A novel AAV1 variant for transduction of Japanese quail neurons in vitro and in vivo

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Abstract

The widespread use of rodents in neuroscience has prompted the development of optimized viral variants for transduction of brain cells, *in vivo*. However, many of the viruses developed are less efficient in other model organisms, with birds being among the most resistant to infection by current viral tools. Resultantly, the use of genetically-encoded tools and methods in avian species is scarce; markedly holding the field back. We sought to bridge this gap by developing custom viruses towards the transduction of brain cells of the Japanese quail. We first develop a unique protocol for culturing primary neurons and glia from quail embryos, followed by characterization of cultures via immunostaining, single cell mRNA sequencing and patch clamp electrophysiology. We then leverage the cultures for the rapid screening of various viruses, only to find that all yielded very poor to no infection of cells *in vitro*. However, few infected neurons were obtained by AAV1 and AAV2. Scrutiny of the sequence of the AAV receptor found in quail led us to rationally design novel AAV variants, with one variant (AAV1-T593K; AAV1*) showing improved transduction efficiency *in vitro* and *in vivo*. Together, we present a novel culturing method, unique transcriptome profiles of quail's brain cells and a custom-tailored AAV1 for transduction of quail neurons *in vitro* and *in vivo*.

Introduction

Avian species are a powerful experimental organism in neuroscience (e.g.,^{1–7}). Birds display sophisticated cognitive capabilities and specialized behaviors, such as long-distance navigation⁸, imprinting, homing, food-caching, song learning, etc.^{9,10}. Interestingly, these complex cognitive capabilities are presented by birds despite their diverging neuroarchitecture in comparison to mammals^{4,11}. These provide unique opportunities for comparative studies of cellular mechanisms leading to behavior³. Indeed, these realizations are behind the increased interest in avian neuroscience in recent years^{1,2,7,12–17}.

Of the various avian species commonly studied, we focused our attention on domestic Japanese quail (*Coturnix japonica*). Quails are relatively small animals and therefore require smaller housing and animal facilities, reach sexual maturity quickly (significantly shorter than mice and chicken) and females lay ~one egg per day; ideal for routine experimentations¹⁶. A particular benefit of this model over other birds is their ground-dwelling nature, which may simplify various behavioral studies (such as spatial navigation¹⁸) by reducing dimensionalities (e.g.,¹⁹). Despite the latter, (and despite extensive use in developmental biology^{16,20,21}), quails are rarely used in neuroscience^{6,18}. In part, we deem this to result from lack of tools and methods for monitoring and examining neurons and/or glia in quails *in vivo*²². More precisely, whereas mammalian neuroscience heavily relies on viral tools for delivery of genetic optical probes for interrogating the brain (e.g., GCaMP and ChR^{23,24}), the use of viruses in quails has been primarily, if not exclusively, employed for transgenesis of primordial germ cells (and solely by lentiviruses)^{6,16,25–29}. Thus, whether transduction of other cells types can be obtained by lentivirus or other viruses (e.g., adeno associated virus; AAV) remains unknown¹⁶. Notably, the shortage (or complete lack) of viral tools is not limited to the quail model, rather is a recurring theme in the avian field^{30–33}. Resultantly, the avian neuroscience community is largely focused on a handful of species (e.g., finches³⁴, pigeons³⁰ and canaries⁷).

We sought to bridge this gap by screening for suitable viral vectors for transduction of neurons of Japanese quails. To screen numerous viral candidates, we develop a unique protocol for culturing of primary neuronal cultures from quail and chicken embryos. Systematic characterization of cells in cultures demonstrated that the cultures are viable, contain over 11 different cellular populations, including two populations of mature neurons. We use the cultures for screening of multiple viruses commonly used in rodents and other avian species, but none show efficient transduction. We then reverted to rational engineering of two viral capsids and produce a single infectious AAV1-variant (AAV1-T593K; AAV1*) exhibiting improved infection efficiency *in vitro*. This variant also proved suitable for transduction of primary chicken cultures. Lastly, and importantly, we find that AAV1* yields better transduction and dispersion *in vivo*.

Results

AAV1 does not infect quail neurons *in vivo*— It is well accepted in the avian field that viral-transduction of neurons *in vivo* is very challenging³⁰. However, a handful of recent reports show partial success following screen of common recombinant adeno associated viruses (rAAV; AAV for brevity), for instance AAV1 in pigeons³⁰ and barn owls¹⁵. Thus, we firstly examined whether AAV1 would also be suitable for transduction of neurons of Japanese quails *in vivo*. We produced and injected YFP-expressing AAV1 (titers are described in **methods**) into the Wulst of two months old quails, followed by search for YFP-positive cells over the course of three to eight weeks (**methods**). Unfortunately, we found no expression in three animals, with a fourth animal exhibiting very restricted YFP expression past seven weeks of expression *in vivo* (**Suppl. 1** and see below). The poor infection, and extreme variability between different animals (even when injected with the same virus batch), deterred us from performing similar exploration with added viral serotypes, as this endeavor would require the use of a very large cohort of animals and months to completion.

Development and characterization of primary cultures from Quail embryos— To rapidly screen multiple different viruses, we sought to examine *in vitro* transduction of cultured cells instead. However, we were immediately struck by the scarcity in protocols for producing primary neuronal avian cultures^{31,35} and — complete absence — in the case of Japanese quails. To develop primary neuronal cultures from embryos or post hatch quails, we first explored the most common methods for culturing primary neurons from rodents and chickens^{36,37}. We dissected forebrains from seven to nine days *in ovo* (DIO) embryos or post-hatched chicks; mechanically dissociated and enzymatically digested the tissue, followed by plating of cells onto poly-D-lysine (PDL) covered glass coverslips and grown in standard growth media and conditions (see **methods and Table 1**). During the first hours after the procedure, we could distinguish cell bodies, some even showing extending neurite-like processes (**Suppl. 2a, arrowheads**), but only in cultures produced from embryos.

