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RESEARCH ARTICLE

TECHNIQUES AND RESOURCES

Enhanced selective gene delivery to neural stem cells *in vivo* by an adeno-associated viral variant

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ABSTRACT

Neural stem cells (NSCs) are defined by their ability to self-renew and to differentiate into mature neuronal and glial cell types. NSCs are the subject of intense investigation, owing to their crucial roles in neural development and adult brain function and because they present potential targets for gene and cell replacement therapies following injury or disease. Approaches to specifically genetically perturb or modulate NSC function would be valuable for either motivation. Unfortunately, most gene delivery vectors are incapable of efficient or specific gene delivery to NSCs *in vivo*. Vectors based on adeno-associated virus (AAV) present a number of advantages and have proven increasingly successful in clinical trials. However, natural AAV variants are inefficient in transducing NSCs. We previously engineered a novel AAV variant (AAV r3.45) capable of efficient transduction of adult NSCs *in vitro*. Here, to build upon the initial promise of this variant, we investigated its *in vitro* and *in vivo* infectivity. AAV r3.45 was more selective for NSCs than mature neurons in a human embryonic stem cell-derived culture containing a mixture of cell types, including NSCs and neurons. It was capable of more efficient and selective transduction of rat and mouse NSCs *in vivo* than natural AAV serotypes following intracranial vector administration. Delivery of constitutively active β-catenin yielded insights into mechanisms by which this key regulator modulates NSC function, indicating that this engineered AAV variant can be harnessed for preferential modulation of adult NSCs in the hippocampus. The capacity to rapidly genetically modify these cells might greatly accelerate *in vivo* investigations of adult neurogenesis.

KEY WORDS: Adeno-associated virus, Gene delivery, Neural stem cell

INTRODUCTION

Neural stem cells (NSCs) are characterized by the capacity for self-renewal and differentiation into different neural cell types, including neurons, astrocytes and oligodendrocytes (Gage, 2000; Temple, 2001). Within the adult brain, active NSC populations exist in the subventricular zone (SVZ), the striatum (in humans) and the subgranular zone of the dentate gyrus of the hippocampus (Ernst et al., 2014; Gage, 2000). In the subgranular zone, neurogenesis begins with the activation and division of quiescent Type 1 NSCs (which express nestin, Sox2 and Gfap) to generate Type 2a mitotic NSCs (which express nestin and Sox2, but not Gfap) (Lugert et al., 2010; Suh et al., 2009). As differentiation proceeds, Type 2a NSCs develop into Type 2b neuronal precursors (expressing Sox2 and Dcx), which later mature into Type 3 neuroblasts (expressing Dcx but not Sox2), migrate into the granule cell layer, differentiate into mature neurons [expressing NeuN (Rbfox3)] and integrate into the neural network (Lugert et al., 2010; Mira et al., 2010; Suh et al., 2009). Type 1 NSCs are also capable of differentiating into mature hippocampal astrocytes (expressing Gfap and S100β) (Bonaguidi et al., 2011). Adult neurogenesis has been shown to play key roles in learning and memory in mammals, including hippocampal-dependent spatial navigation learning, spatial pattern discrimination and contextual fear conditioning (Deng et al., 2010; Ming and Song, 2011).

Efficient and preferential gene delivery would offer a versatile and rapid means to study regulatory mechanisms of NSC quiescence, proliferation, self-renewal and differentiation. Nestin-CreER^{T2} transgenic mice, which express tamoxifen-inducible Cre recombinase under the control of the nestin promoter, have been heavily utilized to track NSCs and their progeny *in vivo* (Ashton et al., 2012; Bonaguidi et al., 2011; Lagace et al., 2007). These mouse lines have enabled a number of basic advances in NSC investigations; however, deriving a new line to study each new gene is highly time- and labor-intensive, taking months to years (Haruyama et al., 2009). In addition to basic studies, gene delivery could be harnessed for gene or cell replacement therapies to treat neurodegenerative disease or injury; for example, via the overexpression or knockdown of genes that modulate the generation of new neurons. Also, gene delivery to NSCs has been harnessed to express neurotrophic factors for protection from neurodegenerative diseases (Blesch et al., 2002), and restoration of fragile X mental retardation protein expression specifically in adult NSCs rescued mice from learning deficits in a murine model of fragile X syndrome (Guo et al., 2011).

There have been several efforts to deliver genes to adult NSCs *in vivo*. Hashimoto and Mikoshiba used replication-defective adenoviral vectors to deliver genes to progenitor cells in the developing brains of mice at embryonic days 10.5-14.5; these vectors enabled tracking of the differentiation of the progenitor cells, but delivery to adult NSCs has not been demonstrated (Hashimoto and Mikoshiba, 2004). Falk et al. administered polyethyleneimine (PEI) complexes, containing plasmids driving reporter gene expression via enhancer elements from the second intron of the human nestin gene, to the lateral ventricle of mice and showed some selective delivery to NSCs in the SVZ, although the efficiency was limited (Falk et al., 2002). In another study, Lemkine et al. used PEI-DNA complexes and showed low specificity towards mouse SVZ NSCs as compared with globular cells following delivery to the lateral ventricle (Lemkine et al., 2002). Additionally, van Hooijdonk et al. (van Hooijdonk et al., 2009) used a vesicular stomatitis virus G glycoprotein-pseudotyped lentivirus to target

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neural progenitor cells and immature neurons in the subgranular zone of the dentate gyrus of the mouse hippocampus. Although the lentivirus preferentially transduced neuronal progenitor cells and immature neurons, only 11% of cells infected with the virus were nestin$^+$ 1 week after administration (van Hooijdonk et al., 2009). Finally, retroviral vector administration to the mouse hippocampus is useful for targeting mitotic neural progenitors and neuroblasts (Jessberger et al., 2008), but early stage stem cells rarely divide (Bonaguidi et al., 2011).

