavelas) overnight at 4°C. After further washing in PBS, sections were incubated in 4´,6-Diamidino-2-phenylindole dihydrochloride (DAPI) to visualize cell nuclei. Free-floating sections were mounted onto microscope slides, allowed to dry and then coverslipped using Mowiol Mounting Medium (gift of A. Cariboni and J. Parnavelas). The same protocol was used to stain cultured neurons. As fixation

with 4% PFA greatly reduces the fluorescent signal, constructs carrying NpHR-EYFP were always stained with primary antibodies against EYFP and secondaries labelled with AF 488 to detect expression of the vector, constructs carrying ChR2-mCherry were counterstained with primaries against dsRed or mCherry and secondaries labelled with AF 561 or Tetramethylrhodamine. For colocalization studies, primary antibodies against GABA, CCK and Camk2a were labelled with secondaries conjugated to a red fluorophore (for the NpHR-EYFP expressing slices) or a green fluorophore (for the ChR2-mCherry expressing slices). Slices from adult male GAD67-GFP mice expressing GFP in interneurons were used as positive controls for anti-GAD67, anti-CCK and anti-Camk2a antibodies (Supplementary Fig. 1).

**Morphological Analysis**

The evaluation and acquisition of morphological data were performed on a Leica DM2500 Upright confocal microscope with a 40x oil immersion objective and the following excitation laser lines: 488 nm, 532 nm, 405 nm. Slices were first analyzed by fluorescence microscopy for expression of the fluorochrome in the targeted area. Image acquisition was performed with Leica LAS AF software. The colocalization of opsin expressing cells and neuronal or glial cell markers (GABA, CCK, Camk2a) was analyzed in the cortex and hippocampus. To compare the extent of viral vector expression volumes of different constructs, the size of the area exhibiting EYFP fluorescence was measured at 5–10-fold magnification in the cortex and hippocampus. Volumetric analysis was performed using Octave open source analysis software. Immunostained sections were digitized by means of a high-resolution video camera. All stained sections were evaluated. Image analysis and cell counting was performed using GIMP (GNU Image Manipulation Program) and Image J open source software.

## 3. Results

GAD67- and CCK-driven plasmids were first tested in human embryonic kidney (HEK) cells by transfection, to ensure that cloning of the GAD67 and CCK promoters allowed transcription of the downstream opsin-fluorophore construct (Supplementary Fig. 2). Transduction of viral particles was then assessed in neuronal cultures.

**CCK- and GAD67-driven NpHR-EYFP expresses in ganglionic eminence neuronal cultures**

We transduced neuronal cultures from the ganglionic eminence (GE; kindly provided by A. Zito and W. Andrews, Parnavelas lab, UCL) as a culture model rich in inhibitory cells (Fig.1). In rodents, GAD67 mRNA is expressed from embryonic day 10.5 (E10.5) [32], CCK mRNA from E12.5 [33]. E14 cultures were transduced at days 5-7 in vitro with $10^6$ copies/ml LVV per well and imaged two weeks later. Fluorescence was visible for LV-GAD67-NpHR2.0-EYFP and LV-CCK-NpHR2.0-EYFP: ~90 % of cells expressing LV-CCK-NpHR2.0-EYFP also stained for anti-CCK antibody

(19/21 cells, 4 visual fields (VFs)). 40% of LV-GAD67-NpHR2.0-EYFP expressing neurons were also anti-GABA antibody positive(+) (73/181 cells, 4 VFs). Our culture data suggests that LV-CCK-NpHR2.0-EYFP may be useful in targeting CCK+ interneurons in GE cultures.

**GAD and CCK driven opsin vectors are not specific for interneurons in vivo**

Fluorescence was visible in unstained, acutely sliced sections of rodent brain injected with LVV encoding NpHR2.0-EYFP under either Camk2a, GAD67 or CCK-promoters and AAV-eNPAC (Table 1, Supplementary Fig. 3, Supplementary Table 1), the highest achievable viral titre for each construct was used (ranging from 3 – 5 x $10^8$ copies ml for LVV and $10^{12}$ vg/ml for AVV) in both cortex and hippocampus. Conversely, LV vectors expressing ChR2-mCherry under the Camk2a, GAD67 and CCK promoters were only visible on stained sections (Fig. 2 and 4). This may have been due to low expression levels, or to protein misfolding, allowing ChR2-mCherry to become visible only after immunostaining.

The vector LV-GAD67-ChR2-mCherry was not selective for interneurons in CA1 and CA3: only 7.5% (5/67) of cells expressing LV-GAD67-ChR2-mCherry were also GABA positive (Fig.2a-d), whilst of all GABA+ interneurons, only 2.6% (5/193) expressed LV-GAD67-ChR2-mCherry. 2.4% (27/1121) of pyramidal cells also expressed the transgene (Fig.2e).

In a separate set of experiments we tested the vector LV-GAD67-NpHR2.0-EYFP: 82% (91/110) of LV-GAD67-NpHR2.0-EYFP expressing neurons were positive for Camk2a (Fig. 3a-c, g), 21% (43/207) were GABA+ (Fig. 3d-f, h). Approximately 15% (43/279) of GABA+ interneurons expressed LV-GAD67-NpHR2.0-EYFP.

