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Vesicular Glutamate Transporters in Astrocytes as Potential New Therapeutic Targets: Astrocyte-targeted Viral Vectors Expressing Inhibitory Nanobodies

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Abstract: Astrocytes, the main central nervous system (CNS) glial cell type, actively release transmitters, including glutamate, and thereby participate in physiological brain information processing. However, dysregulated transmitter release from astrocytes can contribute to CNS disease pathogenesis and progression. Therefore, targeting astrocyte glutamate release is a promising new therapeutic strategy in hyper-glutamatergic brain conditions, as it does not directly block glutamatergic neurotransmission. Based on the evidence that astrocytes express vesicular glutamate transporters (VGLUT), in collaboration with other NCCR TransCure partners, we developed an innovative approach for astrocyte-selective delivery of nanobodies inhibiting VGLUT. We inserted the anti-VGLUT nanobody constructs in astrocyte-directed viral vectors that were administered peripherally, crossed the blood–brain barrier and led to successful cell-specific CNS-wide expression of the nanobodies.

Keywords: Astrocyte · Cell-specific viral vectors · Nanobody · VGLUT

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Roberta de Ceglia obtained her PhD in Translational Medicine at University of Milano-Bicocca studying multiple sclerosis (MS) neuro-immunological disease. In 2016, she joined the neuroscience group of Andrea Volterra at Univ. Lausanne as post-doctoral fellow. She focused her research on untangling the role of the brain glial cells astrocytes in the modulation of specific brain circuitries. As member of the TransCure NCCR, she teams up with experts in chemistry and structural biology in a multidisciplinary project aimed at targeting vesicular glutamate transporter (VGLUT) in astrocytes.

1. Introduction: VGLUTs in Astrocytes

An emerging view in neuroscience is that astrocytes, the main glial cell population in the central nervous system (CNS), actively release modulatory transmitters such as glutamate, D-serine, ATP/adenosine, GABA and dopamine, and thereby contribute to information processing during normal brain function. Moreover, dysregulated transmitter release from astrocytes may contribute to CNS disease pathogenesis and progression (for reviews see ref. [1]). Therefore, astrocytes emerge as unexpected crucial players in the active control, and even orchestration, of synaptic networks in physiological and pathological conditions.[1–3] Glutamate, the main excitatory neurotransmitter in the brain, is loaded in synaptic vesicles *via* one of three different vesicular glutamate transporter (VGLUT) isoforms, VGLUT1, VGLUT2 or VGLUT3, encoded respectively by solute carrier (SLC) family genes *SLC17a7*, *SLC17a6* and *SLC17a8*. The three VGLUT isoforms are functionally identical but differently distributed and are responsible for loading glutamate in synaptic vesicles in all glutamatergic neurons and also in some non-glutamatergic ones. VGLUT1 and VGLUT2 are the most abundant VGLUT isoforms, and are differentially expressed in forebrain and midbrain, respectively.[4,5] Deletion of VGLUT1 or 2 is lethal, whereas VGLUT3 deletion produces functional impairments.[6] While the presence and function of VGLUTs in neurons is well established, studies from our group and others utilizing immuno-electron microscopy (EM) and single-cell PCR, reported their expression also in astrocytes.[7] Notably, post-embedding immunogold EM experiments conducted in the molecular layer of the hippocampal dentate gyrus, identified the presence in astrocytes of small and clear vesicular organelles (synaptic-like microvesicles, SLMVs) co-expressing VGLUTs (mainly VGLUT1), VAMP SNARE proteins and L-glutamate.[7–9] These astrocytic SLMVs were often seen at sites near the plasma membrane directly in front of sites in the extra-synaptic portion of the perforant path (PP) fiber terminals incoming from the entorhinal cortex and making