Adeno-associated virus (AAV) is a nonpathogenic, non-enveloped virus that is a member of the parvovirus family. The AAV icosahedral protein capsid encloses a 4.7 kb single-stranded DNA genome that contains flanking inverted terminal repeats (ITRs), which serve as the origin of replication and signal for the genome to be packaged (Knipe and Howley, 2007). Between the ITRs, the *rep* open reading frame (ORF) encodes four nonstructural proteins that are responsible for viral replication in the presence of a helper virus, transcriptional regulation of the *rep* and *cap* ORFs, site-specific integration into the AAVS1 locus and virion assembly (Knipe and Howley, 2007). The *cap* ORF encodes three structural proteins (VP1, VP2 and VP3) that assemble to form the 60-mer viral capsid (Knipe and Howley, 2007). The amino acid sequence translated from the *cap* ORF determines the gene delivery properties of AAV, including antibody binding, cell surface receptor binding, glycan binding and endosomal escape, and currently eleven naturally occurring serotypes and over 100 variants of the AAV capsid have been identified (Kotterman and Schaffer, 2014; Schaffer et al., 2008; Wu et al., 2006).

In the recombinant versions of AAV used for gene delivery, *rep* and *cap* are replaced by a gene of interest that is inserted between the ITRs. To produce the gene delivery vector encoding the gene of interest, a plasmid containing *rep* and *cap* and additional helper viral genes are provided to the packaging cells (Flotte, 2004). Recombinant AAV vectors are capable of transducing both dividing and non-dividing cells, and stable transgene expression is possible for years in postmitotic tissue. To date, no natural AAV has been associated with any human disease, which, along with their high efficiency on some cell types, is a key reason why recombinant AAV has emerged as an attractive vector for gene therapy (Knipe and Howley, 2007).

Unfortunately, the use of naturally occurring AAV serotypes has revealed a number of challenges to their widespread use in clinical gene therapy. These include significantly lower transduction in the presence of neutralizing antibodies (Jaski et al., 2009; Manno et al., 2006), lack of specific and/or efficient distribution to many potential target tissues (Zincarelli et al., 2008), lack of efficiency (Manno et al., 2003; Moss et al., 2007; Wagner et al., 2002) and incapacity for targeted delivery to specific cell types. These issues arise because the properties that mediate successful natural viral infections are distinct from those required for success in basic biological or biomedical applications, and viruses did not evolve for the latter. In particular, none of the natural AAV serotypes is capable of efficient gene delivery to NSCs (Jang et al., 2011) and many instead show highly specific tropism for mature neurons (Bartlett et al., 1998; Kaspar et al., 2002; Ortinski et al., 2010).

Directed evolution is a high-throughput molecular engineering approach that has been successfully harnessed to generate AAV variants with altered receptor binding, neutralizing antibody-evasion properties and novel cell tropism (Asuri et al., 2012; Excoffon et al., 2009; Koerber et al., 2008; Maheshri et al., 2006). As is the case with natural evolution, directed evolution utilizes an iterative process in which genetic variants undergo cycles of additional diversification and increasing selective pressure to allow for the emergence of key mutations that improve function for a specific application. The coupling of random diversification and highly tailored selection enables the generation of significantly improved functionality even if the mechanism of action is unknown. Recently, we applied directed evolution to isolate an AAV variant capable of efficient NSC transduction *in vitro* (Jang et al., 2011). Specifically, selection for the capacity to infect cultured adult rat hippocampal NSCs yielded AAV r3.45, an AAV2 variant with a seven-amino-acid peptide insertion at position 588. AAV r3.45 demonstrated 50-fold increased transduction of rat NSCs *in vitro* as compared with wild-type AAV2 and AAV5. This variant AAV was also capable of significantly increased transduction of murine NSCs, human fetal NSCs and human embryonic stem cell (hESC)-derived neural progenitor cells compared with AAV2 (Jang et al., 2011). In addition to improved transduction of NSCs, AAV r3.45 significantly improved homologous recombination-based gene correction: its use resulted in a fivefold increase in targeted gene correction in NSCs compared with wild-type AAV2 and AAV5 (Jang et al., 2011). When AAV r3.45 was immobilized onto elastin-like peptides, delivery to human NSCs was further enhanced (Kim et al., 2012b).

Although the majority of evolved AAV variants have been created using *in vitro* selections, some of these variants have demonstrated success when translated to an *in vivo* model. For example, two variants evolved by Koerber et al. for the ability to infect primary human astrocytes in culture also transduced 3.3- and 5.5-fold more astrocytes, relative to neurons, than AAV2 within the striatum following intracranial injection in rats (Koerber et al., 2009). Furthermore, *in vivo* analysis revealed that another variant from the astrocyte selection was capable of highly specific and efficient infection of Müller glia when compared with AAV2 and AAV6 (Klimczak et al., 2009). The success of AAV variants created through *in vitro* selection to evade neutralization by human antibodies has also translated to increased antibody evasion in a mouse model of immunity (M.A.K., Bum-Yeol Hwang, Daniel Stone, James T. Koerber and D.V.S., unpublished).