Cultures from post-hatched chicks did not yield any viable cultures. Nevertheless, embryonic cultures progressively waned, and most cells died past ~four days *in vitro* (DIV), even when higher PDL concentrations were used (Suppl. 2b, c). Fortunately, we noticed viable cells at the borders of the wells (i.e., beside the glass coverslip) but, again, only in cultures produced from chick embryos. We therefore proceeded to examine growing the cultures directly on the tissue-culture plate itself (also coated by PDL), instead of glass coverslips. We also substituted the MEM-based growth medium typically used in rats and chicken culturing protocols^{31,35}, to an enriched Neurobasal medium employed for growing rodent organotypic slices (Table 1)³⁸. These collective modifications allowed the cultures to thrive, particularly cultures produced from 9 *DIO* embryos, and remain viable past three weeks (Fig. 1a). Cultures produced from an entire hemisphere (plated onto 60 mm culture plates) yielded a rich network of highly interconnected and branched cells, most of which (>90%) showed positive NeuN- and Neurotrace-staining, thereby representing neurons (Fig. 1b, c and Suppl. 2d). Nevertheless, many cells did not exhibit the mature neuronal morphology.

To assess the culture's viability and examine whether we could detect mature neurons, we examined the electrical properties of various cells in culture via whole cell patch clamp recordings. Indeed, cells showing typical neuronal morphology displayed action-potential firing and, under voltage-clamp conditions, prototypical barrages of excitatory post synaptic currents (EPSCs) (Fig. 2a). We thereby explored several added features of quail neurons and find quail neurons to be highly analogous to age-matched cultured neurons produced from mouse embryos (Fig. 2b, Suppl. 3a), but cultured quail neurons display lower firing rates in response to current injections, suggesting these are of lower intrinsic excitability (Fig. 2c, d and Suppl. 3b). Thus, our culturing protocol yields viable cultures with various cell types, importantly with mature neurons displaying prototypical electrical activity.

Single cell mRNA-sequencing of embryonic quail cultures— To better classify the various cell-types observed in the cultures, we employed single cell mRNA sequencing (sc-mRNA seq.³⁹) of cultured cells produced from 9 *DIO* embryos (grown for 7 DIV; methods^{40–42}). Unsupervised clustering of the data (based on the significant top-ten or top-200 differentially-expressed-genes; DEGs) acquired from ~10,000 cultured cells revealed 15 noticeable clusters of cell types (clusters #0-14; Fig. 3a, b and Suppl. 4a). We immediately noted that many transcripts could not be easily (and automatically) identified. We had to manually curate many genes (e.g., Fig. 3a, red annotations). However, dozens of other genes could not be easily identified despite multiple reference genomes (e.g., ensembl.org), including the recently published (though partially-sequenced) quail genome⁴³. Nevertheless, to try to identify the clusters, we queried multiple cell-type specific or enriched gene markers, as previously described^{44–46} (along online atlases, e.g., proteinatlas.org) to classify most populations of cells. The largest group of cells (2400 cells) displayed a broad and dispersive expression of most top DEGs (Suppl. 4a). This group shows a combination of neuronal (STMN2, CDH2) and glial markers (FABP7, PROM1, SLC1A3), but also moderate expression of NES (neuroepithelial marker) and VIM (radial glia marker), representing very early Radial glial (RG) cells expressing early neuronal markers, a type of neural committed progenitor cells that precedes neurons, pro-neuronal Radial glial (Fig. 3c, cluster #0, see summary in Table 2)^{47–49}. This observation is supported by our staining results showing the very high abundance of neuronal (or neuronal-like) cells (see Figs. 1, Suppl. 2)⁵⁰, as well as by the presence of two *bona fide* RG populations in the cultures (Fig. 3c, clusters # 1 and 2). Cluster #1 could be best classified as apical-RG owing to PROM1 expression⁵¹ (along other glial markers, namely FABP7 and SLC1A3), whereas cluster #2 shows expression of NES and dispersive expression of other markers (reminiscent of cluster #0); suggesting a slightly earlier differentiation state of non-apical RG⁵¹. We could easily distinguish two mature neuronal populations, both expressing specific pan-neuronal markers STMN2, SYT1 and NEUROD6 (Fig. 3c, clusters 6 and 7). The inhibitory neuronal population exclusively expressed GAD1 and 2, DLX1 and 2, and moderate levels of SOX11, whereas excitatory neurons showed strong expression of GABBR2, GRIN (1 and 2B) and EPN2 and, uniquely, SLC17A6. We did not detect other neuron types in this brain region (e.g., cholinergic). We further find three distinct microglia populations (clusters #5, 8 and 13), with one cluster (cluster #13, n= 130 cells) showing added proliferation markers, explicitly HMGB2, CENPF, TOP2A and CCNB2^{52,53}. These imply that cluster #13 represents dividing microglia. These are consistent with reports showing the complex heterogeneity in microglia populations especially during early development, compared with adult stages^{54,55}. The smallest cluster of cells (#14, n=125 cells) is distinguished as oligodendrocyte precursor cells owing to expression of PTPRZ1, PMP2 and PDGFRA (Fig. 3c, PDGFRA; manually annotated). Cluster #10 lies at the interface between clusters 0, 4 and 14, with neuronal progenitor cells (NPC) markers and additional division markers as seen in cluster 13, specifically HMGB2 and TOP2A^{52,53}, therefore depicts non-quiescent (i.e., dividing) neuronal progenitor stem cell population. We also find that the cultures contain ~14% fibroblasts (clusters # 3, 9, 11; n=1415 cells) by their variable expression of COL1A1⁵⁶. These cells arise from remaining meninges and perivascular system⁵⁷. Interestingly, one fibroblast population (#11) shows very weak expression of neuronal markers (e.g., SOX11 and ENC1). This is a unique scenario for which we could not find any mention in the literature. In fact, inclusion of SOX11 (a transcription factor) in fibroblasts (postnatal skin fibroblasts) is used to differentiate the cells into cholinergic neurons⁵⁸. Identification of clusters #12 and 4 proved more challenging. Cluster #4 (residing adjacent to clusters 0-2) expresses RGs markers (SLC1A3, VIM, NES, PROM1; similar to cluster #1), along expression of MFGE8, GJA1 and ID3; astrocytic markers in the mouse^{46,59}. However, this cluster shows no other astrocytic markers, especially GFAP⁶⁰ (Suppl. 4b). We confirmed this by detecting no staining against GFAP, whereas control rat cultures showed extensive labeling (Suppl. 4c). Furthermore, cluster #4 shows weak, albeit significant, expression of RELN (neuronal marker). This unexpected transcriptome signature led us to hypothesize that this population represents an earlier differentiation state than RGs, namely RG-Progenitors (RGP)^{61,62}. Indeed, ~65% of cells within cluster #4 showed enriched levels of SFRP1; a prominent marker for RGP (Fig. 3d). Lastly, cluster #12 (n= 135 cells) shows strikingly overlapping transcriptional signatures with cluster #0, but with higher expression of inhibitory neuronal markers (SOX11, ENC1), though with no other GABAergic markers. Its location between clusters # 0, 1 and 6 suggests it to represent a slightly more NPC-RG differentiated state towards inhibitory neurons⁶³. Together, we find that the quail embryonic cultures provide a very rich repertoire of cell types⁶⁴, ideal for studying stem cells of the brain. We further suggest that the rich abundance of the latter, along easy access provided by the monolayered culture, provides opportunities for exploring their electrophysiological properties.

