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ORIGINAL ARTICLE

Spinal nociceptive circuit analysis with recombinant adeno-associated viruses: the impact of serotypes and promoters

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Abstract

Recombinant adeno-associated virus (rAAV) vector-mediated gene transfer into genetically defined neuron subtypes has become a powerful tool to study the neuroanatomy of neuronal circuits in the brain and to unravel their functions. More recently, this methodology has also become popular for the analysis of spinal cord circuits. To date, a variety of naturally occurring AAV serotypes and genetically modified capsid variants are available but transduction efficiency in spinal neurons, target selectivity, and the ability for retrograde tracing are only incompletely characterized. Here, we have compared the transduction efficiency of seven commonly used AAV serotypes after intraspinal injection. We specifically analyzed local transduction of different types of dorsal horn neurons, and retrograde transduction of dorsal root ganglia (DRG) neurons and of neurons in the rostral ventromedial medulla (RVM) and the somatosensory cortex (S1). Our results show that most of the tested rAAV vectors have similar transduction efficiency in spinal neurons. All serotypes analyzed were also able to transduce DRG neurons and descending RVM and S1 neurons via their spinal axon terminals. When comparing the commonly used rAAV serotypes to the recently developed serotype 2 capsid variant rAAV2retro, a > 20-fold increase in transduction efficiency of descending supraspinal neurons was observed. Conversely, transgene expression in retrogradely transduced neurons was strongly reduced when the human synapsin 1 (hSyn1) promoter was used instead of the strong ubiquitous hybrid cytomegalovirus enhancer/chicken β-actin promoter (CAG) or cytomegalovirus (CMV) promoter fragments. We conclude that the use of AAV2retro greatly increases transduction of neurons connected to the spinal cord via their axon terminals, while the hSyn1 promoter can be used to minimize transgene expression in retrogradely connected neurons of the DRG or brainstem.

Keywords: dorsal horn, rAAV-mediated gene transfer, sensory circuits, spinal cord.

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Recombinant adeno-associated virus (rAAV) vector-mediated gene transfer for expression of effector proteins such as pharmacogenetic receptors, bacterial toxins, or channelrhodopsin is a powerful tool for specific manipulation of neurons (Schon *et al.* 2015; Fu and McCarty 2016; Montgomery *et al.* 2016). Local injection of rAAV vectors

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Abbreviations used: CAG, hybrid cytomegalovirus enhancer/chicken β-actin promoter; CGRP, calcitonin gene-related peptide; C-LTMR, c-low threshold mechanoreceptor; CMV, cytomegalovirus; DRG, dorsal root ganglia; eGFP, enhanced green fluorescent protein; hSyn1, human synapsin 1 promoter; L4/5, lumbar segment 4/5; NF200, neuro-filament 200; PFA, paraformaldehyde; rAAV, recombinant adeno-associated virus; RVM, rostral ventromedial medulla; S1, somatosensory cortex; TH, tyrosine hydroxylase.

provides a fast approach to the analysis of neuronal morphology and function at well-defined sites. When combined with the FLEX system (a Cre LoxP-based system for irreversible inversion and activation of coding sequences contained in rAAV vectors) it also enables the manipulation of specific genetically defined subsets of neurons (Atasoy *et al.* 2008). In addition, by injecting rAAV.FLEX vectors only at adult stages into Cre transgenic mice, undesired more widespread recombination, for example, resulting from embryonic activity of a Cre driver, can be avoided. Using intraspinal injection of Cre-dependent rAAV vectors into Cre transgenic mice it has thus become possible to specifically identify and interfere with dedicated spinal sensory circuits. Several groups have addressed the function of genetically defined dorsal horn neuron populations by ablating, silencing, or activating specific spinal neuron-subpopulations via rAAV vector-mediated gene transfer. (Foster *et al.* 2015; Peirs *et al.* 2015; Petitjean *et al.* 2015; Cui *et al.* 2016; Francois *et al.* 2017).

Spinal injection of rAAVs can be used to interfere with neurons located at the injection site or, via retrograde transduction, with neurons that project to the injection site. Therefore, depending on the type of study, retrograde transduction should either be maximized or avoided. In case of studies that specifically address the function of local dorsal horn neurons, retrograde transduction of dorsal root ganglia (DRG) neurons is a particular concern. We therefore tested whether undesired expression of viral transgenes in pre-synaptically connected neurons can be reduced through the use of specific serotypes or promoters. We found that all tested rAAV serotypes not only showed similar transduction efficiencies of spinal neurons and spinal neuron subtypes but were also capable of transducing axon terminals of DRG neurons and descending projection neurons located in the rostral ventromedial medulla (RVM) or somatosensory cortex (S1) with similar efficacies. Conversely, drastically reduced numbers of retrogradely labeled DRG neurons and descending projection neurons were found for rAAVs in which transgene expression was driven by the human synapsin 1 promoter (hSyn1) instead of the hybrid cytomegalovirus enhancer/chicken β-actin promoter (CAG) or cytomegalovirus (CMV) promoters. On the other hand, by far the most efficient AAV in transducing supraspinal descending projection neurons was the recently developed AAV serotype 2 capsid variant rAAV2retro (Tervo *et al.* 2016).

Materials and methods

Mice

All injections were carried out in C57B/6 mice. Mice were originally purchased from The Jackson Laboratory and colonies were maintained by the Laboratory Animal Services Center (LASC) of the University of Zurich. Mice of both sexes were used in this study. Animals were kept in a 12 h light/dark cycle and received food *ad libitum*. No animals had to be excluded from the analysis. All animal experiments were approved by the Swiss cantonal veterinary office (Zurich).

Immunohistochemistry and image analysis

Immunohistochemistry was essentially performed as previously described (Punnakkal *et al.* 2014). In brief, mice were transcardially perfused with 80 mL of 4% ice-cold paraformaldehyde (in 0.1 M Sodium phosphate buffer, pH 7.4). Lumbar spinal cord, brain tissue, and the part of the vertebral columns containing the lumbar DRGs were immediately dissected and post-fixed for 2 h with 4% paraformaldehyde on ice. Post-fixed tissue was briefly washed with 0.1 M Sodium phosphate buffer (pH 7.4) and then incubated in 25% sucrose (in 0.1 M Sodium phosphate buffer, pH 7.4) overnight at 4°C. Cryoprotected tissue was cut at 16 µm (DRGs) or 35 µm (spinal cord or brain) on a Hyrax C60 Cryostat; Zeiss, Oberkochen, Germany, mounted on superfrost plus glass slides and then incubated with the respective combinations of primary antibodies in 5% donkey serum in phosphate-buffered saline (PBS) over night at 4°C. After brief washes in PBS, sections were incubated with the respective secondary antibodies for 30–45 min at 23–25°C and briefly rinsed in PBS, before mounting with coverslips and DAKO fluorescent mounting media; Dako, Carpinteria, CA, USA. All primary antibodies used are listed in the Table 1. Secondary antibodies raised in donkey were purchased from Jackson Immuno-Research, West Grove, PA, USA (RRID:SCR_010488).

Z-stacks of fluorescent images were acquired on a Zeiss LSM710 Pascal confocal microscope; Zeiss, Oberkochen, Germany. Numbers of immunoreactive cells in z-stacks were determined using the ImageJ (RRID:SCR_003070) Cell Counter plugin (Kurt De Vos, University of Sheffield, Academic Neurology).