Based on these successes, we investigated the *in vivo* transduction properties of AAV r3.45 and demonstrated its utility for targeted genetic modification of adult NSCs *in vivo*. In particular, AAV r3.45 exhibited efficient and selective transduction of adult mouse, rat and human NSCs, both *in vitro* and *in vivo*. In addition, to investigate its utility for basic biological investigation, AAV r3.45 was harnessed to deliver constitutively active β-catenin to NSCs in the mouse hippocampus in order to study the mechanisms by which β-catenin signaling increases neurogenesis.

RESULTS

AAV r3.45 enables increased selectivity towards hESC-derived NSCs *in vitro*

Jang et al. previously described the directed evolution of a novel AAV2 variant, AAV r3.45, which contained a LATQVGQKTA peptide insertion at amino acid 587 with a V719M mutation (Jang et al., 2011). The variant mediated enhanced gene delivery to rat, mouse and human NSCs *in vitro* compared with several wild-type AAV serotypes (Jang et al., 2011). However, efficiency is distinct from selectivity, and *in vitro* is different from *in vivo*. To initially assess selectivity, an hESC-derived culture containing a mixture of cells, including neural progenitor cells, neurons and astrocytes, was infected at an MOI of 10,000 with GFP-encoding AAV r3.45, its parental wild-type AAV2 serotype, or wild-type AAV6 (the most effective natural serotype tested on NSCs *in vitro*) (Jang et al.,

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2011). Forty-eight hours later, the percentage of infected cells in each culture that stained positive for the neural stem and progenitor cell marker nestin was significantly higher for AAV r3.45 compared with wild-type AAV2 and AAV6 (Fig. 1). Furthermore, AAV r3.45 was the only virus to transduce a higher proportion of NSCs relative to neurons. In conjunction with the *in vitro* data reported by Jang et al., this experiment indicates that AAV r3.45 is capable of general and selective neural stem and progenitor cell infection.

AAV r3.45 enables increased selectivity towards and infectivity of adult NSCs in the rodent brain

Several AAV variants generated via *in vitro* directed evolution systems have proved successful when translated to an *in vivo* model (Klimczak et al., 2009; Koerber et al., 2009), and we therefore investigated the transduction properties of AAV r3.45 *in vivo*. This AAV variant encoding GFP was initially administered via intracranial injection to the dentate gyrus of the rat hippocampus. Consistent with previous reports, AAV2 showed strong tropism for mature neurons (Bartlett et al., 1998; Kaspar et al., 2002; Ortinski et al., 2010) in the hilar region. In clear contrast to wild-type AAV2, AAV4 and AAV6, a two- to fivefold higher fraction of cells infected by AAV r3.45 expressed the markers nestin and Sox2 (Fig. 2A-C; supplementary material Fig. S1A). Specifically, ∼65% of the cells infected by AAV r3.45 expressed nestin and Sox2, as compared with ∼33% NeuN-expressing neurons and 1% Gfap- and S100β-expressing glia (Fig. 2B), demonstrating selective infectivity in the brain. AAV r3.45 also infected a 1.5- to threefold larger fraction of the resident Type 1 (nestin$^+$/radial morphology) and Type 2a (nestin$^+$/Sox2$^+$) neural stem and progenitor cells in the subgranular zone (∼41% and ∼60%, respectively) than any wild-type AAV serotypes tested, indicating that it is also capable of efficient NSC transduction *in vivo* (Fig. 2D).

To extend these results to a murine model, GFP-encoding AAV r3.45 was administered to the mouse hippocampus. Three weeks post-administration, ∼82% of cells infected by AAV2 and 67% of cells infected by AAV6 were neurons (Fig. 3A,B; supplementary material Fig. S1B). By comparison, AAV r3.45 selectively transduced murine Type 2a cells relative to wild-type AAV2 and AAV6, although it appeared to transduce neurons and NSCs with similar selectivity (Fig. 3A,B). However, NSCs continuously undergo proliferation and differentiation into neurons over a period of days to weeks (Ashton et al., 2012; Kim et al., 2012a), and it is thus possible that GFP$^+$ neurons visualized 3 weeks after injection originated from cells that were NSCs at the time of AAV administration. To determine whether the difference in NSC versus neuronal transduction between mouse and rat could be attributed to the more rapid timescale for stem cell differentiation in mice (Duan et al., 2008), a second cohort of animals received BrdU to label proliferating stem cells following injection of AAV r3.45. Of the infected neurons present 3 weeks post-injection, a significantly higher percentage were BrdU$^+$ in mice administered with AAV r3.45 than in mice administered AAV2 or AAV6 (Fig. 3C), indicating that these cells may have been stem and progenitor cells at the time of infection.

To investigate the timing of AAV transgene expression and NSC differentiation in greater detail, hippocampi were analyzed 3 days, 1 week and 2 weeks post-injection with AAV r3.45. At each of these three earlier time points, a significantly higher percentage of NSCs