Viral screening *in vitro* and rationale evolution of viral capsid to infect quail brain cells — Having established the identity of cells in our cultures, we next proceeded to screen for transduction of primary quail cultures by various viruses. We focused on common adeno-associated viruses currently in use:

AAV1, AAV2 (and variants), AAV9, AAV-PhP-B⁶⁵, Baculovirus (baculo.)⁶⁶, lentivirus (lenti.)^{25,26}, and an avian AAV (A3V³¹). We produced YFP-expressing viruses (AAVs, A3V and lenti.) and purchased a commercial GCaMP-(genetically-encoded calcium indicator⁶⁷) expressing baculovirus (see **methods**). We infected the cultures in parallel to cultured mammalian cells (HEK293t cells), followed by assessment of fluorescence after three DIV (**Fig. 4a**). Whereas all viruses robustly infected mammalian cells (except for A3V which showed no infection of HEK293t, as described⁶⁸), we observed very sparse infection and weak expression in quail cells. Nevertheless, and importantly, few positively-infected neurons (assessed by morphology) were observed following infection by AAVs, specifically AAV1, AAV2-SL1 and AAV-PhP-B, and by the lentivirus, though slightly less cells (**Fig. 4**). Baculovirus infection was highly toxic to these cultured cells (**Fig. 4, bottom row**).

The persistent failures of common viruses to infect the cultures motivated us to try to engineer tailored viral vectors for quail cells. To do so, we first examined the entry routes of AAVs into mammalian cells and note that most AAVs do so by binding to non-specific proteoglycans at the membranes of cells⁶⁹. However, AAV1 and AAV2 also require the AAV-receptor (AAVR, also known as Dyslexia-associated protein KIAA0319-like protein) as a co-receptor for entry into cells^{70,71}. Fortunately, cells in our cultures express this transcript, especially microglia and neurons (**Fig. 5a**). The AAVR contains five polycystic kidney disease (PKD) repeat domains, from which PKD2 is recognized by AAV1 and AAV2 (**Fig. 5b**). Sequences of the PKD2 domain from various species show very high homology (**Fig. 5c**), though the very few differences between the sequences of quail and mammalian AAVR (qAAVR and mA AVR, respectively) occur specifically in residues that are essential for the interactions with AAV1 and AAV2, namely Q432 and K464 (mA AVR numbering) (**Fig. 5b, c, Table 3**). Of note, the divergent residues in qAAVR do not appear to be completely random, rather mirror the residues found in the viral capsid. For instance, whereas mA AVR contains a lysine (K464) that interacts with a threonine (T593) found in capsids of AAV1 protomers (**Fig. 5b, green and magenta**), the qAAVR has a threonine (T204) instead. Similarly, the mA AVR contains a glutamine (Q432) that interacts with an arginine of the AAV2 capsid while the qAAVR mirrors AAV2's capsid with an arginine of its own (R172). We thereby firstly hypothesized that the mirroring residues (T-T for AAV1 and R-R for AAV2) may repel one another and disturb the interaction between qAAVR and viral capsids. To potentially restore these interactions, we rationally mutated the residues in the capsids of AAV1 or AAV2 to match the residues found in the mammalian AAVR. Thus, this limited our mutagenesis towards residues that solely contain one interacting partner (**Table 3**). We thereby produced AAV1 with a single mutation (T593K, denoted AAV1*) and two AAV2-variants (AAV2 and AAV2-SL1⁷², an optimized AAV2 variant) bearing two mutations (R471Q and T592K), denoted AAV2* and AAV2-SL1*, respectively (**methods**).

To test our hypothesis, we initially tested AAV1* on primary rat neuronal cultures and found that AAV1*'s infection efficacy was diminished ~two-fold (**Suppl. 5a-c**). This supports the importance of this residue for AAV's ability to infect mammalian cells⁷⁰. We proceeded to infect the primary quail cultures by AAV1* and by AAV2* and AAV2-SL1*. Whereas the AAV2-variants yielded no infection of cells in multiple experiments (**Suppl. 6**), AAV1* exhibited three-fold significant improvement (1.6% to 5.6%, $p < 0.001$) in transfection efficiency of neurons (i.e., Neurotrace-positive cells), and more than two-fold (32% to 68%, $p < 0.001$) transduction of non-neuronal cells (see **Methods** and **Fig. 6a, b**). These observations show that AAV1* maintains its tropism towards non-neuronal cells, which matches our sc-mRNA seq. data showing enriched expression of qAAVR in microglia than mature neurons (**Fig. 5a**). We patched YFP-positive cells and indeed find that whereas most cells show very silent (or atypical) electrical activity, we could easily detect infected neurons by morphology, and these exhibited very prominent action potential firing and synaptic activities (**Fig. 6c, d, respectively** and see **Fig. 2**).

We were then interested in testing whether AAV1* would be suitable for expressing GCaMP at sufficient amounts for monitoring activity in cultured cells. We produced AAV1* expressing GCaMP6m and infected 9 DIV cultures, grown for a week *in vitro*. We find that, unlike baculovirus expression of GCaMP (see **Suppl. 5a, b**), AAV1* transduction of GCaMP in these cells isn't cytotoxic (**Fig. 6e**). We next monitored activity from dozens of cells simultaneously, and could distinguish a variety of different Ca^{2+} -activities, such as slow waves of calcium, long-lasting calcium rises (reminiscent of glial activity^{73,74}) and very sharp Ca^{2+} -spikes likely representing action potential firing (**Fig. 6f, g**). Together, our results demonstrate the positive, though not exclusive⁶⁹, role of the pairing residues between AAVR and AAV1 for infection of cells *in vitro*⁷⁰. We find that AAV1* shows improved infection efficiency of quail cells *in vitro*. Lastly, demonstrate that AAV1* is highly suitable for delivering optical probes (such as GCaMP) for monitoring activity from various cell types in culture.