Quantification

For quantitative analyses, sections were prepared from at least three animals and at least three (maximal 5) sections per animal were analyzed. No sample size calculation was performed. No blinding was performed. Percentages in Figs 1, 4, 5, and 6 represent percentages of the neuron population labeled with the indicated marker (e.g., NeuN, labeling all neurons or tyrosine hydroxylase (TH); labeling the c-low threshold mechanoreceptor (C-LTMR) subset of DRG neurons). In Fig. 3 the total number of neurons found after quantifying every third section of the injected mice is depicted. The eGFP fluorescence intensity in Fig. 7 was determined by first taking images of spinal cord slices under non-saturating conditions and then applying the same settings to image acquisition of DRG neurons. Next, using the ImageJ software, cell bodies were determined as regions of interest and mean gray values of regions of interests were determined. Mean gray values from 3 to 4 sections of three animals per condition/viral construct were determined.

Applied statistics

A one-way ANOVA with Bonferroni post hoc correction was performed in order to assess differences in the transduction efficiency of DRG neurons among the different serotypes used in this study (Fig. 4d). Student's *t*-tests were performed to assess differences in retrograde transduction when comparing AAV9 and AAV2retro (Fig. 3f), to assess differences in the transduction efficiency of DRG neurons comparing AAV9.CAG.eGFP and

AAV9.hSyn1.eGFP (Fig. 5g) and to assess differences in enhanced green fluorescent protein (eGFP) expression levels evoked by AAV9.CAG.eGFP or AAV9.hSyn1.eGFP (Fig. 7d).

Virus production and injections

Viruses were obtained from the resources indicated in the Table 1. AAV2retro-CAG-tdTomato-WRPE-pA was generated from the plasmid pAAV-FLEX-tdTomato (Edward Boyden/Addgene plasmid # 28306) by the VVF Zurich. Virus injections were made in adult mice (6–12 weeks) anesthetized with 2–5% isofluorane and immobilized on a motorized stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA and Neurostar, Tübingen, Germany). Vertebral column was fixed using a pair of spinal adaptors and lumbar spinal cord at L4 and L5 was exposed. Injections (3 × 300 nL) spaced approximately 1 mm apart were made at a rate of 30 nL/min with glass micropipettes (tip diameter 30–40 μm) attached to a 10 μL Hamilton syringe. On average spinal injections lead to viral transduction of neurons located in spinal Laminae I–IV in the vast majority of animals. For the injections of L4 DRGs lumbar/sacral vertebrae L6 and S1 were partially removed and injections of 500 nL were performed as above.

Results

Recombinant AAV vectors are available in a number of different naturally occurring serotypes and many of them have been used to transduce neuronal cells. In studies addressing spinal circuits, at least two different serotypes have been used successfully for intraspinal delivery of transgenes [i.e., 1 and 8 [ref. Azim *et al.* 2014; Foster *et al.* 2015; Peirs *et al.* 2015; Petitjean *et al.* 2015)]. To better understand the differences and similarities between commonly used AAV serotypes, we set out a study comparing local (at the site of delivery) and retrograde (axonal) transduction efficiencies of seven different

Table 1 Resource table

Reagent	Resource	Identifier
Antibodies (dilution)		
647-IB4 (1 : 500)	Molecular Probes, Eugene, OR, USA	I32450
Rabbit anti-CGRP (1 : 1000)	Immunostar, Hudson, WI, USA	RRID:AB_572217
Rabbit anti-GFAP (1 : 2000)	DakoCytomation	RRID:AB_10013382
Chicken anti-GFP (1 : 1000)	Thermo Fisher Scientific, Waltham, MA USA	RRID:AB_2534023
Rabbit anti-GFP (1 : 1000)	Molecular Probes	RRID:AB_221570
Rabbit anti-Iba1 (1 : 1000)	Wako Pure Chemical Industries, Osaka, Japan	RRID:AB_839504
Guinea pig anti-Lmx1b (1 : 10 000)	Dr Carmen Birchmeier	(Muller *et al.* 2002)
Mouse anti-NeuN (1 : 1000)	EMD Millipore Corporation, Temecula, CA, USA	RRID:AB_2298772
Rabbit anti-NeuN (1 : 1000)	Abcam, Cambridge, UK	RRID:AB_10711153
Rabbit anti-NF200 (1 : 1000)	Sigma, St Louis, MO, USA	RRID:AB_477272
Goat anti-Pax2 (1 : 400)	R & D Systems, Minneapolis, MN, USA	RRID:AB_10889828
Rabbit anti-Pax2 (1 : 400)	Invitrogen, Carlsbad, CA, USA	RRID:AB_2533990
Rabbit anti-Pkcγ (1 : 1000)	Santa Cruz Biotechnology, Dallas, TX, USA	RRID:AB_632234
Sheep anti-TH (1 : 1000)	Millipore	RRID:AB_90755
Viruses		
CAG(CB7)-eGFP		
AAV1.CB7.CI.eGFP.WPRE.rBG	Penn Vector Core (Philadelphia, PA, USA)	AV-1-PV1963
AAV5.CB7.CI.eGFP.WPRE.rBG	Penn Vector Core (Philadelphia, PA, USA)	AV-5-PV1963
AAV8.CB7.CI.eGFP.WPRE.rBG	Penn Vector Core (Philadelphia, PA, USA)	AV-8-PV1963
AAV9.CB7.CI.eGFP.WPRE.rBG	Penn Vector Core (Philadelphia, PA, USA)	AV-9-PV1963
AAVrh10.CB7.CI.eGFP.WPRE.rBG	Penn Vector Core (Philadelphia, PA, USA)	AV-10-PV1963
CMV-eGFP		
AAV6.CMV.PI.eGFP.WPRE.bGH	Penn Vector Core (Philadelphia, PA, USA)	AV-6-PV0101
AAV7.CMV.PI.eGFP.WPRE.bGH	Penn Vector Core (Philadelphia, PA, USA)	AV-7-PV0101
CAG-eGFP		
AAV8-CAG-EGFP-WPRE-SV40p(A)	Viral Vector Facility (Zurich, Switzerland)	v24
AAV2retro-CAG-tdTomato-WRPE-pA	Viral Vector Facility (Zurich, Switzerland)	Custom production
AAV9-CAG-EGFP-WPRE-SV40p(A)	Viral Vector Facility (Zurich, Switzerland)	v24
hSyn-eGFP		
AAV9-hSyn1-EGFP-WPRE-SV40p(A)	Viral Vector Facility (Zurich, Switzerland)	v81
Mice		
C57BL/6J	Institute of Pharmacology (Zurich, Switzerland)	RRID:IMSR_JAX:000664
Plasmids		
pAAV-FLEX-tdTomato	Addgene (RRID:SCR_002037)	28306

serotypes (1, 5, 6, 7, 8, 9, rh10) available from the Penn Vector Core virus service facility. Retrograde transduction efficiencies were assessed by quantification of the number of transduced neurons at synaptically connected sites such as the DRGs and the somatosensory cortex, which provide input to but do not receive input from the dorsal spinal horn. All rAAV vectors used in this initial comparison contained an enhanced green fluorescent protein (eGFP) coding sequence followed by a Woodchuck hepatitis B virus post-transcriptional regulatory element (WPRE) and a rabbit beta-globin (rBG) polyadenylation signal sequence. The expression of eGFP was transcriptionally controlled by strong ubiquitous promoters, either the hybrid cytomegalovirus enhancer/chicken β-actin promoter (CAG aka CB7, CBA), along with a chicken β-actin intron (CI) or the cytomegalovirus promoter (CMV), along with a synthetic Promega intron (PI). We performed three injections per animal into the lumbar spinal segments L3–L5. The number of injected vector genomes (vg) per animal was kept constant when comparing the different serotypes (1×10^9 vg per injection = 3.3×10^9 vg per animal).