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excitatory synapses onto dentate granule cells (GCs). At these sites facing astrocytes carrying SLMVs, PP terminals express the NMDA receptor subunits GluN2B and GluN3A.[10,11] Single-cell RT-PCR experiments confirmed the presence of VGLUT1 and VGLUT2 transcripts in astrocytes of the molecular layer of the hippocampal dentate gyrus. Both EM and PCR experiments were performed in specimens from the adult brain and their results indicated that VGLUTs were expressed in just part (20–30%) of the astrocytes.[7] However, these observations were countered by later negative transcriptomic and proteomic data reporting lack of VGLUTs and SNARE mRNA/proteins expression in astrocytes[12–14] and leading to an intense debate about the existence and role of glutamatergic exocytosis in astrocytes.[15–17] Recently, more refined studies of RNAseq analysis from individual brain regions and at various ages ranging from the developmental period to the aged brain, reported detectable levels of VGLUTs and SNAREs (SNAP25, VAMP2, *etc*.) transcripts in astrocytes and showed that the levels ranged from below to above detection, with significant differences depending on region and age (*e.g*. ref. [18], see online database http://igc1.salk.edu:3838/astrocyte_aging_transcriptome/). More sensitive single-cell RNAseq studies added the critical extra information that VGLUTs and SNAREs transcripts are present in subpopulations of astrocytes and that these subpopulations have different regional and even sub-regional distributions (*e.g*. ref. [19], see online database http://dropviz.org/). Thus, astrocytes expressing VGLUTs or SNAREs transcripts appear to be a subgroup of the total astrocytic population and for this reason were probably missed by the initial bulk transcriptomics/proteomic studies that analyzed astrocytes as a single population from a large portion of the brain at a given age, thereby most likely diluting out the subtle differences in expression of subsets of transcripts that characterize the local heterogeneity of astrocytes and its dynamics now revealed by single cell studies. Our own initial data are totally in line with the current view that most likely only part of the astrocytes release glutamate and exert circuit-specific synaptic regulatory effects.

2. Astrocyte-specific VGLUT Targeting: NCCR TransCure Collaborations

In the above context, we considered the following three levels of information: (a) VGLUTs are expressed in sub-populations of astrocytes;[7] (b) vesicular glutamate release from astrocytes has a neuromodulatory role in given circuits;[10,20] (c) its alteration leading to excess glutamate release from astrocytes[21] contributes to malfunction of such circuits supporting behavioral impairments, *e.g*. in the sphere of cognitive function.[22] Such information drove our interest in developing an astrocyte-restricted VGLUT targeting as a promising new therapeutic strategy that would act on the 'neuromodulatory' astrocytic component of glutamatergic synaptic transmission without directly affecting the core neuronal transmission itself.

With this idea in mind, we took advantage of the complementary competences in medicinal chemistry and structural biology of other groups participating to the National Center of Competence in Research (NCCR) TransCure, funded by Swiss National Science Foundation, and made collaborative achievements that have led to the development of two innovative strategies for astrocyte-selective VGLUT targeting. The first one consists in the generation of an astrocyte-specific chemistry, jointly elaborated with the group of Jean-Louis Reymond at University of Bern, to chaperone VGLUT inhibitors selectively to astrocytes upon peripheral administration. The second strategy consists in exploiting inhibitory VGLUT nanobodies (Fig. 1a,b) produced by the group of Raimund Dutzler at University of Zurich and jointly characterized by our two groups.[23] We brought them to further pharmacological development *via* insertion in astrocyte-directed viral vectors for peripheral administration and CNS cell-specific delivery. In the present review we will focus on this latter approach, while the former will be the subject of a future *ad hoc* article.

3. VGLUT Nanobodies

Our interest in VGLUT nanobodies with capacity to inhibit the transporter activity (anti-VGLUT NBs) came as a spin-off of work done in parallel by the group of Raimund Dutzler within NCCR TransCure. Their goal was to crystallize VGLUTs, which proved extremely challenging, probably due to high flexibility of the transporter in detergent and because of the lack of suitable crystal contact sites. In crystallography, these limitations have frequently been overcome by specific binding proteins termed crystallization chaperones. Most crystallization chaperones that have been successfully used to date are derived from immunoglobulins.[24] Among these, nanobodies, the binding domains of single-chain camelid antibody fragments, are a particularly promising scaffold.[25] Different from classical immunoglobulins, their smaller antigen binding site can effectively access buried epitopes, such as clefts or substrate entries of membrane transporters. Moreover, they were selected because potentially useful as conformation-specific biosensors or for trapping certain states of a membrane protein transport cycle, thus interfering with protein function. With these goals in mind, following the immunization of llamas, the Dutzler group identified four NBs that bind the cytosolic face of VGLUTs, and found that two of them act as potent inhibitors.