AAV1* infects chicken neurons- Quails are close relative of chickens (*Gallus gallus*), which are a more widely used experimental model. We therefore sought to examine infection efficiency of AAV1* of chicken neurons. As noted above, chicken AAVR (chAAVR) only show one divergent residue from mammalian AAVR (T464 instead of K464) (**Fig. 5c**). We first applied our culturing method to chicken embryos and find our method to be similarly suitable for this species, extending the usability of our protocol towards primary chicken cultures (**Suppl. 7a**). We infected chicken cultures with AAV1, AAV1*, AAV2* and AAV2-SL1*. In contrast to quail cultures, AAV1 showed a slightly higher transfection efficiency of cells *in vitro* but, as in quails, AAV1* significantly increased infection efficiency of neurons (2.1% to 5.2%) (**Suppl. 7a-c**). Furthermore, as seen in quail cultures, AAV2-variants were completely non-infectious (**Suppl. 7d-f**).

In vivo AAV1* transduction of neurons in young and adult quails – We proceeded to assess AAV1* transduction *in vivo*. We injected AAV1* (encoding CAG-eYFP or CAG-GCaMP6m) into the Wulst of two young (4 – 5 weeks old) and three adult quails (2 months old). After seven to eight weeks, brains were removed, fixed, and sectioned. Consistent with our *in vitro* results, AAV1* showed significantly higher infection efficiency (**Figs. 7a, b**). Cells also tended to exhibit higher YFP-fluorescence compared to AAV1, though it did not reach significance (**Fig. 7c**). AAV1* also showed better dispersion than AAV1 *wt* (**Suppl. 8 and Table 4**). Interestingly, most infected cells appeared to be neurons (assessed by morphology and presence of spines), despite the ubiquitous promoter employed (CAG⁷⁵) and the pan-tropism observed in culture. Very few cells appeared glial (**Fig. 7 and Suppl. 8, white arrowheads**). These observations demonstrate that AAV1* significantly outperforms AAV1 for delivery of genetically-encoded tools to neurons of the Japanese quails *in vivo*, thereby making it the first tailored-AAV for this bird species.

Discussion

Avian species provide unique opportunities for studying the brain (e.g., ^{2,7,13,76}). The Japanese quail, in particular, presents several advantages over other avian species currently in use, for instance faster sexual maturity, ground dwelling, and females lay ~one egg per day¹⁶. We have recently used this model to explore spatial navigation by use of electrodes, only to find that quails contain head-direction cells, as in other mammalian species, but we could not detect place or border cells in their hippocampal formation¹⁸. This is striking, as spatially-modulated cells are at the core of mammalian navigation not to mention recently described in a species of food-caching bird⁷⁷. We thereby suggest that this model provides unique opportunities to elucidate how the brain achieves spatial specialization across different species⁷⁸. Despite the latter, genetic and molecular tools¹⁶ (or transgenic lines^{6,21,25–27}) for interrogating the brains of quails are much less abundant or completely absent²². These are behind the motivation to concentrate our efforts towards the design of viral vectors for transduction of quail brain cells.

Scrutiny of the literature shows that the use of viruses in avian models is relatively uncommon^{7,15,30–34}, especially in comparison to mammalian models (e.g., ^{65,79}). In the case of quails, there are even fewer descriptions and, to the best of our knowledge, in all instances lentiviruses were exclusively employed for germline transgenesis^{27,80–82}. Thus whether—and which—viruses can directly infect and transduce brain cells of the quail has yet to be demonstrated. We were particularly interested in testing whether AAVs can be used for transduction of neurons in quails, for the reasons that AAVs are small, replication-deficient DNA viruses (safe to handle) that, despite their limited payload (<4.7kb), are minimally toxic to cells, provide long-lasting expression of the gene(s) and can be produced at very large titers (~10¹³)⁷⁹. Importantly, many AAV serotypes show better spread in the brain and improved tropism towards neurons compared to other viruses (e.g., lenti.)^{79,83}. We initially assessed AAV1 *in vivo* because of its reported suitability in other birds (e.g., pigeons³⁰ and barn owls¹⁵). Nevertheless, AAV1 yielded very discouraging results, namely only one bird showed localized expression in the quail's brain (**Fig. 1**). To quickly sift through a larger list of viruses, we developed a primary culturing protocol from brains of quail embryos (**Fig. 2**). This protocol yielded viable cultures consisting of a very large fraction of neuronal progenitor cells (**Figs. 2 and 3**). This is consistent with previous reports showing that cells from early embryonic stages contain mostly (>95%) neuronal progenitors⁸⁴. Our culturing method also proved suitable for embryonic chicken cultures (**Suppl. 7**). The quail cultures proved to be a treasure trove of brain cell types (11 different cell types) (**Fig. 3**). By comparing our sc-mRNA seq. data with reported transcriptomes^{44,47,51,85,86}. In fact, the cultures provide cells from most neural developmental stages (from multipotent to committed neurons)⁵¹. Despite similarities between reported transcriptomes, we also find several interesting differences. For example, we find no mature astrocytes in our cultures, rather precursors of RG⁵⁹, and detected a unique fibroblast population showing weak expression, though detectable, of neuronal markers (e.g., SOX11).