LV-CCK-ChR2-mCherry stained very few neurons in the hippocampal pyramidal layer, and expression was difficult to find by microscopy despite repeated experiments. Eight LV-CCK-ChR2-mCherry - positive neurons were identified in four visual fields, seven of which co-stained with anti-GABA antibodies (Fig. 4a-d). The difficulty in visualizing ChR2-mCherry in vivo was thought to be due to either low expression levels or to opsin-fluorophore misfolding, as the antibodies targeted to mCherry demonstrated that the protein had been expressed.

26% (9/34) of LV-CCK-NpHR2.0-EYFP expressing neurons were positive for GABA antibody. LV-CCK-NpHR2.0-EYFP stained 2% (2/98) of anti-Camk2a (Fig.5a-c, g), and 47% (9/19) of anti-GABA (Fig.5d-f, h) positive neurons.

**Expression volumes of GAD/CCK driven constructs are small**

The total volume of viral vector expression was calculated for a whole injection area per vector by summing the fluorescent volume of each slice (area exhibiting strongly fluorescent neurons under 5x or 10x magnification multiplied with thickness of the slice). In the hippocampus, expression volumes of GAD67 promoter driven constructs were larger than those of CCK-driven vectors. The control vector AAV-eNPAC achieved the largest expression volumes (Fig.6 and Table 1). In the cortex, expression volumes were again largest for AVV injected animals (Supp. Fig. 3 and Supp.

Table 1).

## Discussion

### GAD67 Promoter-driven Opsins

Expression of the GAD67 promoter-driven opsin constructs was not specific for interneurons both in cultures and slices. The GAD67 promoter construct used in our vectors contains 3.7 kb of 5' upstream sequences ('short' GAD67 promoter [21, 22]), containing the first intron and part of the second exon of the GAD67 promoter. This 'short' promoter has been previously used to target GABAergic interneurons in the nucleus of solitary tract in organotypic slice cultures [22]. If increasingly longer 5' upstream regulatory sequences are included in the GAD67 promoter construct [21], the expression of reporter genes becomes more specific. However, transgenic mouse lines containing up to 7 kb of 5' upstream sequences ('medium' and 'long' promoters) of the GAD67 promoter still showed variable staining of hippocampal interneurons [21], as well as ectopic expression in hippocampal pyramidal neurons that express low levels of GAD mRNA [34]. Only the longest construct (9 kb of 5' upstream sequences) correctly labelled cerebellar Purkinje cells, whilst still showing patterns of mixed correct and ectopic expression both in hippocampus and cortex [21]. In concordance with these observations, the 'short' GAD67 promoter we used, may contain all regulatory sequences for correct expression in brain regions of high GABA content, which develop early [21], but may not be specific for the targeting of interneurons in the hippocampus or cortex.

### CCK Promoter-driven Opsins

In cultures from the ganglionic eminence Nearly all cells expressing CCK-driven NpHR-EYFP were positive for CCK, although only a limited number of cells was tested. In vivo, however, CCK-driven expression levels were small and less specific, making these vectors unsuitable for electrophysiology applications. The CCK promoter (gift of K. Ressler) has been used to label interneurons of the hippocampus and the basolateral amygdala [23, 24], which were counterstained with CCK-mRNA in situ hybridization techniques. In our experiments, LV-CCK-NpHR2.0-EYFP achieved good expression in the polymorphic layer of the dentate gyrus, but stained only few cells in CA1 which were undoubtedly pyramidal, both in their morphology and antibody staining pattern (Fig. 3d). One possible explanation for the discrepancy of these results may be that neurons expressing CCK-mRNA are different or more numerous than those also expressing the CCK octopeptide, e.g. CCK-mRNA is found in the somata of neurons in many brain regions including CA3 pyramidal cells [35]. Staining for the CCK peptide, rather than using in situ hybridization, may be a more specific way to detect expression of the actual neurotransmitter.

**Choice of Viral Vectors**

AVVs serotype 2 and 5 are known to transduce mostly neurons [36] and our opsins delivered with AVV were strongly expressed within the hippocampus and cortex, and in a larger volume than constructs delivered with lentiviruses. AVV preparations are known to achieve higher titres than LV-vector preparations, and due to their smaller size (~ 25nm) [36], AAV vector particles (size ~100 nm) spread over a larger volume than LV-vectors. A diffuse spread of vector has the additional advantage that a single injection may be sufficient for experimental or future therapeutic purposes. The motivation to use lentiviral vectors, was their large transgene capacity (8-10 kb in LV vs 4.5 kb in AAV), which allowed us to clone the relatively large GAD67 and CCK promoter sequences (3.5 and ~3 kb, respectively) ahead of the opsin-fluorophore constructs. An alternative approach may involve splitting large transgenes onto 2 complementary AAVs, but the techniques are complex, and the success rate in generating the constructs may be lower. A further disadvantage of AVVs is their potential for inducing neutralizing antibodies. Conversely, LVVs are relatively invisible to the immune system, as viral proteins are not expressed on the surface of transduced cells. This is a clear advantage if considering viral vectors as future therapeutic tools.