3.1 *Generation of VGLUT Nanobodies*

To generate NBs against rVGLUT1, a truncated and non-glycosylated mutant of the transporter was used, which was designed for crystallization experiments. The truncated protein lacks the entire N-terminus and a large part of the highly flexible C-terminus (starting from Gly 58 and ending at Gln 515). Furthermore, the sites for N-linked glycosylation, Asn 92 and Asn 93, were mutated to alanine and glutamine respectively. This construct was overexpressed and purified from tsA201 cells, a transformed human embryonic kidney (HEK) 293 cell line stably expressing an SV40 temperature-sensitive T antigen apt to produce high levels of recombinant proteins. The homogeneity and lack of glycosylation of the purified protein was verified by size exclusion chromatography, SDS-PAGE, and mass spectrometry. After immunization of a llama (*Lama glama*) with the DDM-solubilized construct, eleven ELISA-positive NBs could be retrieved by phage-display from a library generated from an isolated blood sample. These NBs were successfully expressed as His-tag fusion proteins in the periplasm of *E. coli* and purified *via* Ni-NTA chromatography. The binding of the purified NBs to the construct was validated by monitoring the co-elution of both proteins on size-exclusion chromatography. Out of the eleven NBs, complex formation was confirmed for four of them (*i.e.* NBs 3, 7, 9 and 10).

3.2 *VGLUT NBs Recognize Native VGLUT1 and VGLUT2 and Bind to a Cytosolic Epitope*

Since the NBs were selected with a truncated and non-glycosylated transport protein, it was then tested if they would recognize native VGLUT1 in neurons. For that purpose, C-terminally myc-tagged NBs 3 and 9 were purified and their staining pattern was analyzed by immunocytochemistry in primary cortical neurons cultures. Both NBs stained synaptic boutons and faithfully reproduced the staining pattern of a guinea pig anti-VGLUT1 antibody, whereas their denaturation largely abolished specific labeling. Thus, NBs 3 and 9 bind native VGLUT1 in synaptic vesicles, which makes them suitable tools for immunocytochemistry. To address the question whether binding occurs on the cytosolic or luminal side of rVGLUT1, a crude preparation of synaptic vesicles (LP2 fraction) was isolated from rat brains. Purified myc-tagged NBs 3 and 9 were added in the presence or