A main focus of this study was to determine the ability of different serotypes to retrogradely transduce neurons that provide synaptic input to the spinal cord either from the periphery (such as DRG neurons) or from supraspinal sites such as the brainstem or cerebral cortex. We and others have found that serotype 1 retrogradely transduces DRG neurons after intraspinal injection (unpublished results and (Cui *et al.* 2016)). We therefore used AAV1.CAG.eGFP to determine an incubation time that leads to maximal transduction in DRG neurons. To this end, we compared the number of transduced neurons after 10 days and 4 weeks. Four weeks after intraspinal injection of AAV1.CAG.eGFP a significant increase in the number of transduced DRG neurons could be observed as compared to 10 days post injection (22.3 ± 3% of eGFP+ DRG neurons after 10 days vs. 37.9 ± 4.2% of eGFP+ DRG neurons after 4 weeks $p < 0.05$). We therefore decided to do all subsequent analyses at 4 weeks after injection. Next, we determined the percentage of transduced spinal neurons and whether non-neuronal cells can become transduced as well after intraspinal injection of each serotype. At the site of injection, titer-matched serotypes 1, 5, 6, 7, 8, 9, and rh10 showed very similar transduction efficiencies (80–95% Fig. 1a and d). Only AAV5 displayed slightly reduced transduction efficiency (70%). None of the tested serotypes displayed significantly different tropism for either inhibitory (Pax2+) or excitatory (Lmx1b+) spinal dorsal horn neurons (Fig. 1b, c, e and f). We also found that all serotypes were able to transduce GFAP+ astrocytes, albeit at lower levels (Fig. 2 not quantified).

Next, we addressed the question whether spinal injection of rAAV vectors would also lead to the transduction of neurons descending axon terminals from supraspinal CNS areas. We analyzed eGFP expression at supraspinal sites known to provide descending input to neurons of the lumbar spinal cord, such as the RVM and the primary somatosensory cortex (S1). All tested serotypes led to eGFP expression in the RVM and in S1 (Fig. 3a–c). Quantification of the number of eGFP+ neurons in S1 indicated that serotypes 6 (63 ± 17 neurons/animal) and 9 (33 ± 12 neurons/animal) displayed the highest capability to retrogradely transduce terminals of descending supraspinal neurons (Fig. 3c). In order to better understand which proportion of descending projection neurons became transduced by the respective serotypes, we compared the transduction efficiency of serotype 9 to the recently published serotype 2 capsid variant rAAV2retro. rAAV2retro has been modified in order to maximize retrograde transduction capabilities (Tervo *et al.* 2016). Comparing serotype 9 to rAAV2retro, we found a > 20-fold increase (19 ± 8 vs. 502 ± 42 neurons/animal, respectively) in the number of labeled neurons in S1 when using rAAV2retro (Fig. 3d–f). This indicates that less than 5% of descending supraspinal neurons displayed eGFP expression when using any of the other AAV serotypes.

Another site of the nervous system that provides synaptic input to spinal neurons is the peripheral nervous system, that is, neurons of the DRG. These neurons are primary detectors of somatosensory and proprioceptive stimuli and convey this information to the dorsal spinal cord. We therefore compared the transduction efficiency of DRG neurons after intraspinal injection of the different AAV serotypes. All tested rAAV vectors were capable of transducing DRG neurons via their central axon terminals. However, the degree of transduction was significantly different between serotypes (one-way ANOVA, $F(6, 16) = 6.039$, $p = 0.0019$), (Fig. 4a and d). Serotypes 1 and 9 were most efficient, displaying average transduction rates of > 40%. Clearly reduced transduction rates could be observed after the injection of serotypes 7, 8, and rh10 (15 ± 3%, 12 ± 4%, and 16 ± 6%, respectively). When looking at the identity of the infected DRG neurons, we found that all major subtypes of DRG neurons became transduced [neuro-filament 200 (NF200)+ myelinated neurons, calcitonin gene-related peptide (CGRP)+ peptidergic neurons, IB4+ non-peptidergic neurons, and TH+ c-low threshold mechanoreceptors]. However, we observed that transduction of NF200+ and CGRP+ DRG neurons was much more efficient than that of other types (Fig. 4b, c, e and f).

We were wondering whether the observed bias might be because of differences in the surface area of the axon of a particular DRG neuron subtype present in the spinal cord. There is indeed evidence that vGlut1+ terminals are larger than terminals of C fibers (Todd *et al.* 2003). We therefore reasoned that, if we increased the viral particle number per injected area, we might overcome this bias. We tested this idea by injecting high titer of AAV9.CAG.eGFP (1.5×10^{13} vg/mL = 1.35×10^{10} vg per animal) into the spinal cord and analyzed the identity of the transduced DRG neurons. We found that under this condition, NF200+, CGRP+, IB4+, and