**Conclusion**

The ability to study and influence the activity of excitatory and inhibitory interneurons separately, is one of the main goals of modern neuroscience and a requirement for future gene therapy of nervous system diseases. Opsins are ideal candidates to accomplish this task, but targeting separate neuronal populations with GAD67 and CCK promoter-carrying vectors is not possible in their current form. More work is required to understand, at the molecular level, which regulatory elements and epigenetic mechanisms determine promoter function and how cell-type specific expression is achieved in inhibitory interneurons.

## Figure Legends

**Figure 1 CCK- and GAD67-driven opsins express in ganglionic eminence neuronal cultures. LV-CCK-NpHR2.0-EYFP (a-c and g)**: Approximately 90% (19/21) of LV-CCK-NpHR2.0-EYFP expressing neurons (**a**, in green) also stain for anti-CCK antibody (**b**, in red). Overlay in **c**. Arrows depict examples of colocalizing neurons, arrowheads non-colocalizing neurons. **LV-GAD67-NpHR2.0-EYFP (d-f and h)**: 40% (73/181) of LV-GAD67-NpHR2.0-EYFP expressing neurons (**d**, in green) also stain for GABA-antibody (**e**, in red). Overlay in **f**. Arrows and arrowheads used as above. (Antibody (Ab), positive (+).)

**Figure 2 Immunofluorescence study of LV-GAD67-ChR2-mCherry expression in hippocampus and cortex. (a)** LV-GAD67-ChR2-mCherry-expressing neurons (in red, arrowheads) do not colocalize with **(b)** anti-GABA positive interneurons (in green). **(c)** overlay (with DAPI). Experiments with anti-GABA and anti-CaMKIIa antibodies were not performed in the same slices. **(d)** 7.5% of vector expressing cells (5/67) were positive for GABA (n=3 slices, 20 VF counted, 1 animal, total number of cells in brackets). The remaining vector expressing neurons may have been non GABA-ergic neurons, e.g. excitatory principal neurons, cholinergic non-GABAergic non-principal cells, granule cells.**(e)** 30% (27/91) of vector expressing cells were excitatory neurons and stained with Camk2a antibody (n=4 slices, 22 VF counted, 3 animals, total number of cells in brackets). The remainder of vector expressing cells may have been GABAergic neurons, cholinergic non-GABAergic non-principal cells, principal cells expressing a different CaMKII isoform, granule cells or neurogliaform cells. Whilst the % of positive neurons will depend on the viral titre, we used the highest titre achievable in our hands, which resulted in non-specific staining. Lower titres were not tested as they were predicted to achieve even lower expression. The scale bar is 25 μm for all images. Stratum radiatum (rad.), pyramidale (pyr.; dotted lines) and oriens (or); positive (+); visual field (VF).

**Figure 3 Colocalization of LV-GAD67-NpHR2.0-EYFP with Camk2a or GABA in hippocampus. (a-c, g)** LV-GAD67-NpHR2.0-EYFP-expressing neurons in green, anti-Camk2a positive pyramidal cells in red and overlay with DAPI to visualize cell nuclei. Arrows point to colocalizing, arrowheads to non-colocalizing neurons. 82% (91/110) of LV-GAD67-NpHR2.0-EYFP expressing neurons are pyramidal cells (12 VF, 2 animals, total number of cells in brackets). **(d-f, h)** LV-GAD67-NpHR2.0-EYFP-expressing neurons in green, anti-GABA positive interneurons in red and overlay with DAPI (23 VF, 3 animals, total number of cells in brackets). Arrows and arrowheads used as above. The scale bar is 25 μm for all images. Stratum radiatum (rad.), pyramidale (pyr.; dotted lines) and oriens (or); visual field (VF).

**Figure 4 Immunofluorescence and colocalization of LV-CCK-ChR2-mCherry in stratum oriens (or). (a)** LV-CCK-ChR2-mCherry-expressing neurons in red (arrows), **(b)** anti-GABA positive cells in green and **(c)** overlay with DAPI to visualize cell nuclei. (Arrows indicate colocalizing, arrowheads not co-localizing cells). **(d)** 7/14 anti-GABA antibody stained neurons also expressed LV-CCK-ChR2-mCherry (4 VFs, 1 animal). 7/8 LV-CCK-ChR2-mCherry positive cells stained for anti-GABA antibody. Numbers of cells counted in brackets. The scale bar is 25 μm for all images; visual field (VF).

**Figure 5 Colocalization study of LV-CCK-NpHR2.0-EYFP in hippocampus. (a-c, g)** LV-CCK-NpHR2.0-EYFP-expressing neurons in green, anti-Camk2a positive pyramidal cells in red and

overlay with DAPI to visualize cell nuclei (n=7 VF, 3 slices, 2 animals). Arrowheads points to LV-CCK-NpHR2.0-EYFP-expressing neurons, which do not colocalize with anti-Camk2a+ cells. **(d-f, h)** LV-CCK-NpHR2.0-EYFP-expressing neurons in green, anti-GABA positive interneurons in red and overlay with DAPI (n=3 VF counted from 2 animals). Arrowheads points to LV-CCK-NpHR2.0-EYFP-expressing neurons, which do not colocalize with anti-GABA+cells. Numbers of cells counted in brackets. Stratum radiatum (rad.), pyramidale (pyr.; dotted lines) and oriens (or); visual field (VF).