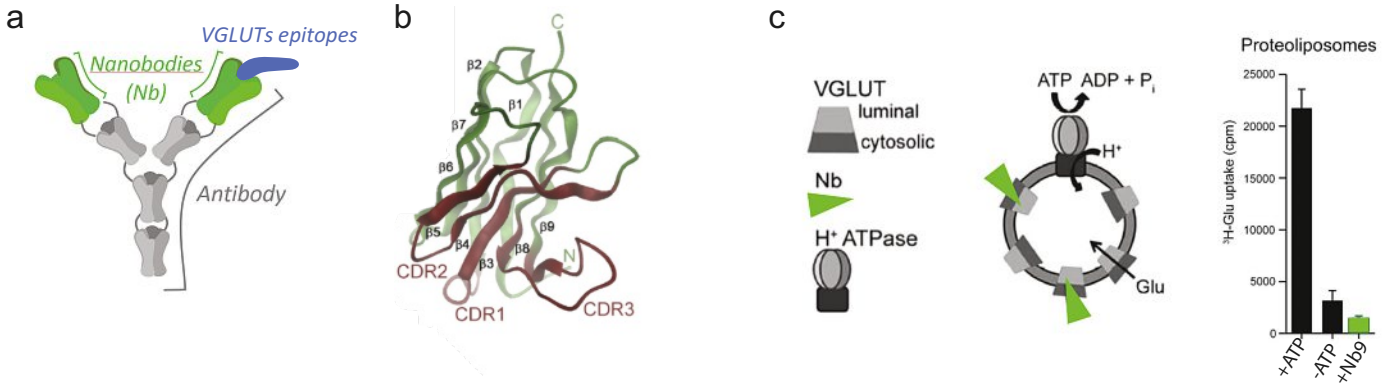

Fig. 1. a) Schematic representation of nanobodies (Nb, green), small fragments of antibodies (grey) recognizing specifically VGLUT epitopes (blue). b) 3D structure of the VGLUTs inhibitor, NB9, represented as ribbon with constant (green) and variable (red) regions, respectively. Termini locations of the variable complementary regions (CDRs) and secondary structure elements are indicated. c) High-affinity nanobodies (NBs, green) potently inhibit ^{3}H-glutamate uptake into proteoliposomes containing purified WT rVGLUT1 co-reconstituted with a proton pump. Proteoliposomes were mixed in three-fold stoichiometric excess with NB9 prior to co-reconstitution. Reprinted adapted with permission from Schenck *et al., Biochem.* **2017**. Copyright 2017. American Chemical Society.

absence of detergent to suspended LP2 vesicles. Magnetic beads coated with anti-VGLUT1 or anti-VGLUT2 antibodies were then used to pull-down either the intact synaptic vesicles (SV) or the solubilized transporters. Co-immunoprecipitated NBs were subsequently detected by Western blotting with an anti-myc antibody. Detection of the SV-resident protein Synaptophysin served as a control for the completeness of SV solubilization. In case of a luminal epitope, NBs would only be detectable in the presence of detergent. However, both NBs co-immunoprecipitated with intact SVs, which is compatible with binding to a cytosolic epitope in VGLUTs. Since the NBs were also detected in the VGLUT2 pulldown with solubilized SVs (although with weaker signal due to the lower abundance in total brain preparations), they likely do not discriminate between the two major VGLUT isoforms. This is expected, given the high degree of identical residues (87.5%) between VGLUT1 and VGLUT2 in the core region of both transporters excluding both termini.

3.3 *VGLUT NBs Inhibit Glutamate Transport*

It was next tested if the addition of NBs would block the transport of glutamate into reconstituted liposomes and native SVs (Fig. 1c). For this purpose, wild type rVGLUT1, expressed in and purified from tsA201 cells, was co-reconstituted with a thermophilic ATP-Synthase (to generate VGLUT1-TF0F1-liposomes)[26] either in the absence of NBs or after incubation with NBs 3, 7, 9 or 10. In the respective samples, NBs were added in 3-fold molar excess to rVGLUT1 prior to the formation of the proteoliposomes to ensure that all transporters would be bound, independent of their orientation in the membrane. All four NBs decreased the uptake of glutamate into proteoliposomes with a close to complete inhibition by NBs 3 and 9. To exclude that this effect was due to interference with the reconstitution of VGLUT1, it was tested if transport inhibition occurred upon adding NBs 3 and 9 to the outside of preformed VGLUT1-TF0F1-liposomes prior to the uptake measurements. Also in this case, uptake was strongly decreased, thus indicating that the inhibition was due to a true blockage of the transporter. Since NBs 3 and 9 bound to intact SVs, glutamate uptake into isolated crude SVs (LP2 fraction) from rat brains was tested after addition of either NB. Also with SVs, it was confirmed the inhibition of glutamate uptake in the presence of NBs 3 or 9, further supporting their inhibitory activity upon binding to a cytosolic epitope of the transporter.

3.4 *Intracellular Expression of VGLUT NBs Fused to Fluorescent Proteins*

Since NBs 3 and 9 bind the cytosolic face of VGLUT1 and VGLUT2, it was then tested whether they could be expressed as fusions to fluorescent proteins in the cytosol of tsA201 cells, and could label co-expressed VGLUT inside the cells. Upon expression of the NBs in tsA201 cells as GFP or mCherry fusion proteins, a cytosolic localization of the fusion proteins was apparent. When co-expressed with rVGLUT1 (devoid of a GFP tag), the cytosolic distribution of the NB-GFP fusion proteins changed to a more punctuate staining resembling the localization of an rVGLUT1-VenusYFP fusion protein. Upon co-expression of rVGLUT1-VenusYFP with mCherry tagged NB9 (NC-NB9), near-perfect co-localization was observed, which was absent when the NB was co-expressed with N-terminally VenusYFP-tagged rVGAT. These data confirm that NBs fused to fluorescent proteins retain VGLUT-specific targeting.