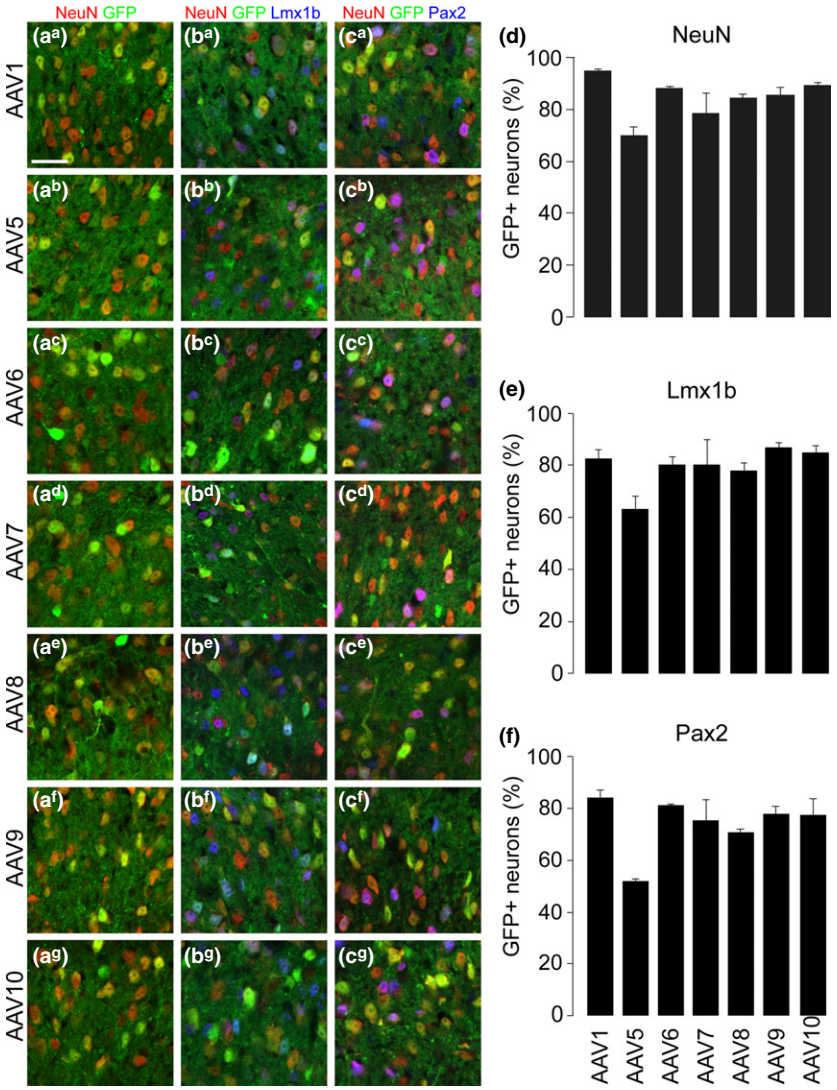


Fig. 1 Transduction efficiencies of spinal neurons by different adeno-associated virus (AAV) serotypes. recombinant AAVs (rAAVs) vectors composed of enhanced green fluorescent protein (eGFP) expression cassettes contained within the capsids of the indicated serotypes were injected into the spinal cord of mice. Sections of the respective spinal cords were reacted with antibodies against NeuN and GFP (a^a–a^g) to determine the fraction of neurons that became transduced, NeuN, GFP and Lmx1b (b^a–b^g) to determine the fraction of excitatory dorsal horn neurons that became transduced and NeuN, GFP and Pax2 (c^a–c^g) to determine the fraction of inhibitory spinal neurons that became transduced by the respective AAV serotype. The immunohistochemical analysis of spinal cord sections indicates that close to the injection site, the vast majority of spinal neurons express some degree of eGFP. (d–f) Quantification of the fraction of either all spinal neurons (d), or excitatory dorsal horn neurons (e) or inhibitory dorsal horn neurons (f) located close to injection site, which expressed eGFP 4 weeks after intraspinal injection of the indicated AAV serotype. Scale bar: 50 μm, error: ± SEM.

TH+ DRG neurons became transduced with similar efficiencies (Fig. 5a–c and h). These results indicate that the initially observed low number of eGFP-expressing IB4+ and TH+ neurons after intraspinal injection was likely as a result of an uptake of fewer virions relative to NF200+ neurons but not because of an inability to become transduced by rAAV vectors.

Unlike in this study, recent reports suggested that certain AAV serotypes would avoid infection of DRG neurons. When comparing our findings, we noticed that in some of these studies, different promoters have been used. Peirs *et al.* (2015) found no transduction in vGlut3+ DRG neurons when expression was driven from the hSyn1 promoter. We therefore compared expression of eGFP after intraspinal injection of either AAV9.CAG.eGFP or AAV9.hSyn1.eGFP (at 1.5×10^{13} vg/mL). Injection of AAV9.hSyn1.eGFP lead to a significantly reduced number of eGFP+ neurons in the DRGs as compared to AAV9.CAG.eGFP (8 ± 2% vs. 49 ± 5%, respectively), while there was no significant difference in number of transduced spinal neurons (98 ± 1% vs. 97 ± 1.5%, AAV9.CAG vs. AAV9.hSyn1) at the site of injection (Fig. 5a–g). In addition, we found a biased expression in NF200+ and CGRP+ DRG neurons (Fig. 5h). When analyzing transduction of DRG neurons after intraspinal injection of AAV9.CAG.eGFP, we also found that transduction was biased toward NF200+ and CGRP+ neurons. This difference could be overcome by increasing the viral titer, which likely led to an increase in the average number of viral particles taken up per DRG neuron. As we could not further increase the titer, we injected AAV9.hSyn1.eGFP directly into the L4 DRG. We reasoned that, if the hSyn1 promoter is just weaker than the CAG promoter, then increasing the number of viral particles per cell through direct injection into the DRG should increase the number of transduced neurons. Determining the number and the identity of the transduced neurons, we found a significant increase in the number of transduced neurons (7.9 ± 1.8% after intraspinal injection vs. 40.6 ± 4% after injection into the L4 DRG, $p < 0.001$) but also that the bias for expression in NF200+ and CGRP+ neurons remained

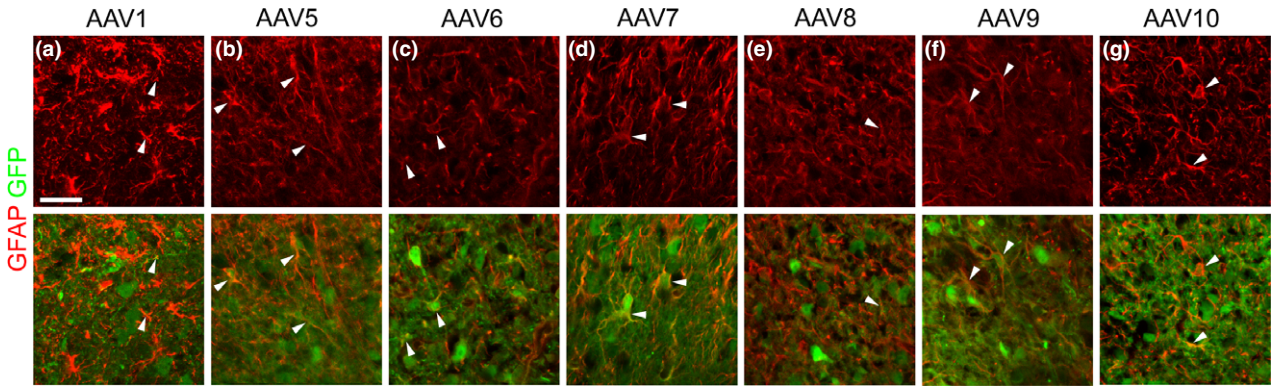


Fig. 2 All tested recombinant adeno-associated virus (rAAV) serotypes are capable of transducing astrocytes. Immunohistochemical analysis of spinal cord sections of mice which have been injected with the indicated AAV serotypes. (a–g) An antibody against GFP (green) reveals transduction by the respective enhanced green fluorescent protein (eGFP)-encoding rAAV vector. Co-expression of immunohistochemically detected eGFP and GFAP (red) is indicated by arrowheads and suggests transduction of astrocytes. Scale bar: 50 μm.

(Fig. 6a–d). This indicated to us that the hSyn1 promoter not only has a lower general activity than the CAG promoter but is also more active in NF200+ and CGRP+ neurons than in unmyelinated TH+ or IB4+ neurons.

The hSyn1 promoter has been used to drive functional expression of effector proteins in different subtypes of spinal cord neurons (Foster *et al.* 2015; Peirs *et al.* 2015). In a final set of experiments, we therefore wanted to understand how the relative expression achieved in DRG neurons after intraspinal injection of AAV9.hSyn1.eGFP and AAV9.CAG.eGFP compares to the AAV9.hSyn1.eGFP evoked relative expression obtained in the spinal cord. We therefore compared the relative eGFP fluorescence in DRG neurons transduced with either AAV9.hSyn.eGFP or AAV9.CAG.eGFP to the eGFP fluorescence in spinal neurons after transduction with AAV9.hSyn.eGFP. We found that in DRG neurons, the average hSyn1-driven eGFP fluorescence is 3.8 times lower than the average CAG-driven eGFP fluorescence (Fig. 7a, b and d). We then compared the eGFP expression levels in DRG neurons obtained with either the CAG or the hSyn1 promoter to that of hSyn1-driven eGFP at the spinal injection site. Average eGFP expression driven by the CAG promoter in DRG neurons was 2.3 times lower and hSyn1-driven average eGFP expression in DRG neurons was 8.9 times lower than hSyn1-driven eGFP expression in the spinal cord (Fig. 7a–d). Our data thus suggest that intraspinal injection of viral constructs using the hSyn1 promoter leads to strongly reduced expression in DRG neurons while maintaining high expression levels in intrinsic dorsal horn neurons.