Nevertheless, cells in culture were resistant to infectivity by most viruses tested (**Fig. 4**). These observations were somewhat unexpected as: 1) several AAVs have been shown provide high infection efficiency of avian neurons *in vivo*^{15,30}, 2) A3V has been tailored for infection of chicken neurons⁴³ and 3) the specific lenti. we have tested has been employed for transgenesis of quail germ cells²⁵. The reason(s) behind this observation is unknown but, in the case of AAV1 and AAV2, we suspected the involvement of the AAVR. We mined our sc-mRNA seq. data and find our cultures to express qAAVR (**Fig. 5a**), but the sequences between the mammalian and the quail AAVRs diverge at key residues responsible for binding of the AAV1 and AAV2's capsids (**Fig. 5c**)^{71,87–89}. We also note that different bird species have different AAVRs, and these slight differences could be part of the reason why different AAVs are suitable for one species but not another^{15,30,90,91}. Interestingly, whereas the viral capsids make slightly different interactions with residues of the AAVR, both viruses require two residues that are different in quails (**Table 2**). Not only are those residues different in the qAAVR, but they also appear to mirror the residues in the capsids. To reintroduce these interactions, we inverted residues in the capsid, that make single contacts with the AAVR, to those that are naturally found in the mA AVR. To our surprise, though this scheme did not enable AAV2 variants to infect the cultures, it did significantly improve the infection efficiency of AAV1 *in vitro* and *in vivo* (**Figs. 6, 7 and Suppl. 6, 8**). AAV1* yields transduction of ~6% of Neurotrace-positive cells (**Fig. 6a,b**). However, based on our sc. mRNA seq. results, we now know that most of cells in culture are not mature neurons. In fact, mature neurons (clusters 6 and 7, n= 514 and n=455 cells, respectively) represent less than 10% of all cells. Thus, the estimation of transduction efficiency of mature neurons is greatly underestimated. Another unique feature of AAV1* stems from the fact that, whereas successful transduction is typically reported only when extremely high titers of AAVs are used (10¹³) (e.g., ^{7,15,30}, it can be used at lower titers (10^{11–12}). Lastly, we find that AAV1* is also suitable for transduction of chicken cultures (**Suppl. 7**). chAAVR and mA AVR diverge at a single residue, precisely residue K464 (mammalian numbering, **Fig. 5c**), at which position chAAVR has a T (as does qAAVR). Thus, improvement in transduction of chicken cultures supports our hypothesis regarding the relevance of mirroring residues, otherwise we would have expected major reduction in infection, as observed in rat cultures (**Suppl. 5c**). Thus, we suggest that AAV1* may be compatible with other bird species but, more importantly, demonstrate that our simple strategy may be used by other labs to quickly tailor AAVs to their avian model. This is in stark contrast to viral evolution methods that require highly specialized labs and are time consuming.

In conclusion, our study provides the complete description of the development of a novel quail-tailored AAV1; starting from development of culturing protocol, through diverse molecular characterization of the embryonic cellular landscape of the avian brain and, finally, to the rational engineering of a viral capsid. Together, these enabled us to produce an improved AAV1 variant for transduction of quail's brain cell *in vitro* and *in vivo*. These efforts expand the available toolbox for interrogating the brains of a new animal model, likely to increase interest and use of this unique avian model.

Methods

Primary quail neuronal cultures — Neuronal cultures were prepared from forebrains of seven, eight- or nine-day quail embryos. In a laminar hood, shells from post-fertilized eggs were gently removed (by breaking the shell at its upper tip). Embryos were isolated from the yolk and placed in Dulbecco's Modified Eagle Media/Nutrient Mixture F-12 (denoted dissociation medium). Tissues were then enzymatically and mechanically dissociated as previously described³⁸, with several key modifications (see **Table 1**). Briefly, skin, skull and meninges were removed by scissors and tweezers, and forebrains were isolated and placed in a 15 ml conical tube containing dissociation media enriched with Papain (30 U/ml) and DNase I (57 U/ml), and placed at 37 °C for 30 min. Following incubation, the solution was gently removed without disturbing the forebrains. Digested forebrains were washed three times with PBS and after the last wash, PBS was completely removed and replaced by 2 ml of plating medium consisting of neurobasal medium (Gibco, Cat. 21103049) supplemented with 2% B-27 (Gibco, Cat. 17504044), 1% Pen/Strep and 0.25% Glutamax (Gibco, Cat. 35050061). Then, tissue was manually dissociated by gentle trituration of solution with a fire polished glass pipette (x15), followed by a single trituration with a 1000 ml plastic tip (to ensure complete dissociation). This solution was then applied onto a 40 mm cell strainer (placed on a 50 ml conical tube and pre-washed by 1 ml plating medium). Additional plating medium was added to strainer after passing the entire solution, to elute remaining cells from strainer. The filtered solution, containing the dissociated cells, was transferred to sterile tissue-culture grade plastic plates (Corning, 60 mm, Cat. 430166) pre-coated with PDL, and these were placed for one hour in an incubator (37°C, 5% CO₂). Following incubation, medium was removed and replaced by fresh plating medium (pre-warmed in the incubator). Plates were placed back in incubator and half the media was replaced every two days by fresh and pre-warmed plating medium.

DNA Constructs and Mutagenesis— Helper plasmid pAdDeltaF6 (expressing adenovirus E4, E2A and VA; Cat. 112867); Rep/Cap plasmids – pAAV2/1, pAAV2/9, pAAV2/SL1, PHP-eB, 7m8 (Cat. 112862, 112865, 81070, 103005, 64839, respectively) and Transfer plasmid – pAAV-CAG-eYFP (Cat. 104055) were purchased from Addgene. For the production of Avian adeno associated virus (A3V), all plasmids (Helper, A3V rep/cap, Transfer-RSV-eGFP) were generously provided by Prof. Watanabe (Kyoto University Japan)⁶⁸. For lentivirus production, we used pFUGW, the HIV-1 packaging vector Δ8.9 (pΔNR/8⁹²), the VSVG envelope glycoprotein vector (pVSVG) and and FR(GCaMP6S-p2A-nls-tdTom) as described earlier²⁵, and these were a kind gift from Prof. Lois C. (Caltech, USA). Baculovirus (BacMam) containing CAG-GCaMP7s was purchased from Montana Molecular. Ltd (Montana, USA). Point mutations in AAV rep/cap plasmids were introduced by PCR; using PFU polymerase (Promega, United States). PCR program consisted of 18 cycles of 55°C annealing temperature and 68°C (for 20 min) extension with the following primers:

Mutation	Sense primer	Antisense primer
pAAV2/2 and SL1 R471Q	5'CAGGCCGGAGCGAGTGACATTGAGGACCAGTCTAGGAAGTGGCTT	5'AAGCCAGTTCTAGACTGGTCTGAATGTCACTCGCTCCGGCCTG
pAAV2/2 T592K	5'AGAGGCAACAGACAAGCAGCTAAAGCAGATGTCAACACACAAGGC	5'GCCTTGTGTGTTGACATCTGCTTTAGCTGCTTGTCTGTTGCCTCT
SL1 D592K	5'AGAGGCAACCTAGCAGACCAAAAATACAAAAAAGTCTAGGCAA	5'TTGCCCTAGCAGTTTTTGTGTATTTTTGGTCTGCTAGGTTGCCTCT
pAAV2/1-T593K	5'AGCAGCAGCACAGACCCTGCGAAAGGAGATGTGCATGCTATGGGA	5'TCCCATAGCATGCACATCTCCTTTCGCAGGGTCTGTGCTGCTGCT