4. Packaging VGLUT NBs in Viral Vectors for Astrocyte-specific Expression

The above data show that inhibitory NBs 3 and 9, although originating from secreted proteins and containing a disulfide bridge, can be expressed as functional binders in the cytosol of HEK cells and co-localize with VGLUTs in the boutons of cultured neurons, *i.e.,* represent interesting tools to target VGLUTs inside cells. Indeed, with the help of viruses or by transfection, NBs could be delivered to different cell types, including neurons or astrocytes. By using cell-type specific promoters, the targeted expression of NBs could then be used to block VGLUT activity in the cells of interest.

Based on these ideas, we started to develop a viral strategy for astrocyte-specific expression of the anti-VGLUT NBs (Fig. 2a). We selected for this NB9, one of the two NBs with highest inhibitory potency on VGLUTs transport. The NB9 sequence fused at the N-terminal to a Myc- and SBP-tag and to a green-fluorescent reporter (EGFP), as detailed in section 3.4, was cloned under an astrocytic promoter – the short human GFAP sequence (gfaABC1D), to drive NB9 expression *in vivo* selectively in astrocytes – in a plasmid suitable for adenoviral production. The generated vector was used to create a serotype 5 adeno-associated virus (AAV-5), that has tropism for glial cells infection.[27] EGFP reporter was used for *in vivo* tracking of the NB protein expression and distribution, while Myc and SBP tags were used for pull-down.

We next proceeded to cause *in vivo* infection with this new NB-expressing AAV virus *via* stereotaxic injection in the cerebral cortex (Fig. 2b) of tamoxifen-treated hGFAPcreERT2-tdTomato$^{lox/stop/lox}$ mice. These mice were selected because, upon tamoxifen administration (7 days, 10 mg/kg), they switch on a red fluorescent reporter, tdTomato, specifically in astrocytes, permitting easy co-localization studies with green-fluorescent NB ex-

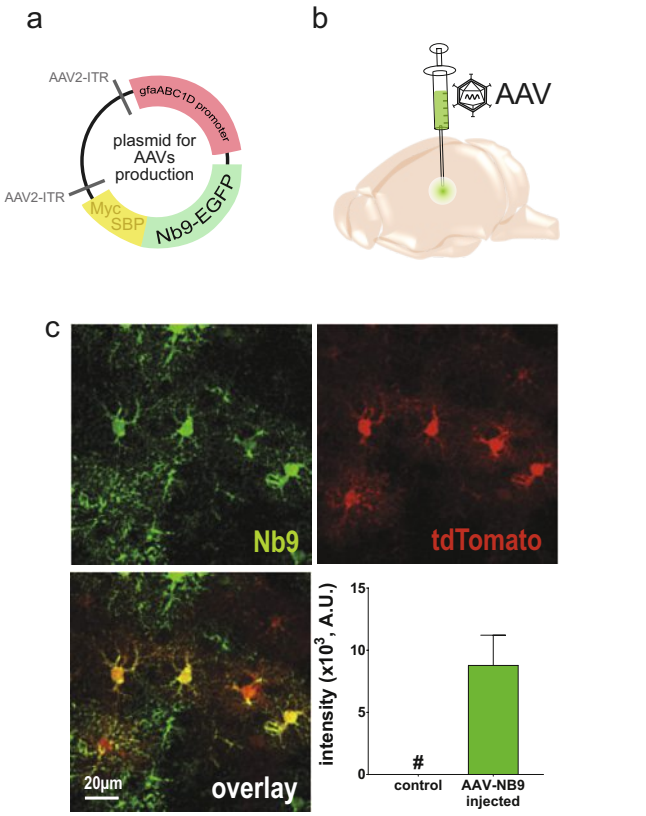

Fig. 2. a) Plasmid map for expressing NB9-EGFP in viral vectors. b) Schematic representation of intra-cerebral injection of astrocyte-specific AAV5-NB9-EGFP. c) validation of NB9 expression *in vivo*. Immunostaining panels show co-localization of green-fluorescent NB9-EGFP expression with red-fluorescent astrocytes expressing the tdTomato reporter in the infected cortical region. Bottom right panel shows NB9 protein detection in the cortical brain homogenate by pull-down of NB9-EGFP through SBP/Myc tags followed by mass-spectrometry analysis. No NB9 fragments are detected in the control, non-injected hemisphere.

pression. Indeed, 4–8 weeks after tamoxifen administration, overlapping green (NB-EGFP) and red (astrocyte-specific tdTomato reporter expression) labeling confirmed successful expression of the NB construct specifically in cortical astrocytes (Fig. 2c).