**Figure 6 Comparison of expression levels in hippocampus.** Fluorescence micrographs showing extent of expression in hippocampus injected with 1.5 µl of either **(a)** LV-CCK-NpHR2.0-EYFP, **(b)** LV-GAD67-NpHR2.0-EYFP (both 10x magnification), or **(c)** 1 µl AAV-eNPAC (5x magnification). Slices were counterstained with anti-GFP antibodies and AF 488 secondary antibodies to amplify the GFP signal; Stratum radiatum (rad.), pyramidale (pyr.; dotted lines) and oriens (or); visual field (VF).

**Table 1 Comparison of expression in hippocampal slices injected with LVVs carrying different promoters and LVV vs. AVV**

## Supplementary Figure Legends

**S1 Immunohistochemistry on control sections**

Fluorescent micrographs from GAD67-GFP mice used as positive controls for anti-GAD67, anti-CCK and anti-Camk2a antibodies. **(a), (d), (g)** Interneurons expressing GFP under the GAD67 promoter. **(b)** Anti-Camk2a stains neurons in the pyramidal layer of the hippocampus (n = 97) and none of the GFP+ neurons (n = 23; arrowheads). **(c)** shows overlay images of the green **(a)**, red **(b)** and blue (DAPI) channels. **(e)** All GABA-antibody stained neurons (n = 14) expressed green fluorescence (arrows). **(f)** showing overlay images of **(d), (e)** and the blue (DAPI) channel. **(h)** CCK-antibody stained neurons in red **(i)** overlay image: 80% (7/9) of CCK+ neurons (red) expressed GFP (green) . One third of GFP expressing interneurons (green) also stained for CCK-antibody (9/27; arrows). Stratum radiatum (rad.), pyramidale (pyr.; dotted lines) and oriens (or).

**S2 Transfected HEK cells express transgene**

Cloned lentiviral constructs and assessment of promoter activity in vitro. **(a-c)** Left panels: schematic of the viral vector; right panels: fluorescence micrographs of transfected HEK cells. Micrograph a2 and b2 were taken with a different filter setting.

**S3 Expression volumes in cortex**

Comparison of expression levels in cortex. Fluorescence Micrographs at 10x magnification

obtained from cortex of animals injected with **(a)** LV-CCK-NpHR2.0-EYFP, **(b)** LV-Camk2a-NpHR2.0-EYFP and at **(c)** 5x magnification showing expression of AAV-eNPAC. Continuous line denotes the area of fluorescence, dashed line the pial surface. Slices were counterstained with anti-GFP antibodies and AF 488 secondaries to amplify the GFP signal; injection volume 1–1.25 μl.

**S Table 1 Comparison of expression in cortical slices injected with LVV vs. AVV.**

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**Conflicts of Interest Statement**

The authors declare that they have no conflicts of interest.

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## Tables

### Table 1

| Hippocampus | LV-CCK-NpHR2.0-EYFP | LV-GAD67-NpHR2.0 EYFP | AAV5-eNpHR3.0-2A-ChR2-EYFP (eNPAC) |
|---|---|---|---|
| Viral Titre | $10^8$ copies/ml | $10^8$ copies/ml | 2 x $10^{12}$ vg/ml |
| Injection Volume | 1.5 µl | 1.5 µl | 1 µl |
| Expression Volume | 0.009 mm³ | 0.013 mm³ | 0.08 mm³ |

### Supplementary Table 1

| Cortex | LV-Camk2a-NpHR2.0-EYFP | AAV5-eNpHR3.0-2A-ChR2-EYFP (eNPAC) |
|---|---|---|
| Viral Titre | $10^8$ copies/ml | 2 x $10^{12}$ vg/ml |
| Injection Volume | 1.25 µl | 1 µl |
| Expression Volume | 0.04 mm³ | 0.09 mm³ |