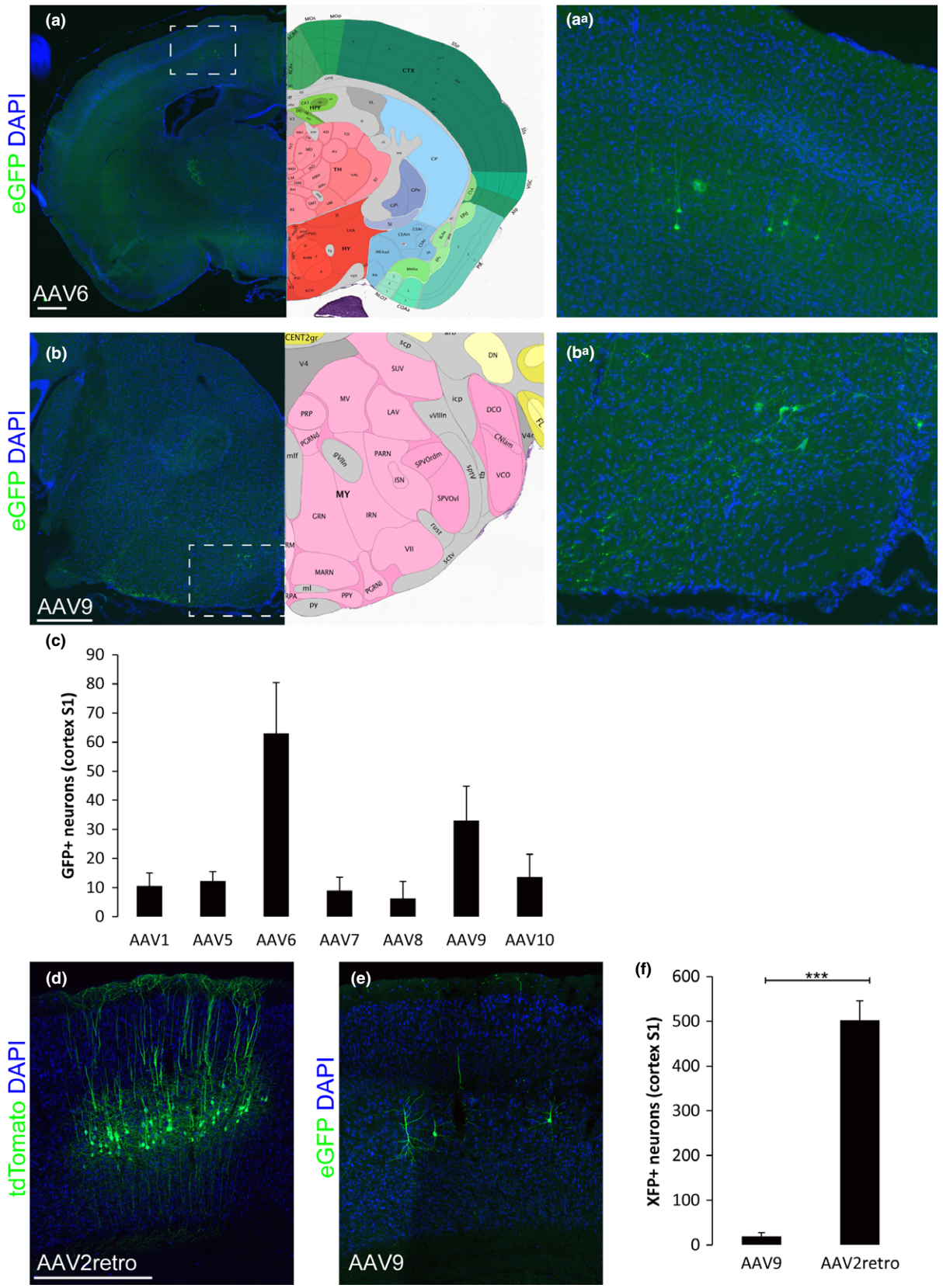
Discussion

rAAV vector-mediated targeted expression has become almost indispensable for interrogation of sensory circuit function. If neurons located at the injection site are to be studied, it is crucial to restrict the expression of an effector protein as far as possible to the targeted site within the nervous system thus avoiding ambiguous results. Here, we show that seven different commonly used rAAV vectors are capable of transducing DRG neurons and descending supraspinal neurons after intraspinal injection. However, we also show that co-expression of the encoded transgene in neurons at the injection site and neurons at synaptically connected sites of the nervous system can be reduced by driving transgene expression from the human synapsin 1 promoter fragment. Conversely, if descending neurons are to be studied, they can efficiently be targeted using rAAV2retro vectors.

rAAV-mediated gene transfer in the nervous system

Numerous studies have addressed the efficiencies of different rAAV serotypes in transducing neurons at various CNS or PNS sites. To the best of our knowledge, this is the first study to specifically compare spinal local and retrograde

Fig. 3 Retrograde (axonal) transduction of descending supraspinal neurons by different recombinant adeno-associated virus (rAAV) serotypes. Analysis of enhanced green fluorescent protein (eGFP) expression in supraspinal CNS areas after intraspinal injection of serotypes 1, 5, 6, 7, 8, 9, rh10 reveals labeled cells in the somatosensory cortex (S1) (exemplified in a and a[a]) as well as in the rostral ventromedial medulla (RVM) (exemplified in b, b[a]). Reference atlas images were taken from the Allen Mouse Brain Atlas/Coronal Atlas (http://mouse.brain-map.org/experiment/thumbnails/100048576?image_type = atlas). (c) Quantification of the number of retrogradely transduced neurons in S1 after intraspinal injection of the indicated serotypes suggests an increased retrograde transduction efficiency of AAV6 and AAV9. (d-f) Comparison of the retrograde transduction efficiency of serotype 9 and the recently described serotype 2 capsid variant AAV2retro indicates a > 20-fold increase in the number of transduced corticospinal S1 neurons following intraspinal injection. Scale bars: 500 μm, error: ± SEM, ***$p < 0.001$.


(a)
eGFP DAPI
AAV6
(aa)
(b)
eGFP DAPI
AAV9
(ba)
(c)
GFP+ neurons (cortex S1)
90
80
70
60
50
40
30
20
10
0
AAV1
AAV5
AAV6
AAV7
AAV8
AAV9
AAV10
(d)
tdTomato DAPI
AAV2retro
(e)
eGFP DAPI
AAV9
(f)
XFP+ neurons (cortex S1)
600
500
400
300
200
100
0
***
AAV9
AAV2retro