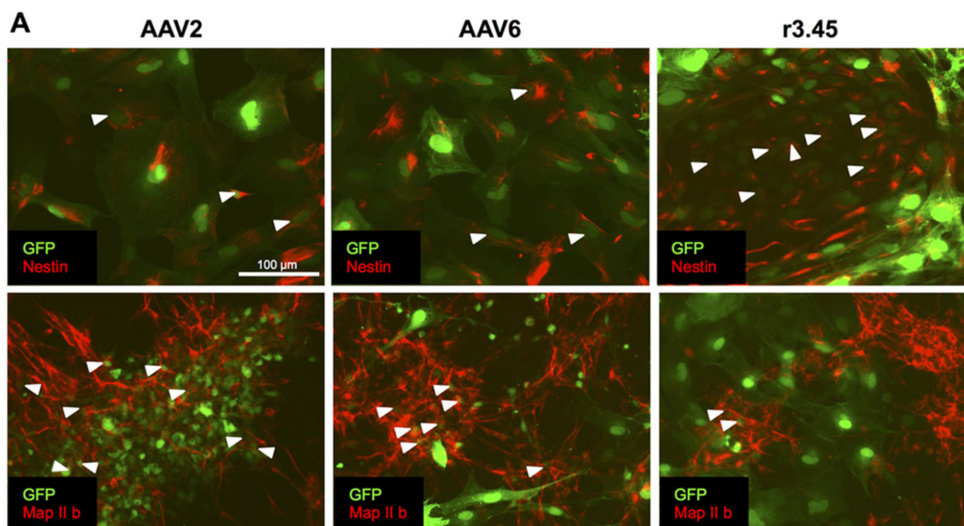

Fig. 1. Selectivity towards hESC-derived NSCs *in vitro*. (A) Representative images of areas of hESC-derived neuronal cultures containing neural stem and progenitor cells (top row, red) or mature neurons (bottom row, red), 48 h post-infection with recombinant AAV2, AAV6 or AAV r3.45 vectors expressing GFP (green). Representative examples of infected cells of each type are marked with arrowheads. Scale bar: 100 µm. (B) The percentage of GFP$^+$ cells co-staining for nestin or MAP2B was quantified to determine the selectivity of each viral vector. Error bars indicate s.d. (n=3); *P<0.01, **P<0.005 (ANOVA).

DEVELOPMENT

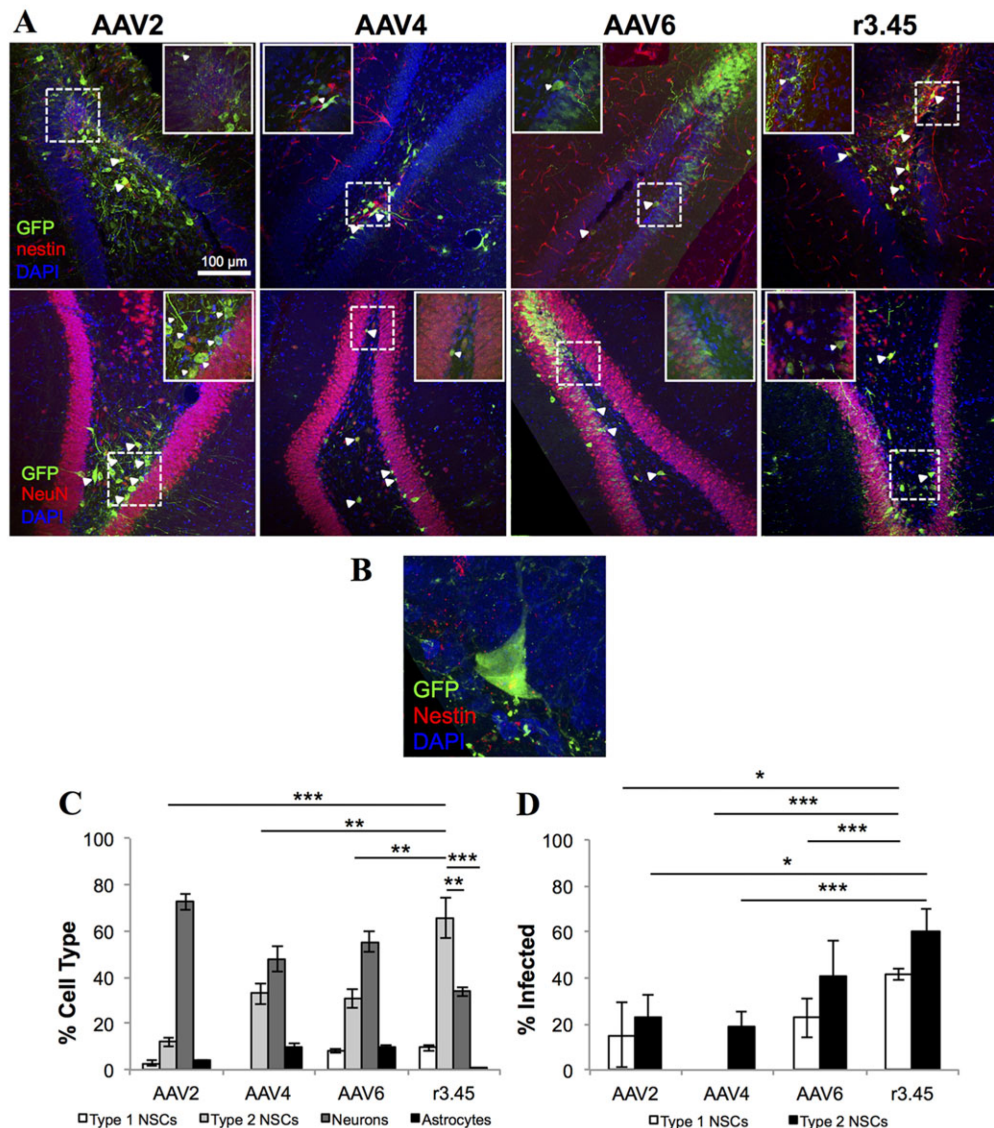

Fig. 2. Selectivity towards and infectivity of adult NSCs in the rat brain. (A) Representative images at low (main, 20×) and high (inset, 100×) magnification of the rat dentate gyrus 3 weeks post-injection of recombinant AAV2, AAV4, AAV6 or AAV r3.45 vectors expressing GFP (green). Brain sections were co-stained for nestin (top row, red) or NeuN (bottom row, red) along with DAPI (blue), and infected cells of each type are marked with arrowheads. Dashed rectangles indicate the regions magnified in the insets. Scale bar: 100 μm. (B) Representative image of a Type 1 NSC infected with AAV r3.45 vector. (C) The percentage of GFP$^+$ cells co-staining for markers of each cell type was quantified to determine the selectivity of each viral vector. (D) The percentage of nestin$^+$/Gfap$^+$ (Type 1) or nestin$^+$/Sox2$^+$ (Type 2a) cells infected by each viral vector was quantified to determine NSC infectivity. Error bars indicate s.d. (*n*=3); **P*<0.05, ***P*<0.01, ****P*<0.005 (ANOVA).