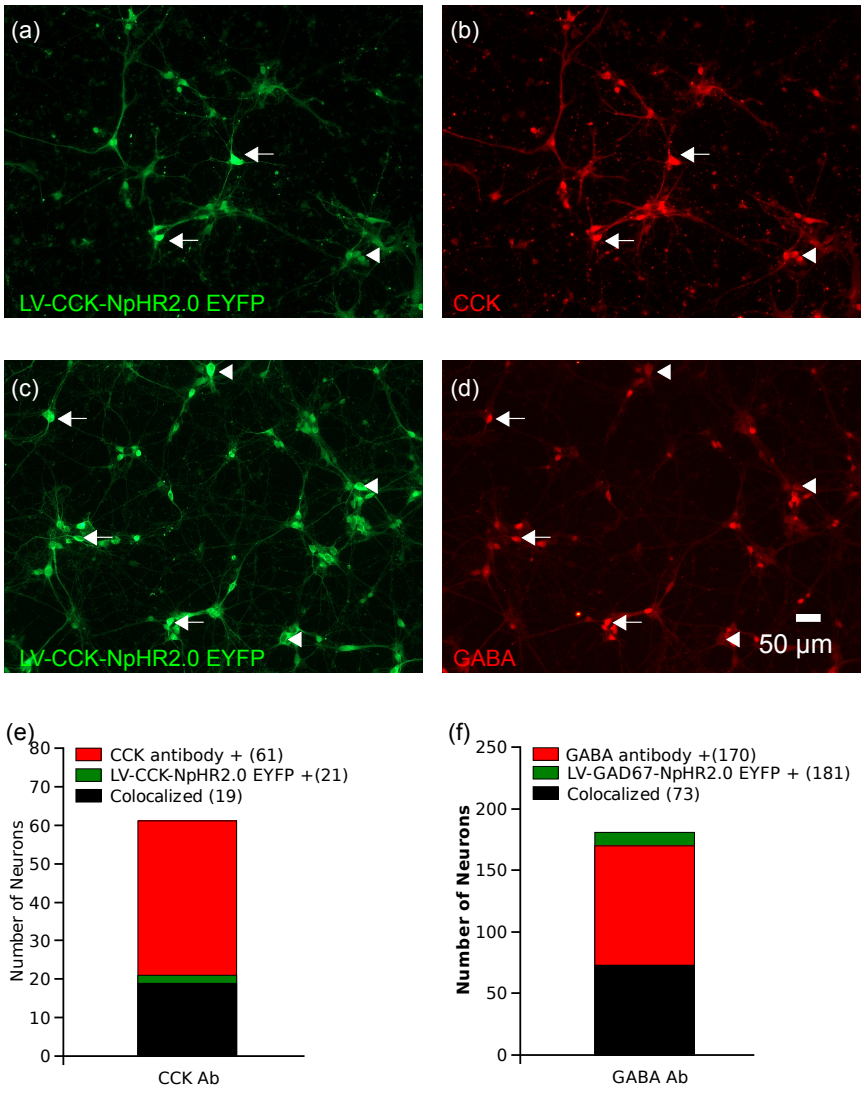



Figure 1 CCK- and GAD67-driven opsins express in ganglionic eminence neuronal cultures.

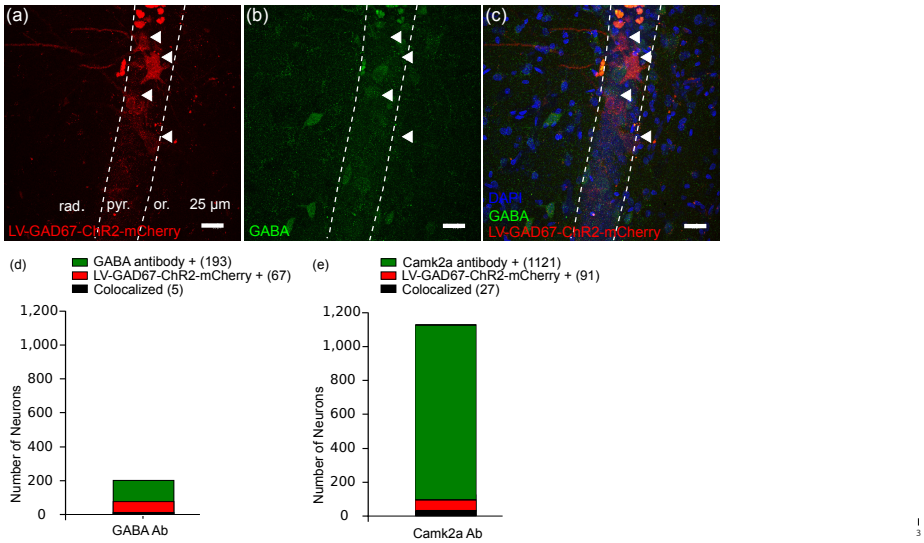



Figure 2 Immunofluorescence study of LV-GAD67-ChR2-mCherry expression in hippocampus and cortex.