To obtain direct proof of the NB9 protein expression, we performed pull-down experiments followed by mass-spectrometric analysis (Fig. 2c) in collaboration with the group of Mark Verheijen at the Free University, Amsterdam, The Netherlands. Cortical tissue from mice infected with NB-expressing AAV virus in one hemisphere was dissected from the regions showing green-fluorescence, indicative of NB-EGFP expression, homogenates were prepared and subjected to two rounds immunoprecipitation, first using GFP-TRAP agarose beads, and then anti-Myc antibodies. Both eluates were subjected to nano-mode mass-spectrometric analysis. Several peptide fragments unique to the NB9 protein were detected in the eluates, representing overall about 35% of the NB9 sequence. Importantly, as control, cortical tissue from the contralateral hemisphere not injected with the NB-expressing AAV virus, was subjected to the same procedure but mass-spectrometric analysis did not reveal the presence of any NB-related peptide fragment. Taken together, the above data let us conclude that our viral approach is valid for Nb9 protein expression and astrocyte selectivity.

5. New Viral Vectors for Peripheral Administration and Brain Delivery with Astrocyte-specific Expression: Towards a Gene Therapy Approach

Reaching proof-of-concept in the use of a viral vector strategy to express anti-VGLUT NBs selectively in astrocytes, led us to move forward towards a more specific gene therapy-oriented approach. Thus, if so far we performed CNS viral infections by local stereotaxic injections in the brain tissue, the recent discovery of AAV capsids with CNS tropism (AAV-9), in particular of engineered AAV9 variants (named AAV-PHP.eB) that exhibit high delivery to the CNS of mice following intravenous (i.v.) peripheral injections,[28] has opened new horizons to a direct therapeutic use of anti-VGLUT NBs. Therefore, as next step, we aimed to generate a new viral construct usable as a pharmacological tool in pre-clinical studies. For this, we based on our own experience using the viral serotype AAV-PHP.eB, a modified adeno-associated virus serotype 9 (AAV-9). Thus, in preliminary experiments, we proceeded to intravenous (i.v.) administration of AAV-PHP.eB viral construct encoding a fluorescent reporter under astrocyte-specific promoter by injection into the retro-orbital sinus of adult mice. As a result, we could observe widespread brain expression of the reporter selectively in astrocytes. This was a very encouraging result because it clearly showed the capacity by the viral construct to: (a) penetrate the blood–brain barrier; (b) retain cell-specific targeting; (c) induce expression covering most brain areas, with the potential for a vast pharmacological effect.

Therefore, we decided to package the anti-VGLUT NB9 sequence fused to EGFP in AAV-PHP.eB viral vector carrying the usual gfaABC1D astrocyte promoter. This viral vector was administered *via* a single peripheral intravenous retro-orbital injection and 6 weeks later the brain was processed to identify green-fluorescent cells indicative of NB9 expression.

As shown in sagittal whole brain sections (Fig. 3), the presence of green-fluorescent cells was widespread throughout the brain, with some difference in density depending on the brain region. Counterstaining with a combination of antibodies for two astrocyte markers, glutamine synthase (GS) and S-100-beta, as well as lack of co-localization with the neuronal marker, NeuN, confirmed astrocyte targeting of the NB expression (Fig. 4). This experiment confirmed that the approach we developed is well suited for a gene therapy strategy.