was infected relative to neurons (Fig. 3D), offering additional evidence that AAV r3.45 preferentially infects NSCs in the mouse hippocampus. Consistent with the results from administration to rats, AAV r3.45 infected ∼38% of mouse NSCs in the subgranular zone *in vivo*, a larger percentage than with the wild-type AAV serotypes tested, showing that efficient transduction of NSCs is conserved across rodent species (Fig. 3E).

β-catenin increases neurogenesis through both proliferation and differentiation of NSCs

NSCs are regulated by cues from the environment, including growth factors, morphogens, neurotransmitters and other signals (Faigle and Song, 2013). For example, the Wnt pathway generally functions in cell-cell communication in both the embryo and adult and has been shown to play a role in stem cell proliferation and differentiation during development and healing (Logan and Nusse, 2004). The canonical Wnt pathway involves the stabilization of β-catenin, which then translocates to the nucleus to act as a transcriptional co-factor of key transcriptional targets. Previous work determined that the canonical Wnt pathway elevates the number of newborn neurons, although the study did not investigate whether this pathway did so via regulation of proliferation or differentiation of NSCs (Lie et al., 2005). Subsequent studies showed through lineage tracing of Wnt-responsive NSCs in Axin2^{CreERT2} transgenic mice, *Wnt7a* knockout mice, and lentivirus-mediated delivery of a β-catenin inhibitor to mouse NSCs *in vivo* that the Wnt pathway regulates the proliferation of NSCs (Bowman et al., 2013; Qu et al., 2010). However, recent work demonstrates that ephrin B2 signals through β-catenin, the activation of which induces neuronal lineage commitment of NSCs *in vivo* (Ashton et al., 2012).

Based on the ability of AAV r3.45 to efficiently and selectively infect NSCs in the mouse hippocampus, we delivered constitutively active β-catenin (CA β-catenin) to NSCs in the mouse hippocampus to study its mechanism of action. Mice were injected with EdU to label proliferating cells 3 days post-AAV administration, at the approximate time when gene expression from the AAV r3.45 vectors is initiated (based on GFP expression in Fig. 3D). Analysis 18 days later showed significant increases in the number of infected cells per hippocampus that were EdU$^+$ (15.00±1.12 cells), EdU$^+$/nestin$^+$ (7.17±0.17 cells), EdU$^+$/Dcx$^+$ (4.56±0.25 cells) and EdU$^+$/NeuN$^+$ (3.28±0.69 cells) (Fig. 4) in the mice administered AAV r3.45 expressing CA β-catenin, as compared with the number of infected cells per hippocampus that were EdU$^+$ (6.89±0.94 cells), EdU$^+$/nestin$^+$ (4.17±0.17 cells), EdU$^+$/Dcx$^+$ (2.28±0.59 cells) and

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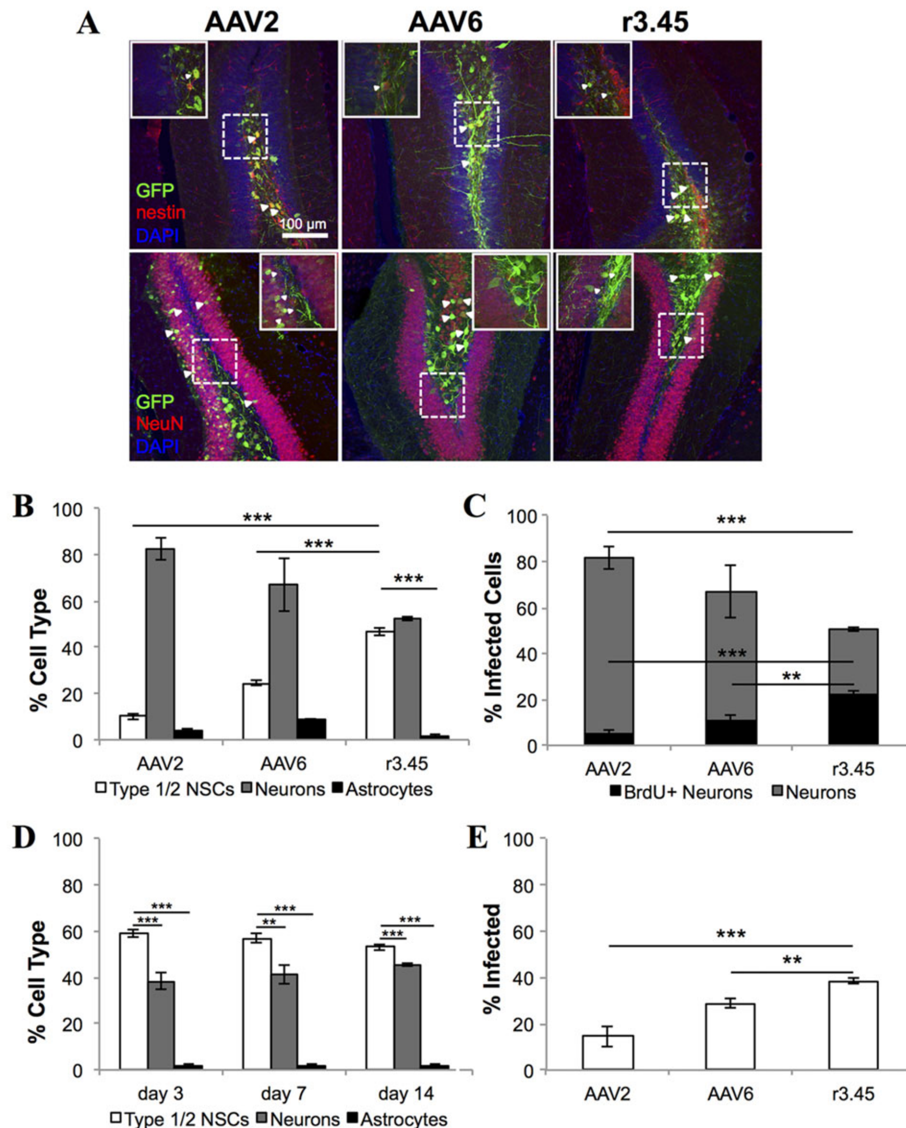