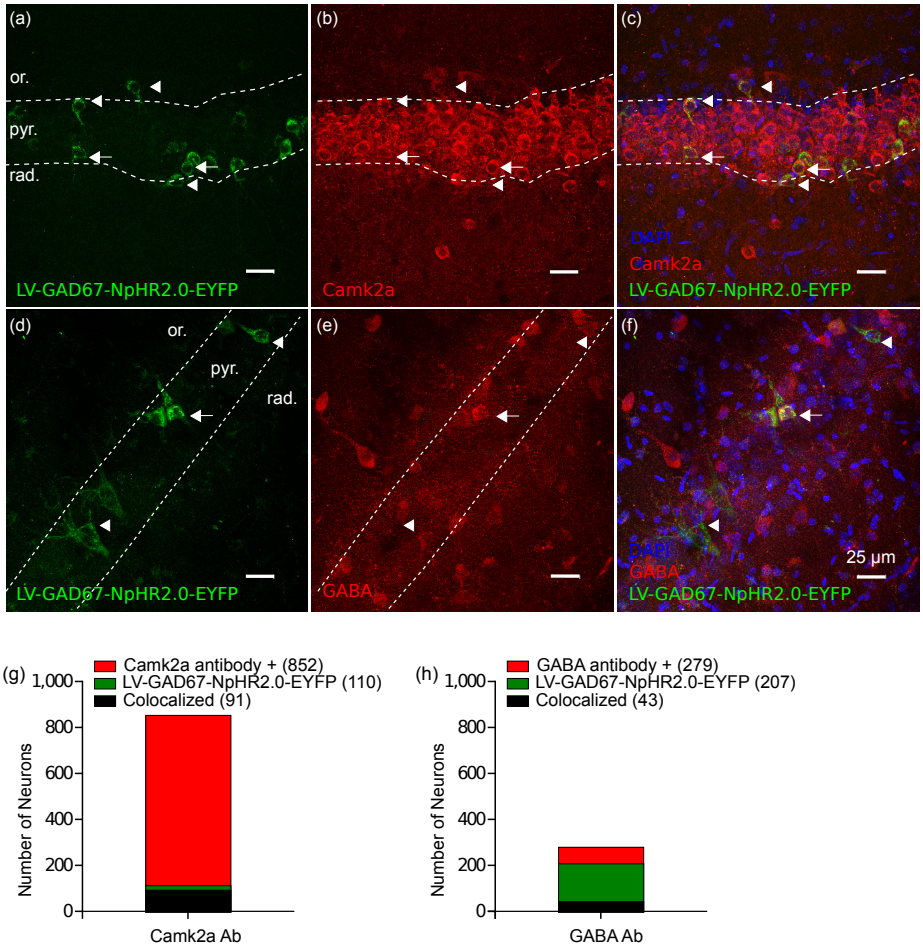



Figure 3 Colocalization of LV-GAD67-NpHR2.0-EYFP with Camk2a or GABA in hippocampus.

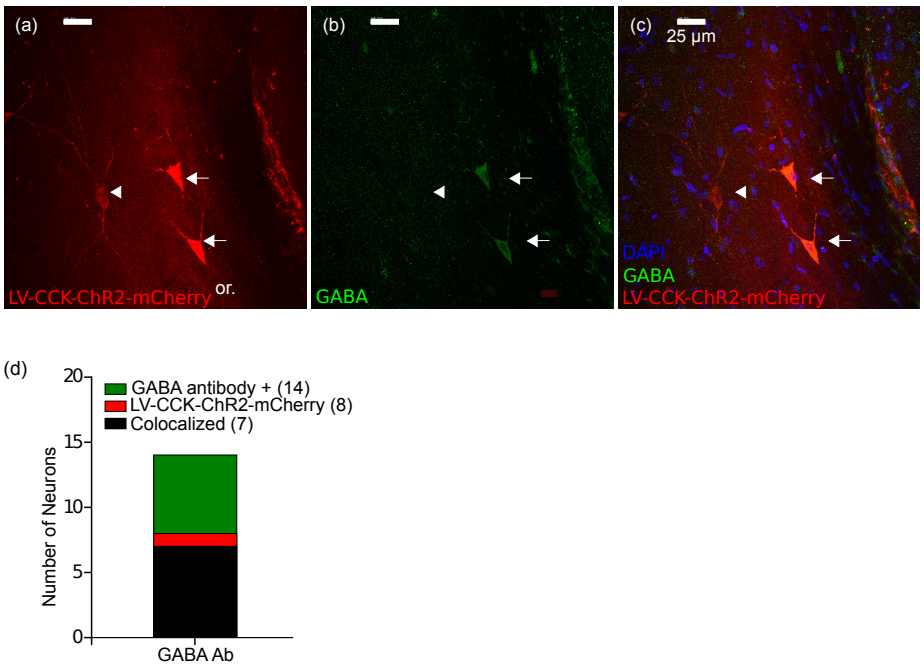



Figure 4: Immunofluorescence and colocalization of LV-CCK-ChR2-mCherry in stratum oriens (or).