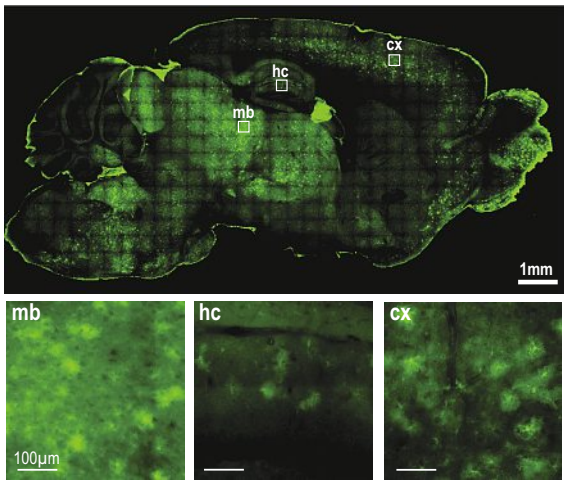

Fig. 3. Top panel: sagittal section of a mouse brain infected with the AAV-PHPeB-NB9-EGFP viral vector showing widespread presence of PHPeB-NB9-EGFP infected cells. Bottom panels: zoom-in of the PHPeB-NB9-EGFP infected cells in selected brain regions: mb = midbrain; hc = hippocampus; cx = cerebral cortex.

6. Future Directions

The next steps will consist in verifying the functional efficacy of NB9 expression in the brain, looking for the NB inhibitory effect on VGLUT-expressing astrocytes *in vivo*, for example by verifying that NB9 expression produces defined behavioral

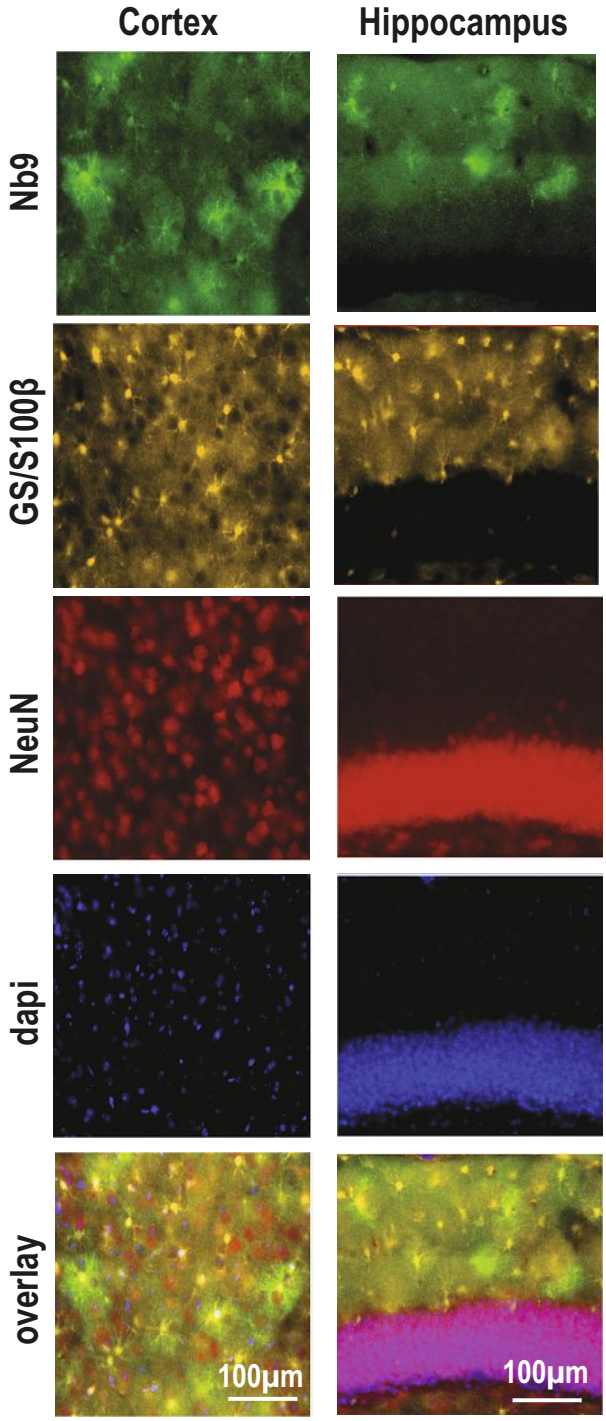

Fig. 4. Immunostaining for NB9-EGFP (green), the astrocyte markers glutamine synthase (GS) and S-100-beta (yellow), the neuronal marker NeuN (red), and nuclei (dapi) in hippocampus and cortex. Overlay shows co-localization of NB9-EGFP-expressing cells with GS/S-100-beta-expressing cells (astrocytes) but not with NeuN-expressing cells (neurons).