Fig. 3. Selectivity towards and infectivity of adult NSCs in the mouse brain. (A) Representative images at low (main, 20×) and high (inset, 100×) magnification of the mouse dentate gyrus 3 weeks post-injection of recombinant AAV2, AAV6 or AAV r3.45 vectors expressing GFP (green). Brain sections were co-stained for nestin (top row, red) or NeuN (bottom row, red) along with DAPI (blue), and infected cells of each type are marked with arrowheads. Dashed rectangles indicate the regions shown at high magnification in the insets. Scale bar: 100 µm. (B) The percentage of GFP$^+$ cells co-staining for markers of each cell type was quantified to determine the selectivity of each viral vector. (C) The percentage of GFP$^+$ cells co-staining for NeuN (a neuronal marker) or NeuN and BrdU (a cell division marker) were analyzed to determine the proportion of GFP$^+$ neurons that had differentiated from infected NSCs prior to sacrifice. (D) The percentage of GFP$^+$ cells co-staining for markers of each cell type was quantified to determine the selectivity of variant AAV r3.45 at 3, 7 and 14 days post-injection. (E) The percentage of nestin$^+$/Sox2$^+$ (Type 2a) cells infected by each viral vector was quantified to determine the infectivity of NSCs. Error bars indicate s.d. (n=3); $^{**}P<0.005$, $^{***}P<0.001$ (ANOVA).

EdU$^+$/NeuN$^+$ (0.44±0.19 cells) when administered control, GFP-encoding AAV r3.45. The EdU$^+$ counts indicate that CA β-catenin acts to increase proliferation of the NSC population. Furthermore, the increase in the number of infected cells that were EdU$^+$/NeuN$^+$ was much greater than the increase in infected cells that were EdU$^+$/nestin$^+$ and EdU$^+$/Dcx$^+$, which indicates that β-catenin may also induce differentiation (Fig. 4C).

These data suggest that the Wnt pathway functions through both proliferation and differentiation mechanisms to induce neurogenesis, consistent with reports that propose a proliferative and a differentiative role (Ashton et al., 2012; Chen et al., 2013; Israsena et al., 2004; Otero et al., 2004). In addition, this study establishes proof-of-principle that AAV r3.45 can be used to deliver transgenes to study NSC regulation in the hippocampus.

DISCUSSION

Adult NSCs, as defined by their capacity for self-renewal and differentiation into mature neural cell types of the brain, contribute to neurogenesis throughout mammalian life. Selective and efficient gene delivery to NSCs *in vivo* offers an opportunity to more rapidly study the basic mechanisms that regulate the quiescence, proliferation, self-renewal and differentiation of NSCs in their *in vivo* environment, and opens potential future avenues to harness NSCs to treat CNS injury or disease. The development of transgenic mouse lines has led to advances in adult neurogenesis research (Ashton et al., 2012; Bonaguidi et al., 2011; Lagace et al., 2007). However, such efforts are labor intensive (Haruyama et al., 2009), and gene delivery by comparison is more rapid and offers translational opportunities (Kotterman and Schaffer, 2014). Prior work in the field established some transduction of neural progenitors, but efficiency was limited and did not include Type 1 NSCs (Falk et al., 2002; Lemkine et al., 2002; van Hooijdonk et al., 2009). AAV vectors have gathered increasing momentum for basic biological investigation (Oh et al., 2014) and for clinical gene delivery (Bainbridge et al., 2008; Kotterman and Schaffer, 2014; MacLaren et al., 2014; Maguire et al., 2008, 2009; Nathwani et al., 2011; Ojala et al., 2015). AAV r3.45 is reportedly capable of efficient transduction of rat, mouse and human NSCs *in vitro* (Jang et al., 2011). This work further establishes that AAV variants engineered *in vitro* can also be successful when translated to *in vivo* models (Klimczak et al., 2009; Koerber et al., 2009).