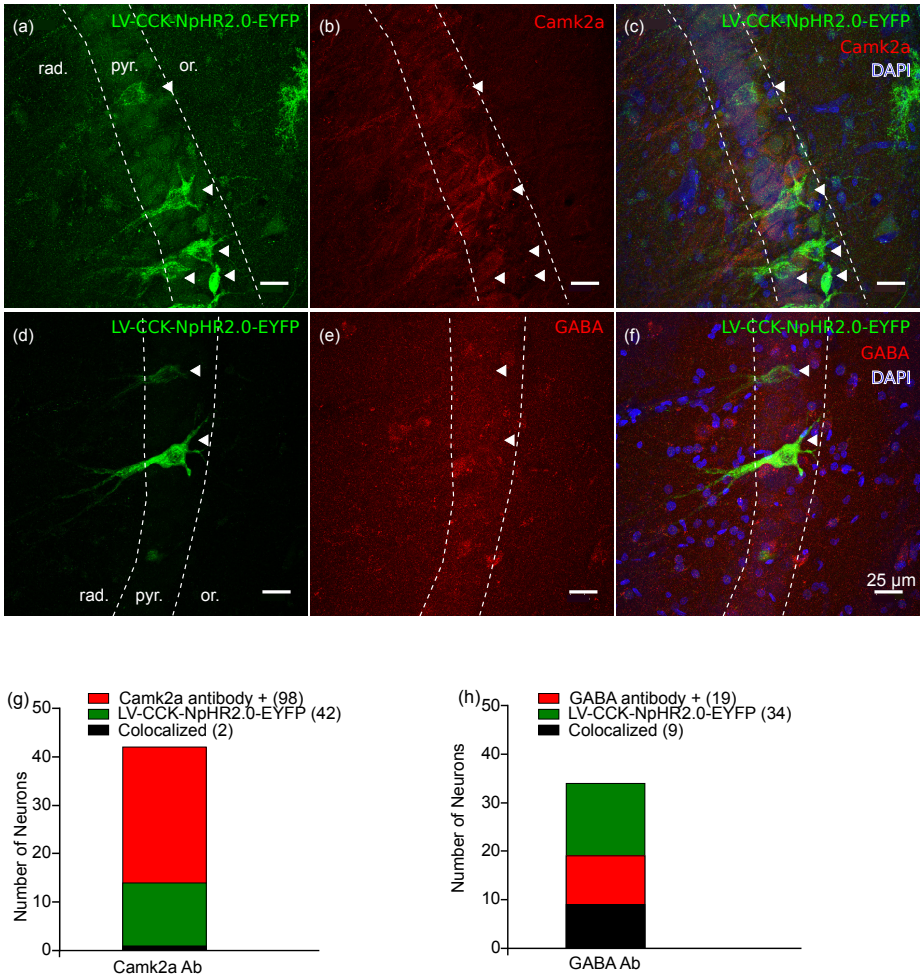



Figure 5: Colocalization study of LV-CCK-NpHR2.0-EYFP in hippocampus.

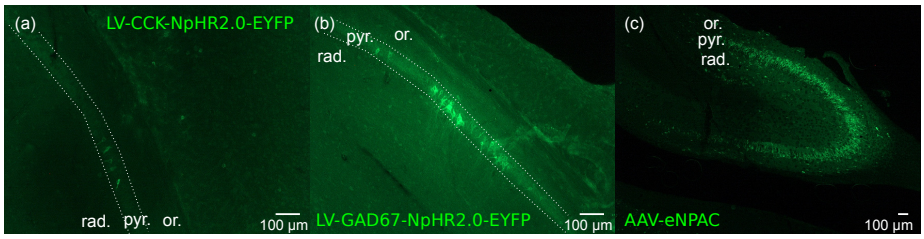
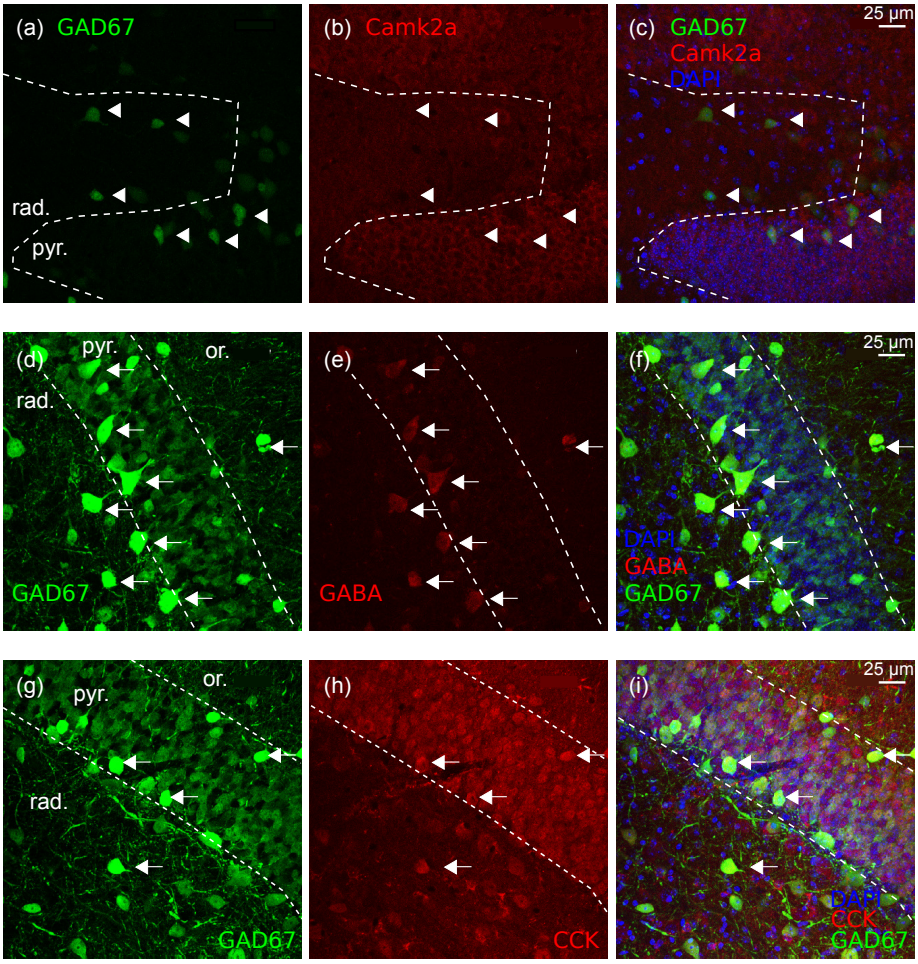