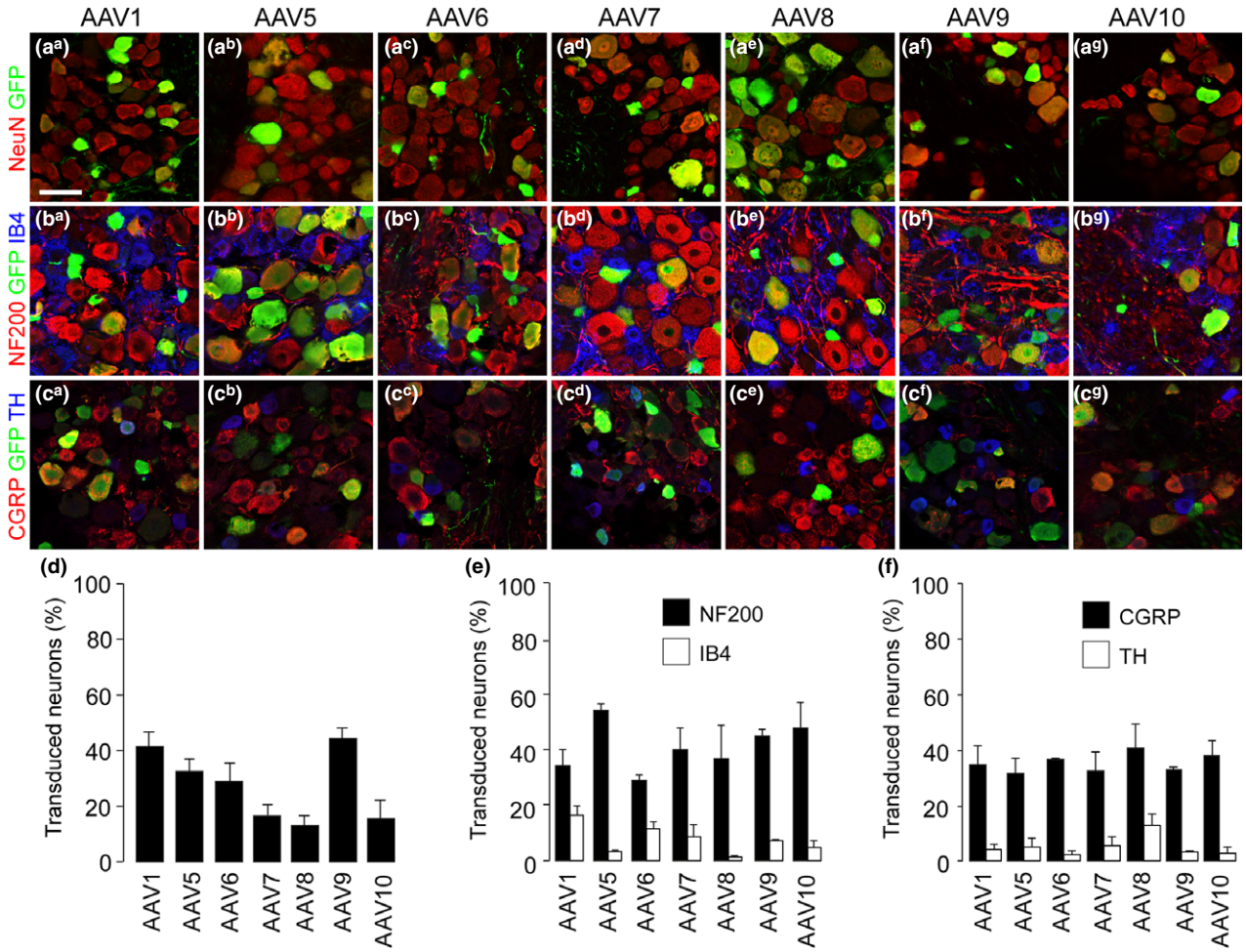
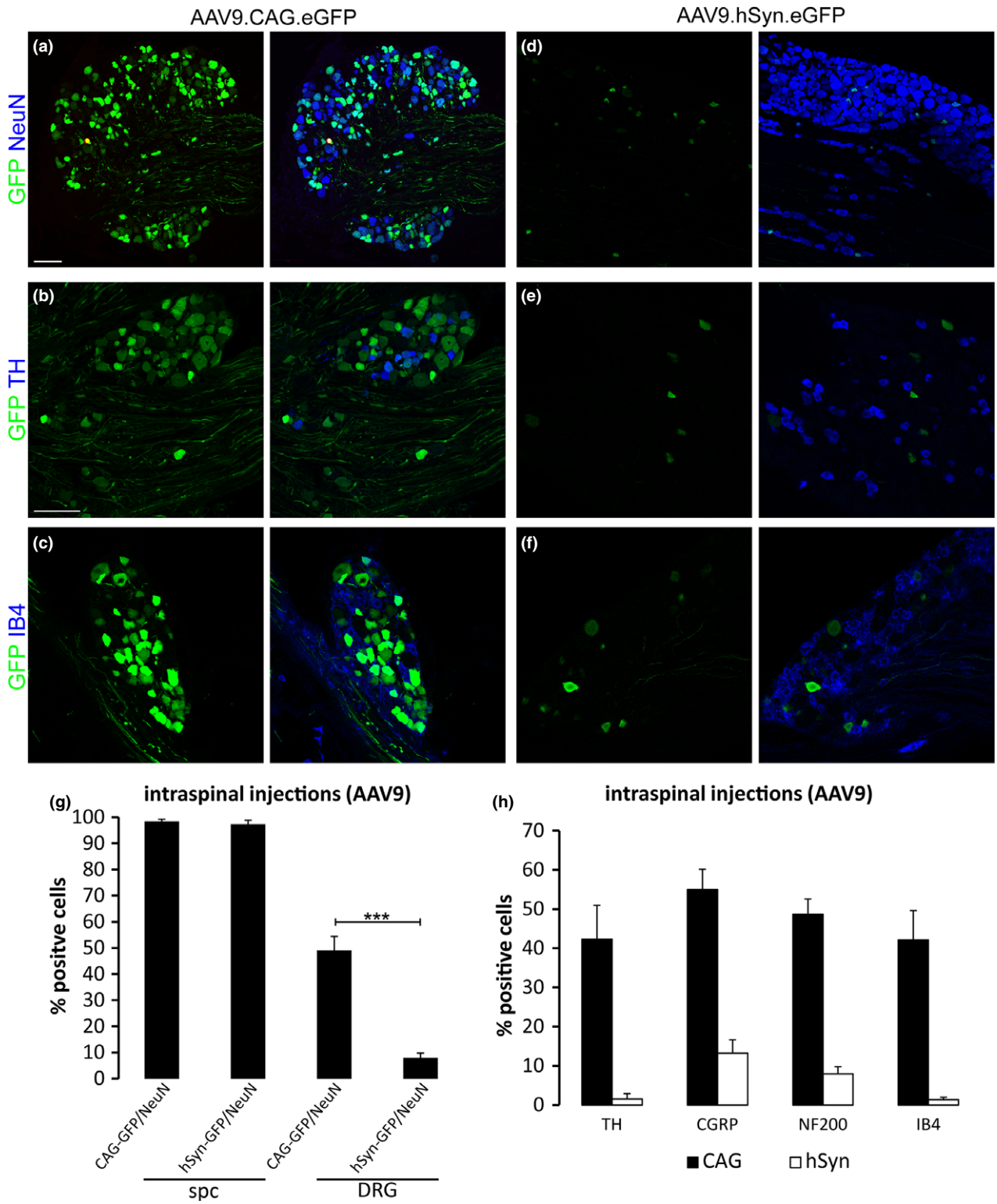


Fig. 4 Retrograde (axonal) transduction of dorsal root ganglia (DRG) neurons and DRG neuron subtypes by recombinant adeno-associated virus (rAAV) vectors. Analysis of eGFP expression in lumbar DRGs after intraspinal injection of serotypes 1, 5, 6, 7, 8, 9, rh10 reveals between 12 and 45% of labeled neurons. (a^a–a^g and d) Imunohistochemical analysis and quantification of the fraction of DRG neurons expressing eGFP after intraspinal injection of rAAV vectors using antibodies directed against GFP (green) and NeuN (red). (b^a–b^g and e) Immunohistochemical analysis and quantification of the fraction of myelinated (neuro-filament 200, NF200+/red) and unmyelinated non-peptidergic (IB4+/blue) DRG neurons that display eGFP expression 4 weeks after intraspinal injection of the indicated AAV serotype. (c^a–c^g and f) Immunohistochemical analysis and quantification of the fraction of peptidergic (calcitonin gene-related peptide (CGRP)+/red) and unmyelinated c-low threshold mechanoreceptors (tyrosine hydroxylase, TH+/blue) that co-express eGFP after intraspinal injection of the indicated AAV serotype. Scale bar: 50 μm, error: ± SEM.

transduction efficiencies of the majority of commonly available AAV serotypes. A previous study conducted in the rat nervous system compared AAV1, 2, and 5 and found that AVV vectors serotyped with AAV capsid 1 or 5 efficiently transduced neurons at various injection sites in the CNS including the spinal cord (Burger *et al.* 2004). We also