Electrophysiology—Patch clamp recordings were obtained by MultiClamp 700B and Digidata 1440A (Molecular Devices), and performed as previously described⁹³. Briefly, cells were voltage-clamped at -70 mV. Borosilicate glass capillaries (i.e., pipettes) were pulled to resistances of 3-7 MΩ and were filled with an internal solution containing (in mM): 135 K-gluconate, 10 NaCl, 10 HEPES, 2 MgCl₂, 2 Mg²⁺-ATP, 1 EGTA, pH = 7.3. Recordings were done in extracellular recording solution containing (in mM): 138 NaCl, 1.5 KCl, 2.5 CaCl₂, 10 D-glucose, 5 HEPES, 0.05 glycine, pH = 7.4. Gap free recording protocol was used to assess spontaneous activity in the culture. For assessing intrinsic excitability, cells were current clamped by injected currents to -60 mV. Then current steps were at 50 pA increments and the number of action potentials was calculated. We analyzed only complete action potentials (not differential potentials). Data were analyzed using the Clampfit software (Molecular Devices, USA).

Histochemistry— Quail cultures (grown directly on plastic plates) were fixed by 4% paraformaldehyde (PFA) for 15 minutes, permeabilized with PBS containing 5% FBS (fetal bovine serum) and 0.1% triton X-100. NeuN-immunostaining was performed by overnight incubation of fixed cells with the antibody (1:1000 in PBS) at 4°C, with 3% FBS and 0.1% triton X-100. The next day, plates were washed three times with PBS, and stained by secondary antibody (Rhodamine Red) in 3% FBS and 0.1% triton X-100 for one hour at room temperature. Plates were then washed by PBS and stained with DAPI (1:1000). Neurotrace 640/660 staining (ThermoFisher) was performed as previously described⁹⁴. Briefly, cultures were fixed as described above, permeabilized with PBS containing 0.1% triton X-100 for 10 minutes, washed, followed by incubation for 30 minutes with PBS containing Neurotrace stain (1:200). Then, cells were washed by PBS and stained with DAPI (1:1000).

Virus production— We have produced viruses by the iodixanol method, as we previously describe⁹⁵. Briefly, HEK293T (Human Embryonic Kidney cells, ATCC #CRL-1573) were grown in Dulbecco's Modified Eagle Media/Nutrient Mixture F-12 (supplemented with 10% Fetal bovine serum and 1% L- glutamate) on 10 ml tissue culture plates (Corning, Cat. 430167), at 37°C and 5% CO₂. Cells were grown to 50 to 70% confluency, transfected with three viral plasmids (Helper, rep/cap, and transfer) using Polyethyleneimine (PEI) at a ratio of 8.1ug, 5.4ug,13.5ug of DNA respectively. After six - eight hours, growth media was replaced by serum free media with 1% Glutamax. Media was collected after 48 and 72 hours following transfection (collected media was preserved in -80°C). The collected media was then filtered and concentrated via the iodixanol step gradient method⁹⁶ to a final volume of 100 – 500 ml. Viral titer was determined by qPCR. Titers below 10¹⁰ were discarded. Viral titers of the viruses used: AAV1 WT – 3.13* , AAV1*TK – 5.25* , AAV2SL1 – 3.92* , AAVphpB – 6.3* , AAV2-7m8 – 4.1* , AAV9 – 9.3* , Baculovirus – 1.3* , A3V – 3*

Cryo-sectioning— After four to eight-weeks following viral injections, anesthetized animals underwent whole animal perfusion fixation, as previously described⁹⁷. Briefly, 4% PFA was perfused to entire body of the animal via the vascular system through the heart of the quail. Whole brains were then dissected and placed in 4% PFA overnight. The following day preserved tissue was treated with 30% sucrose solution and embedded in O.C.T for cryo-sectioning. Brains were sliced (40 μ m sections) via a cryostat.

Fluorescence Imaging— Imaging was performed on a Zeiss Laser Scanning Confocal Microscope (LSM-880 or 900-Airy2; Zeiss, Germany), as previously described^{98,99}. Experiments were conducted using a water immersion objective lens 20x [a water Plan-Apochromat objective lens; 20x/1.0 DIC D = 0.17 (UV) VIS-IR M27 75 mm] with a focal spot diameter of 0.5 μ m ($D = 1.2 \times \lambda/NA$). Brain slices were imaged serially and automatically tiled for reconstructing of brain hemispheres. Live cells (neurons and glia) were imaged in a standard extracellular imaging solution containing (in mM): 138 NaCl, 1.5 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 10 D-glucose, 10 HEPES, pH 7.4. GFP/GCaMP were excited using 488 nm laser; Neu- 561 nm; NeuroTrace- 640 nm, and DAPI- 405 nm. Cultures were imaged after 3 days from viral infection. Change in fluorescence ($\Delta F/F$) was calculated by $(F_t - F_0)/F_0$, where F_t is measured fluorescence (in arbitrary units, a.u.) at a given time t and F_0 is initial baseline fluorescence, typically calculated from averaging the 10 first images, representing the basal fluorescence. In cells showing activity, baseline fluorescence was taken during times of inactivity (or at trough of fluorescence signals). $\Delta F/F = 1$ describes an increase by 100%, equivalent to twofold increase in fluorescence. We did not need encounter near division-by-zero artifacts and bleaching was minimal (most times not observed). Therefore, no corrections were made to fluorescent signals. For analysis of the percent of cells transfected by various AAVs (e.g., Fig. 6b), we imaged multiple (>5) large fields of view, in which we calculated the percent of infected cells by dividing the number of YFP-positive cells from by the total number of cells. We averaged the percentages from multiple experiments, namely from different and independent cultures. Classification of cell types was obtained by staining (DAPI and Neurotrace, see above **Histochemistry**). Neurotrace-positive were counted as neurons, whereas Neurotrace-negative denoted non-neuronal cells. Total number of cells was calculated by counting DAPI-positive nuclei (bright blue staining) in each field of view. This was obtained automatically by the ImageJ software. Assessment of cell density (per mm²) *in vivo* was determined as previously described³⁰, namely by imaging identical fields of view (i.e., identical sizes of images see **Fig. 7 and Suppl. 8**, typically 0.5 x 0.5 mm) in which we counted the number of visible fluorescent somata (we did not count processes as these could project to several planes of view). This was repeated in multiple animals, as noted in the legends.