phenotypic changes and that these changes are in line with those produced by genetic astrocyte VGLUTs deletion. For this phenotypic comparison, mice carrying floxed VGLUT1[29] or VGLUT2[30] sequences are available. In these mice, use of an astrocyte-directed Cre viral vector allows for excision of the floxed sequences and deletion of VGLUT1 or VGLUT2 selectively in astrocytes. If protective phenotypes are observed in these studies, astrocyte-targeted anti-VGLUT NBs will be tested in pathological animal models in which astrocyte VGLUT function is excessive and can produce alterations. In this way, we would reach final demonstration not only that our gene therapy approach is valid, due to the non-invasive route of administration and the verified brain expression and astrocyte-specificity, but also that it can be applied to defined medical conditions. For example, we found that local inflammation/infiltration in the hippocampus of a mouse model of multiple sclerosis causes massive enhancement of cytokine production, which in turn triggers excess glutamate release from astrocytes, dysregulation of excitatory transmission and, ultimately, cognitive deficit.[22] Local inflammatory states leading to excess astrocyte glutamate release could occur also in other neuropsychiatric conditions, notably in Alzheimer's disease[3,31] and represent a primary target of our astrocyte-specific anti-VGLUT strategy.

7. Concluding Remarks

The data presented here provide initial proof-of-concept on the feasibility of producing AAV vectors that can be administered peripherally and delivered to the CNS inducing astrocyte-specific expression of NBs inhibiting VGLUT activity. Our approach aims to reduce excess release and extracellular accumulation of glutamate that is observed in several pathological brain conditions[32] by targeting solely the astrocyte- and VGLUT-dependent mechanisms. This intervention is expected to be significantly milder and more selective than many of the anti-glutamatergic approaches tested so far, several of which failed in clinical trials because they were blocking not only the excess glutamate accumulation but also essential components of the physiological glutamatergic function.[33] Incidentally, the astrocyte-specific targeting approach that we developed using anti-VGLUT NBs packaged in AAV vectors is by no means limited to an anti-glutamate pharmacology and can be similarly designed for any other identified astrocyte target involved in pathological CNS conditions.[34] In this context, the spectrum of attainable NBs, including *via* synthetic methods (dubbed 'sybodies'), is in strong expansion.[35] The use of NBs packaged in an AAV-viral vector is quite novel as a therapeutic strategy, although a few examples of AAV vectors used for targeting NBs to the CNS already exist.[36,37] Despite some challenges and limitations, the approach of AAV viral vector packaging has clear clinical potential, as indicated by the fact that a few gene therapies using recombinant AAVs for *in vivo* delivery have been already approved by EMA and FDA regulatory agencies and that several others exist at the stage of clinical trial.[38] To note that, in terms of AAV packaging, new capsid variants with even better pharmacodynamics than AAV-PHP.eB, *i.e.,* with enriched CNS delivery and reduced off-target liver delivery, have been recently developed.[39] Even the astrocyte-specific targeting approach that we developed can be further improved and rapidly adapted to the emerging knowledge that astrocytes are molecularly heterogeneous.[40] Importantly, in a therapeutic perspective, recent single-cell transcriptomic studies have revealed the presence of molecularly distinct sub-populations of astrocytes in mouse models of Alzheimer's disease (AD) and in AD patients, some of which emerge at early disease stages, and thus are potentially important for disease pathogenesis and progression.[41,42] Targeting these disease-associated astrocyte sub-populations specifically is conceivable, by generating AAV viral vectors that instead of carrying a generic astrocyte promoter such as gfaABC1D, carry an *ad hoc* promoter represented by one of the genes expressed by the disease-associated sub-population but not by the other astrocyte sub-populations.

Overall, the above considerations highlight the interest and potential of the astrocyte-specific targeting therapeutic strategy emerged from our collaborative studies within the NCCR TransCure, as well as the appropriateness of pursuing its further development in the future.

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