Fig. 5 Promoter effects on retrograde expression levels in dorsal root ganglia (DRG) neurons. Spinal cords of mice were injected with high titer serotype 9 coding for enhanced green fluorescent protein (eGFP) driven by either the CAG promoter (a–c/AAV9.CAG.eGFP) or the human synapsin1 promoter (d–f/AAV9.hSyn1.eGFP). (a and d) Immunohistochemical analysis of DRG sections with antibodies against GFP (green) and NeuN (blue). (b and e) Immunohistochemical analysis of DRG sections with antibodies against GFP (green), and tyrosine hydroxylase (TH) (blue). (c and f) Immunohistochemical analysis of DRG sections with antibodies against GFP and IB4. (g) Quantification of the percentage of neurons in the lumbar spinal cord (spc) and lumbar DRGs (DRG) that display eGFP expression after intraspinal injection of either AAV9.CAG.eGFP (CAG-GFP) or AAV9.hSy1n.eGFP (Human synapsin 1 promoter (hSyn1)-GFP). Note that while similar percentages of neurons express eGFP in the lumbar spinal cord, strongly reduced numbers of DRG neurons show eGFP expression after intraspinal injection of AAV9.hSyn1.eGFP as compared to injection of AAV9.CAG.eGFP. (h) Quantification of the fraction of TH+, calcitonin gene-related peptide (CGRP)+, neuro-filament 200 (NF200)+ or IB4+ DRG neurons that express eGFP after intraspinal injection of either AAV9.CAG.eGFP (CAG) or AAV9.hSyn.eGFP (Syn). Scale bar: 100 μm, error: ± SEM, ***$p < 0.001$.



found AAV vectors with serotype 1 to efficiently transduce spinal neurons, but in our hands, serotype 5 was the least efficient serotype for local transduction at the injection site. The study of Burger *et al.* was conducted in rats, whereas our study was conducted in mice. The observed differences might therefore be because of species differences. Our data are in agreement with a previous study indicating that AAV6 is the most efficient naturally occurring capsid variant for retrograde transport (Salegio *et al.* 2013). Nevertheless, consistent with Tervo *et al.* (2016) we find that the capsid

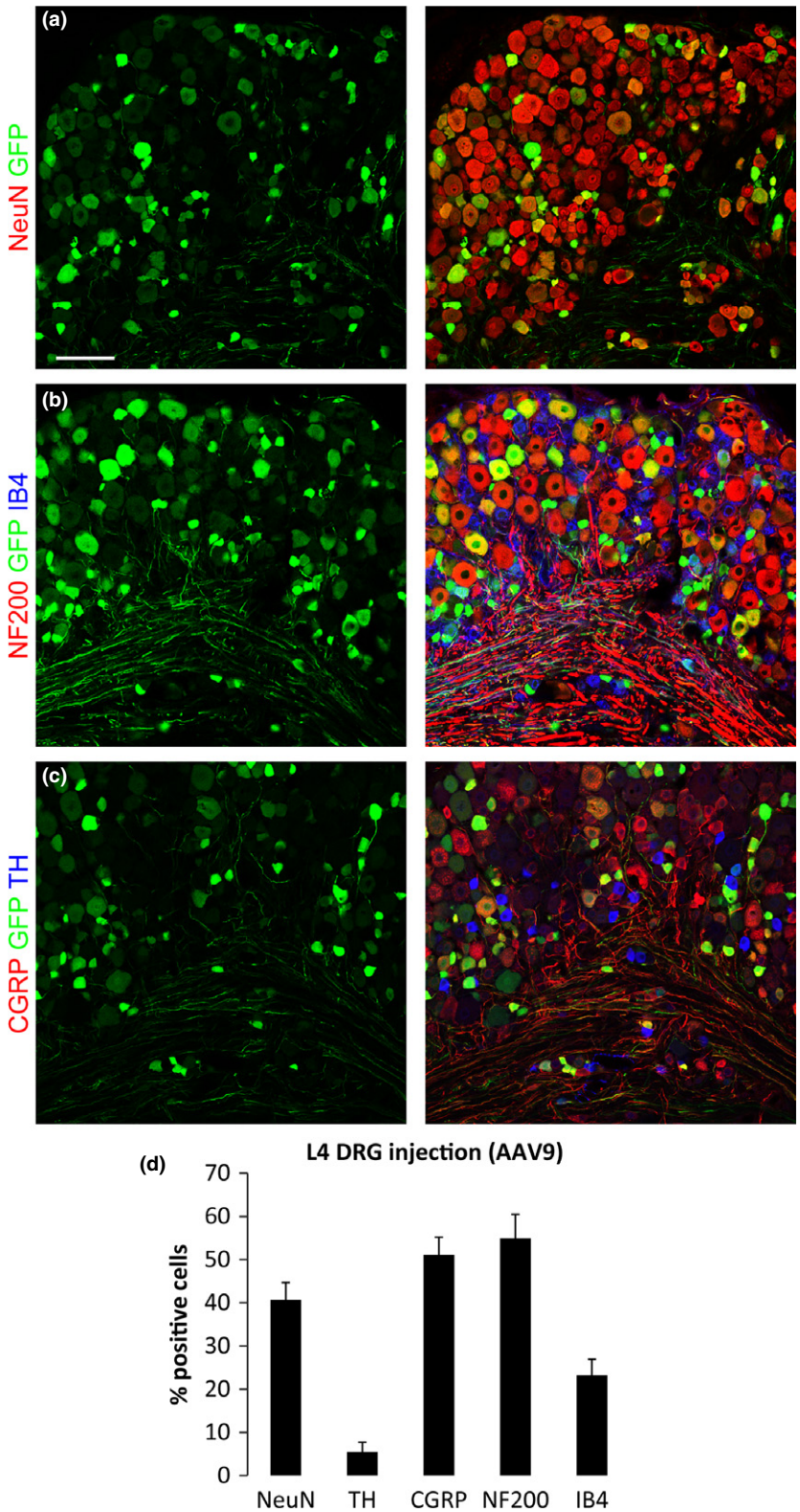


Fig. 6 Human synapsin 1 promoter (hSyn1)-driven enhanced green fluorescent protein (eGFP) expression is reduced in unmyelinated tyrosine hydroxylase (TH)+ and IB4+ dorsal root ganglia (DRG) neurons. Lumbar L4 DRGs were injected directly with AAV9.hSyn1.eGFP at a high titer to increase the vector genomes per cell. (a–c) Immunohistochemical analysis of DRG sections after intra-DRG injection. (a) Determination of the fraction of DRG neurons expressing eGFP using antibodies against GFP (green) and NeuN (red). (b) Immunohistochemical analysis with antibodies against neuro-filament 200 (NF200) (red) and GFP (green) combined with IB4 (blue) labeling. (c) Immunohistochemical analysis with antibodies against calcitonin gene-related peptide (CGRP) (red), GFP (green) and TH (blue). (d) Quantification of the fraction of all DRG Neurons (NeuN), c-low threshold mechanoreceptors (TH), peptidergic (CGRP) myelinated (NF200) and non-peptidergic unmyelinated DRG neurons that express eGFP after intra-DRG injection of AAV9.hSyn.eGFP. Scale bar: 100 μm, error: ± SEM.

variant AAV2retro increases retrograde transduction by more than an order of magnitude. Many other studies have used other routes of administration such as intravenous injection to target a large range of tissues (Foust *et al.* 2009; Hu *et al.* 2010) or intrathecal injection and injection into the sciatic nerve to specifically target DRG neurons (Towne *et al.*