Stereotaxic viral injections *in vivo*— Adult quails were anesthetized by a mixture of 2% isoflurane and 1% nitrous oxide. A small craniotomy was made above the Wulst (on a modified stereotaxic equipment), followed by removal of dura by tweezers and injections of 0.5 mL of each virus (at 50 nl/10 seconds) at multiple depths. Craniotomies were sealed by silicon and C&B MetaBond (Parkell, USA), as previously described¹⁰⁰.

Single cell RNA Sequencing— Cultures produced from nine-day old embryos, grown for a week DIV had their medium replaced with dissection-dissociation medium supplemented with 20 U/ml papain. The plate was incubated for 10 minutes in 37°C, 5% CO₂. Cells were then pipetted thoroughly and moved to 15 ml canonical tube for gentle centrifugation (5 minutes at 300 rcf). Supernatant was removed and replaced with 1 ml DMEM with 10% FBS. Cells were then filtered through a cell strainer (40 μ m nylon filter, LifeGene). Cells were then centrifuged again, and medium replaced as before. Cells were counted using a hemocytometer. Survival assay was performed with trypan blue. Cells were diluted to obtain 1200 cells/ μ l and 40 μ l were processed with 10x scRNAseq with NextGEM v3 (10x Genomics), analyzed further by cell ranger (10x Genomics) and the Seurat pipeline (Version 4)¹⁰¹.

Library preparation and data generation for single cell mRNA sequencing—One RNA single cell library was prepared according to 10X manufacture protocol (Chromium Next GEM Single Cell 3' Library & Gel Bead Kit v3.1, PN-1000268) using 20,000 input cells. Single cell separation was performed using the Chromium Next GEM Chip G Single Cell Kit (PN-1000120). The RNAseq data was generated on Illumina NextSeq2000, P2 100 cycles (R1-28bp, R2-90bp, I1-10bp, I2-10bp) (Illumina, cat no. 20046811).

Data processing bioinformatic analysis—Data was analyzed the data using R (Version 4.0.1) and Seurat R package. Following QC, results we excluded from the dataset cells with <200 and >2,500 genes (potential cell duplets) and a mitochondrial gene percentage of >30%. In addition, genes detected in less than three cells were filtered out. Following these steps, 9,561 cells were left for downstream analysis. Subsequently, PCA was used with all genes as the input and identified significant principal components (PCs) based on the ElbowPlot function. 10 PCs were selected as the input for uniform manifold approximation and projection (UMAP). Cells were clustered by the FindClusters function selecting resolution of 0.5, yielding 15 different clusters (#0-14).

Statistical analysis— All data are presented as mean $\pm$ SEM. Number of cells are indicated by n , whereas number of experiments by N . Statistical significance (Sygmaplot 11 or Prism 8) was obtained by Student t-TEST (two group comparison) or one-way ANOVA for multiple group comparison with post hoc Tukey test. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$; n.s., non-significant.

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Tables

Table 1. Comparison between different culturing protocols for brain cells.

	Mammals (Rats/mouse) ¹⁰²	Chicken ²⁸	Quails (here)
Proteolytic enzyme and conditions	0.5% trypsin, 15 min. at 37 °C (water bath)	0.25% trypsin, 10-15 min. at 37 °C (CO ₂ incubator)	30 U/ml Papain, 30 min. at 37 °C (water bath)
Dissection medium	HBSS + HEPES	DMEM + 10% FBS	DMEM
Neuronal growth medium	MEM (Minimal Essential Media) 5% FBS 2% B27 1% Glutamax 2% D-glucose 0.1% serum extender	NBM (Neurobasal Media) 2% B27 2 mM Glutamax 20 ng/ml NGF X% Pen/Strep	NBM (Neurobasal Media) 2% B27 0.25% Glutamax 1% Pen/Strep
Growth surface & coating	Glass coverslips 0.5 mg/ml PLL	Glass coverslips 0.1 mg/ml PDL	60 mm tissue culture treated plates 2 mg/ml PDL

Table 2. Cell markers for classification of clusters.

Cell type	Markers
Fibroblasts	COL1A1, COL4A1, CALD1, ITIH5, COL6A2, LAMB1 ^{103,104}
Microglia	PTPRC, MAFB, IL1RAP, CD44, MERTK ⁸⁵
Neuronal progenitor stem cells	NOTCH1, TOP2A, HMGB2, CENPF, CDK1, CCNB2, TJP1, CDH2, IGFBP7, VIM, NES, SFRP1, RELN, MFGE8, FABP7, ID3 ^{47,85,105,106}
Oligodendrocyte progenitor cells	PTPRZ1, HEY1, EPN2, PMP2, PDGFRA, OLIG2 ⁸⁵
Precursor of radial glia	SLC1A3, PROM1, SLC1A2, FN1, CDH2, IGFBP7, VIM, NES, SFRP1, RELN, MFGE8, GJA1, FABP7, ID3 ^{51,107}
Glutamatergic neurons	STMN2, SLC17A6, ENC1, SYT1, ATCAY, ZBTB18, GRIN1, SATB2, NEUROD6, GRIN2B, DCX ¹⁰⁸
GABAergic neurons	GAD1, GAD2, DLX2,1,5, SOX11, ERBB4, STMN2, ENC1, SYT1 ⁴⁴
Pro-neuronal Radial glia	SLC1A3, FABP7, VIM, NES, PROM1, SLC1A2, CDH2, IGFBP7, MFGE8, STMN2, NEUROD1 ⁵¹
Radial glia	CDH2, IGFBP7, VIM, NES ⁵¹
Apical RG	SLC1A3, FABP7, VIM, NES, PROM1, SLC1A2, CDH2, IGFBP7 ⁵¹
Intermediate proneuronal radial glia	CDH2, IGFBP7, VIM, NES, SOX11, ENC1 ¹⁰⁹

Table 3. Interacting pairs of residues between hAAVR and qAAVR with capsids of AAV1 and AAV2.