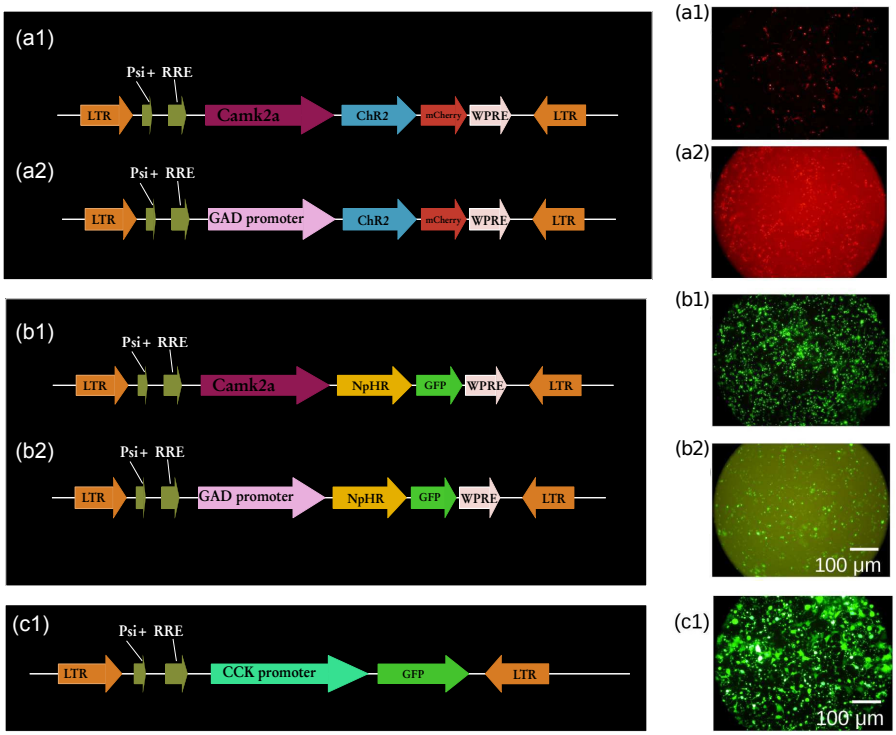

Figure 6: Comparison of expression levels in hippocampus.

Table 1: Comparison of expression in hippocampal slices injected with LVs carrying different promoters and LV vs. AAV .

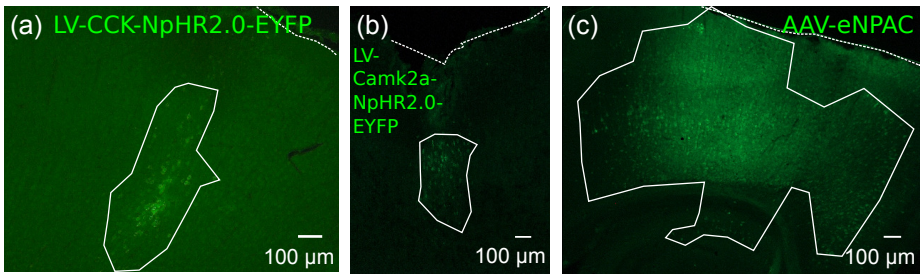
| **Hippocampus** | **LV-CCK-NpHR2.0-EYFP** | **LV-GAD67-NpHR2.0 EYFP** | **AAV5-eNpHR3.0-2A-ChR2-EYFP (eNPAC)** |
|---|---|---|---|
| **Viral Titre** | $10^8$ TU/ml | $10^8$ TU/ml | 2 x $10^{12}$ TU/ml |
| **Injection Volume** | 1.5 µl | 1.5 µl | 1 µl |
| **Expression Volume** | 0.009 $mm^3$ | 0.013 $mm^3$ | 0.08 $mm^3$ |




S1 Immunohistochemistry on control sections




S2 Transfected HEK cells express transgene




S3 Expression volumes in Cortex

S Table 1 Comparison of expression in cortical slices injected with LV vs. AAV.

| **Cortex** | **LV-Camk2a-NpHR2.0-EYFP** | **AAV5-eNpHR3.0-2A-ChR2-EYFP (eNPAC)** |
|---|---|---|
| **Viral Titre** | $10^8$ TU/ml | 2 x $10^{12}$ TU/ml |
| **Injection Volume** | 1.25 µl | 1 µl |
| **Expression Volume** | 0.04 $mm^3$ | 0.09 $mm^3$ |