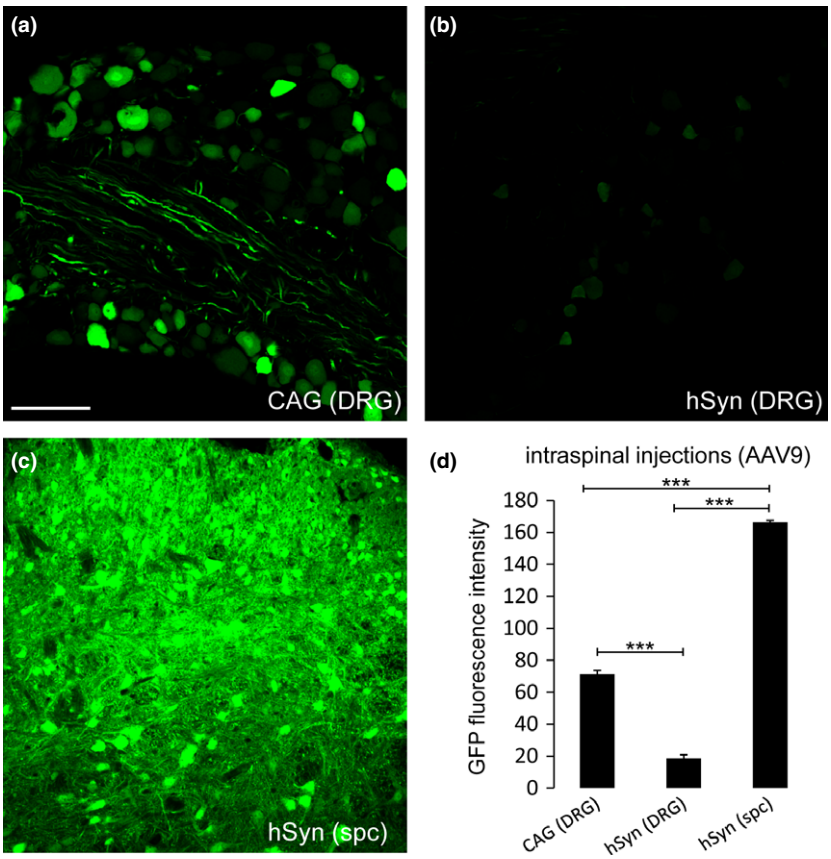


Fig. 7 Human synapsin 1 promoter (hSyn1) drives high level of enhanced green fluorescent protein (eGFP) expression in the spinal cord but low levels in dorsal root ganglia (DRGs) after intraspinal injection. Comparison of eGFP expression levels after intraspinal injection of high titer AAV9.CAG.eGFP or AAV9.hSyn1.eGFP. Sections of DRGs (a and b) or spinal cords (c) were imaged under the same conditions following intraspinal injection of AAV9.CAG.eGFP (a) or AAV9.hSyn1.eGFP (b and c). (d) Quantification of eGFP expression in cell bodies of DRG neurons (DRG) or spinal neurons (spc) after intraspinal transduction by serotype 9 vectors controlling eGFP expression either by the CAG or hSyn1 promoter. Scale bar: 100 μm, error: ± SEM, ***$p < 0.001$.

2009). The transduction achieved by these routes of administration might depend on different mechanisms. Such studies are therefore difficult to compare.

Restricting transduction of pain circuits

Many neuronal subpopulations in the spinal cord share the expression of certain molecular marker genes with other populations at different sites of the nervous system. When such a marker gene is used to drive Cre expression in a transgenic mouse, unambiguous interrogation of the function of the Cre-expressing spinal subpopulation can only be achieved if the activity of effector proteins is limited to the spinal cord. In the context of rAAV vector-mediated delivery of effector proteins, serotypes/capsid variants or promoters have to be used that avoid co-expression in remote but synaptically connected sites. Here, we have shown that intraspinal injection of commonly used rAAV serotypes lead to the transduction of DRG neurons as well as to the transduction of supraspinal CNS sites sending descending inputs to the spinal cord. Transduction efficiency of DRG neurons (and supraspinal neurons) was dependent on the titers applied. The initial comparisons were carried out with titers that were three times lower compared to subsequent applications. These titers were high enough to transduce > 80% of spinal neurons, and 12–44% of all DRG neurons depending on the serotype. Transduction of supraspinal neurons by serotypes 1, 5, 6, 7, 8, 9, and rh10 was less efficient. We found that rAAV2retro labeled > 20-fold more neurons in S1 as compared to AAV9, suggesting that eGFP expression occurred in less than 5% of descending neurons after injection of AAV9. If transduction of supraspinal neurons projecting to the spinal cord is the aim of an experiment, rAAV2retro is clearly superior to the other tested serotypes. Conversely, if co-expression at the injection site and connected sites should be reduced, using the hSyn1 promoter, potentially in combination with an AAV serotype less efficient in retrograde transport than AAV9, is a good option. In fact, we could not detect any eGFP expression at supraspinal sites when injecting AAV9.hSyn1.eGFP intraspinally and furthermore observed a > sixfold decrease in the number of detectable eGFP-expressing DRG neurons and an additional fourfold decrease in eGFP expression levels. In addition, we found that in DRGs hSyn1-mediated transgene expression is preferentially detected in myelinated NF200+ neurons. Our findings are in good agreement with results from Peirs *et al.* (2015) and Petitjean *et al.* (2015), who have found no co-expression in the respective DRG populations (i.e., unmyelinated TH+ and Pvalb+) after intraspinal injection of an AAV8, harboring a hSyn1-driven Cre-dependent expression cassette, into $vGlut3^{Cre}$ or $Pvalb^{Cre}$ animals.

In order to exclude AAV-mediated transgene expression at unwanted sites or to restrict expression to neuronal subsets

using specific short promoters in rAAV vectors has proven successful at other CNS sites (e.g., Cronin *et al.* 2014; de Leeuw *et al.* 2014; Gompf *et al.* 2015; Oh *et al.* 2009). However, while the use of such specific promoters can strongly reduce co-expression in unwanted cell types, as also seen here with the hSyn1 promoter, viral uptake, transport, and unloading of the viral genome remain dependent on the respective AAV capsid. Therefore, the same number of neurons will carry viral genomes regardless of the specific promoter used. As a result, a given promoter, for example, hSyn1, may be too weak to drive reporter gene expression levels beyond detection levels, but may still be sufficient to effectively interfere with neuronal function when highly potent effector proteins such as the bacterial toxins (e.g., diphtheria toxin or tetanus toxin) are used. An alternative, and potentially more powerful, strategy is the use of intersectional genetics which are often based on the use of two recombinases driven by promoters of two different but in their expression partially overlapping marker genes. Marker genes have to be selected in a way that combinatorial expression is restricted to the population of interest and avoided in potentially confounding populations. Using reporter mice intersectional manipulation of neuronal sub-populations has been pioneered by the Dymecki lab and later on successfully employed by the laboratories of Quifu Ma and Martyn Goulding for the analysis of spinal circuits (Awatramani *et al.* 2003; Kim *et al.* 2009; Duan *et al.* 2014; Bourane *et al.* 2015). Meanwhile also intersectional rAAV vector approaches have become available (Fenno *et al.* 2014; Madisen *et al.* 2015), adding the advantage of increased spatial resolution. Further development of both a) cell type-specific short promoters and b) intersectional strategies should greatly enhance our ability to understand and manipulate the function of small functional subpopulations involved in processing of noxious stimuli and thereby contribute to our understanding of the development of acute and chronic pain.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

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