In an hESC-derived culture containing a mixture of cells, including NSCs, neurons and astrocytes, the percentage of cells infected by AAV r3.45 that were NSCs was significantly higher than with

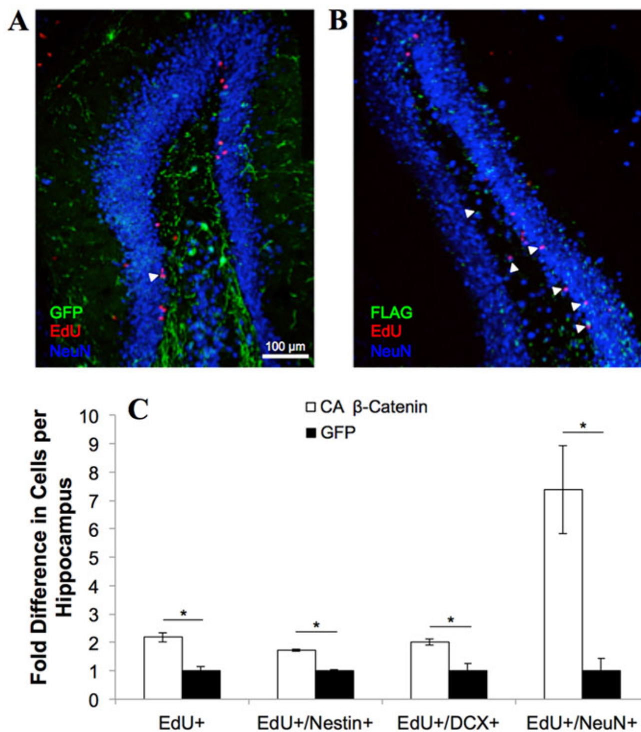

Fig. 4. Proliferation and differentiation of NSCs induced by CA β-catenin. (A,B) Representative images at low (20×) magnification of the mouse dentate gyrus 3 weeks post-injection of recombinant AAV r3.45 vectors expressing (A) GFP (green) or (B) CA β-catenin-2×FLAG (green). Brain sections were co-stained for NeuN (blue) and EdU (red), and infected cells co-staining for NeuN and EdU are marked with arrowheads. Scale bar: 100 µm. (C) Infected cells ($FLAG^+$, β-catenin condition; GFP^+, control condition) co-staining for EdU (a cell division marker), nestin (an NSC marker), Dcx (an immature neuronal marker) and/or NeuN (a neuronal marker) were analyzed to determine the degree to which β-catenin stimulates the proliferation and differentiation of NSCs. Error bars indicate s.d. (n=3); *P<0.001 (ANOVA).

wild-type AAV. Upon characterization of the *in vivo* properties of AAV r3.45, it was discovered that this variant was also capable of efficient and preferential infection of NSCs in adult rat and mouse brain. Approximately 65% of the cells infected in the rat hippocampus by AAV r3.45 were Type 2a NSCs, and 9% were Type 1 NSCs 3 weeks post-injection. Furthermore, overall 60% of Type 2a NSCs and 41% of Type 1 NSCs were transduced. This trend continued in the mouse brain, where ∼38% of Type 2a NSCs were transduced in the hippocampus 3 weeks post-injection. This level of selectivity already offers utility for investigating NSC function, and could be even further refined with promoters or miRNA-binding elements (Brown and Naldini, 2009; Jessberger et al., 2008).

Previous work to elucidate the mechanism of β-catenin-mediated increased neurogenesis indicated two possible effects: (1) the neuronal differentiation of stem cells and (2) the proliferation of later stage transit amplifying cells. Using AAV r3.45 to deliver CA β-catenin to mouse NSCs *in vivo* enabled the study of these pathways. Analysis showed significant increases in the number of infected NSCs, neural progenitor cells and neurons, suggesting that the Wnt pathway functions through both proliferation and differentiation mechanisms to induce neurogenesis, consistent with reports demonstrating each role (Ashton et al., 2012; Chen et al., 2013; Israsena et al., 2004; Otero et al., 2004).

In conclusion, AAV r3.45 exhibits efficient and selective transduction of human NSCs *in vitro* and adult mouse and rat NSCs *in vivo*. This characterization of AAV r3.45 revealed its potential utility in further studies of neurogenesis in the adult brain and in novel gene therapy and cell replacement therapy applications.

MATERIALS AND METHODS

Virus production

HEK293T cells, obtained from the American Type Culture Collection (Manassas), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Invitrogen) at 37°C and 5% CO_2. Recombinant AAV vectors expressing green fluorescent protein (GFP) or CA β-catenin under the control of a CMV promoter were packaged in HEK293T cells using the calcium phosphate transfection method as described, and the viruses were purified by iodixonal gradient centrifugation and Amicon filtration (Excoffon et al., 2009; Maheshri et al., 2006). DNase-resistant genomic titers were determined by quantitative PCR as previously described (Lai et al., 2002).

In vitro infection of human embryonic stem cell-derived mixed neuronal cultures

H1 human embryonic stem cells (WiCell) were cultured on Matrigel-coated cell culture plates (BD Biosciences) in mTeSR1 maintenance medium (Stem Cell Technologies) for growth and expansion. To initiate cortical differentiation of hESCs, cells were seeded in adherent conditions at a density of $5{\times}10^4$ cells/cm^2 in growth medium. At 50% confluence, the medium was gradually changed to NeuroBasal medium (Invitrogen) containing N2 and B27 supplements (Invitrogen). SMAD inhibitors LDN193189 (1 µM, Stemgent) and SB432542 (10 µM, Tocris Biosciences) were added for the first week of neural induction. Cyclopamine (400 ng/ml, Calbiochem) and FGF2 (10 ng/ml, Peprotech) were added on days 3-14 of differentiation. After 12-14 days, cells were mechanically passaged into poly-L-ornithine (Sigma-Aldrich) and laminin (20 µg/ml, Invitrogen) coated plates and allowed to mature for 3-6 weeks. BDNF (10 ng/ml, Peprotech) was added to cultures 1 week after initiation of neuronal maturation.