Human AAVR residues	Quail AAVR residues	AAV1 capsid residues ⁸⁹	AAV2 capsid residues ⁷¹
S413	S	-	E499
P414	P	-	E499
S425	S	D590	Q589
T426	T	D590	Q589
V427	V	T504	Q589, T503
D429	D	W503, T504	W502, T503
sS431	S	W503	S267, W502
Q432	R172	S268, W503	R471
S433	S	S268, N269	S267
T434	T	G266, A267, S268	G265, A266, S267
D435	D	G266, A267, H272	G265, A266, H271
D436	D	A263, H272, S385	Q263, H271, S384
D437	D	H272, S385, Q386	H271, S384, Q385
K438	K	N269, N383, G384	N268, H271, N382, D528
I439	I	N269	N268
Y442	Y	N269	-
I462	I	-	K507
K464	T204	T593	T592

Table 4. YFP expression spread *in vivo*

Variant	Distribution in 'z'
AAV1	270 - 450 μ m
AAV1* (T593K)	360 - 540 μ m

Figures

Figure 1

Development of primary cultures from embryonic quail brains. **a.** Micrographs of cultures produced from 7, 8 or 9 day-old embryos (days in OVO, *DIO*) (lines), grown for 7, 14 and 21 days *in vitro* (DIV) (columns). Cultures remained viable up to 30 days, and likely could be cultured further, though we did explore culturing past one month. **b.** 7 DIV cultures (produced from 9 *DIO* embryos) co-stained with DAPI (blue) and Neurotrace (magenta). The majority of cells (90%) are DAPI and neurotrace positive (magenta arrowheads)- representing neurons, with a smaller fraction (10%) of non-neuronal, denoted glia (pink arrowheads) ; summarized in **c.**

Figure 2

Electrophysiological description of primary cultured quail neurons. **a.** Representative current-clamp (top) and voltage-clamp (bottom) recordings from cultured neurons (left inset- micrograph of patched cell). Current-clamp recording shows resting membrane potential of the neuron (-80 mV), excitatory post synaptic potential (EPSPs) and action potential firing (e.g., dashed rectangle and inset). During voltage-clamp the cell was clamped at -70 mV, during which barrages of excitatory post synaptic currents (EPSCs) could be distinguished. **b.** Summary of electrical properties of quail neurons *in vitro* (n= 12). **c.** Assessment of intrinsic neuronal excitability. Representative voltage traces (top) from a single neuron in response to current injection (bottom protocol) from which we could deduce the maximal firing rates, summarized in **d.**

Figure 3

Single cell mRNA sequencing of cultured cells reveals a large number (15) of cellular populations. **a.** UMAP plot of 9,561 cells, taken from cultures produced from an embryos' entire hemisphere, classified based on the top ten most differentially expressed genes (DEGs), as shown in heatmap in **(b)**. Number of cells within each cluster are noted at left corner of UMAP and clusters' colors correspond to column colors in **b**. Red annotated genes (in **b**) were manually curated (i.e., identity was not automatically found in reference genomes). **c.** Cell type identification. Dotplot shows mean expression levels (blue to red) and percent of cells (size of bullet) in each cluster and for select markers (bottom axis). Red annotated genes were manually curated. **d.** Feature plot showing expression of SFRP1. Mean expression and percent of cells per each cluster are depicted below the plot.

Figure 5

The AAVR is found in quails, but diverges in sequence from the mammalian receptor. **a.** Feature plot of KIAA0319L, the quail AAVreceptor (qAAVR). **b.** (left) Illustration of the binding of the AAVR (grey transmembrane domain and PKD domains in lavender) by an AAV (magenta icosahedron). The PKD2 domain is highlighted (dark lavender) because of its essential role in binding of the AAV's (AAV1 and AAV2) capsid^{89,110}. (right) Atomic structure (by cryo-EM) of AAV1's capsid (magenta) bound to PKD2 (lavender) (pdb: 6JCQ⁸⁹). Key residues in PKD2 that interact with the capsid are highlighted in yellow. The K464 residue (green) in PKD2 interacts with residue T593 (pink) found in the capsid of a second AAV1 protomer. **c.** PKD2's protein sequence alignment between different species shows high conservation (lavender). Residues in the AAVR that directly interact with the AAV1 capsid are highlighted (bold yellow). Two essential residues differ between the quail and mammalian receptor (qAAVR and mAAVR, respectively)—R432 and K464 (bold red). Chicken AAVR (chAAVR).

Figure 6

New AAV1 variant (AAV1*) exhibits enhanced transduction efficiency of quail cultures and enables calcium imaging. **a.** Micrographs of quail cultures infected by AAV1 (left) or by AAV1* (right). Both viruses infect neurons (neurotrace+ + DAPI-positive) and non-neuronal cells (neurotrace negative + DAPI-positive), summarized in **(b)**. **b.** AAV1 infected 1.6% of cells (green slice) that were denoted neurons (blue outline of piechart) and a ~third of non-neuronal cells (orange outline with green slice; 32.2%). AAV1* (right) increases infection efficiency by more than three fold (to 5.6%) of neuronal cells and more than two-fold (to ~68%) of non-neuronal cells. **c, d.** Electrophysiological recordings of AAV1* infected cells (i.e., expressing YFP). **c.** Amorphous cells (inset) show atypical electrical activity (n=4), **d.** mature neurons could be easily assessed by morphology (inset) and show prototypical neural activities (n=5). **e.** Micrograph showing cultures infected by AAV1*-CAG-GCaMP6m and two representative cells showing propagating calcium activities (right). **f.** Calcium dynamics divided into three subgroups: oscillatory (top trace), spiking (middle) and long lasting calcium plateaus (bottom), summarized in heatmap in **g** (N = 5, n = 64).

Figure 7

AAV1* outperforms AAV1 *in vivo* in young quails. **a.** Coronal brain slices of young quails (4 weeks old), produced after 7 weeks from animals injected, side-by-side, with 1 ml of AAV1* (top) or AAV1 (bottom); expressing CAG-eYFP. White arrowheads show non-neuronal cells (likely astrocytes). **b.** AAV1* provides higher density of infected cells (mostly neurons) (top) and tendency for higher expression of within cells (though not significant) (bottom). Each bullet represent one animal (n=5). This side-by-side comparison in young quails supports our initial observations following injections of AAV1 into adult quails' brains, where only one animal showed cells expressing YFP (see **Suppl. 1**). Note that the highest density observed in animals injected with AAV1 is equivalent to the lowest density obtained by AAV1* (b, top).

Supplementary Files

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