Cells were infected at a multiplicity of infection (MOI) of ∼10,000 with recombinant AAV2, AAV6 or AAV r3.45 vectors encoding GFP. Forty-eight hours post-infection, cells were fixed in 4% paraformaldehyde for 15 min, washed three times with phosphate-buffered saline (PBS), and blocked with 1% BSA and 0.1% Triton X-100 in PBS for 30 min. Cells were incubated overnight at 4°C with a mouse anti-nestin (1:500; Abcam, ab6142) or mouse anti-MAP2 (1:500; BD Biosciences, 610460) primary antibody. Cells were then washed three times with PBS and incubated with a fluorescent-conjugated donkey anti-mouse secondary antibody (1:250; Invitrogen, 715-545-150) for 2 hours. Cells were imaged using an Axio Observer.A1 inverted microscope (Zeiss). Quantification of infected cells was performed using the Cell Counter function in ImageJ (NIH).

Stereotaxic injections

Animal protocols were approved by the UC Berkeley Animal Care and Use Committee and conducted in accordance with National Institutes of Health guidelines. Recombinant AAV2, AAV4, AAV6 or AAV r3.45 vectors encoding GFP were stereotaxically injected into the hippocampus (AP, −3.5; ML, ±2.0; V/D, −3.5) of 12-week-old female Fischer 344 rats. Animals were anesthetized with ketamine (Butler Animal Health Supply; 68 mg/kg body weight) and xylazine (Lloyd Laboratories; 38 mg/kg body weight) prior to injection, and 3 µl of $5{\times}10^8$ viral genomes (vg)/µl AAV vector per hippocampus was injected using a Hamilton syringe as described (Lai et al., 2002).

In addition, recombinant AAV2, AAV6 or AAV r3.45 vectors encoding GFP were stereotaxically injected into the hippocampus (AP, −2.12; ML, ±1.5; V/D, −1.55) of 9-week-old female BALB/c mice. The animals were anesthetized with ketamine (Butler Animal Health Supply; 50 mg/kg body weight) and xylazine (Lloyd Laboratories; 50 mg/kg body weight) prior to injection, and 1 µl of $1.5{\times}10^9$ vg/µl AAV vector per hippocampus was injected using a Hamilton syringe as described (Lai et al., 2002). Mice were injected with 50 mg/kg 5-bromo-2′-deoxyuridine (BrdU) for 3 consecutive days pre-stereotaxic injection, then injected with 50 mg/kg

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BrdU every other day until perfusion or injected with 50 mg/kg 5-ethynyl-2′-deoxyuridine (EdU) on days 6-9 post-stereotaxic injection. Three days to 3 weeks post-injection, animals were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde overnight at 4°C and stored in 30% sucrose for cryoprotection.

Histological processing and immunohistochemistry of brain tissue

Brains were mounted onto a Series 8000 sliding microtome (Bright) with Clear Frozen Section Compound (VWR) and frozen with dry ice. Coronal sections (40 μm) were cut, and sections containing the hippocampus were stored at −20°C prior to immunostaining. Brains were stained as floating sections in a 12-well dish. Tissue sections were washed three times for 15 min each in PBS, then blocked in a solution containing 3% donkey serum and 0.3% Triton X-100 for 2 h at room temperature. After blocking, tissue sections were incubated with primary antibodies for 72 h at 4°C. The primary antibodies and dilutions used were: mouse anti-nestin (1:500; Abcam, ab6142), rabbit anti-Sox2 (1:250; Millipore, AB5603), mouse anti-NeuN (1:100; Millipore, MAB377), guinea pig anti-Dcx (1:1000; Millipore, AB2253), mouse anti-Gfap (1:1000; Advanced ImmunoChemical, 2-GFAP), rabbit anti-Gfap (1:1000; Abcam, ab7260), rabbit anti-S100β (1:1000; Abcam, ab52642) and chicken anti-GFP (1:1000; Abcam, ab13970). Tissue sections were washed again three times for 15 min each in PBS, then blocked in a solution containing 3% donkey serum and 0.3% Triton X-100 for 1 h at room temperature. After blocking, tissue sections were incubated with AffiniPure donkey anti-mouse, rabbit, guinea pig and chicken secondary antibodies (1:250; Jackson ImmunoResearch, 715-545-150, 711-545-152, 706-605-148, 703-605-155, respectively) and 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain (Invitrogen) for 2 h at room temperature. Tissue sections were washed three more times in PBS, then mounted onto slides and coverslipped. Images of the sections were taken using an LSM 710 laser scanning confocal microscope (Zeiss). Quantification of infected cells within the sections was performed using an Axio Imager.M1 microscope and analysis system (Zeiss) and Stereo Investigator analysis software (version 8.26, MBF Bioscience). Cells were scored using the following markers: Type 1 NSCs, nestin$^+$/Gfap$^+$; Type 2a NSCs, nestin$^+$/Sox2$^+$; neurons, NeuN$^+$; astrocytes, Gfap$^+$/S100β$^+$.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceived and designed experiments: M.A.K., T.V. and D.V.S. Performed the experiments: M.A.K. Analyzed the data: M.A.K. and D.V.S. Contributed reagents/materials: T.V. Wrote and edited the manuscript: M.A.K. and D.V.S.

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Supplementary material

Supplementary material available online at
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