Arginine-rich cell-penetrating peptide dramatically enhances AMO-mediated *ATM* aberrant splicing correction and enables delivery to brain and cerebellum

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Antisense morpholino oligonucleotides (AMOs) can reprogram pre-mRNA splicing by complementary binding to a target site and regulating splice site selection, thereby offering a potential therapeutic tool for genetic disorders. However, the application of this technology into a clinical scenario has been limited by the low correction efficiency *in vivo* and inability of AMOs to efficiently cross the blood brain barrier and target brain cells when applied to neurogenetic disorders such as ataxia-telangiectasia (A-T). We previously used AMOs to correct subtypes of *ATM* splicing mutations in A-T cells; AMOs restored up to 20% of the *ATM* protein and corrected the A-T cellular phenotype. In this study, we demonstrate that an arginine-rich cell-penetrating peptide, (RXRRBR)₂XB, dramatically improved *ATM* splicing correction efficiency when conjugated with AMOs, and almost fully corrected aberrant splicing. The restored *ATM* protein was close to normal levels in cells with homozygous splicing mutations, and a gene dose effect was observed in cells with heterozygous mutations. A significant amount of the *ATM* protein was still detected 21 days after a single 5 μ M treatment. Systemic administration of an fluorescein isothiocyanate-labeled (RXRRBR)₂XB-AMO in mice showed efficient uptake in the brain. Fluorescence was evident in Purkinje cells after a single intravenous injection of 60 mg/kg. Furthermore, multiple injections significantly increased uptake in all areas of the brain, notably in cerebellum and Purkinje cells, and showed no apparent signs of toxicity. Taken together, these results highlight the therapeutic potential of (RXRRBR)₂XB-AMOs in A-T and other neurogenetic disorders.

INTRODUCTION

Antisense oligonucleotides (AOs) can complementarily bind to a target site in pre-mRNA and regulate splice site selection to reprogram splicing processes. AO-based approaches have been successfully used to correct subtypes of splicing mutations in various genetic disorders (1–4). Therefore, AO-based splicing modulation represents a promising therapeutic strategy for genetic disorders.

Ataxia-telangiectasia (A-T) is a progressive recessive neurogenetic disorder caused by mutations in the *ATM* gene

(*Ataxia telangiectasia mutated*), characterized by progressive neurodegeneration, a high risk of cancer, immunodeficiency and radiosensitivity (5,6). The *ATM* protein plays a critical role in maintaining genome integrity by recognizing DNA damage, regulating cell-cycle checkpoints and repairing DNA (7,8). So far, there is no curative strategy for A-T. Treatment has focused on slowing neurodegeneration progress, devising approaches for the treatment of tumors while minimizing side effects, and treating immunodeficiency with immunoglobulins. Antioxidants may also help patients by reducing cellular oxidation levels (9). As an attempt to

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develop alternative therapeutic approaches, our laboratory has targeted specific splicing mutations with charge neutral forms of antisense morpholino oligonucleotides (AMOs) to correct certain prototypic *ATM* splicing mutations that activate cryptic splicing sites (10). In each case, we were able to induce 10–20% of the functional ATM protein and to restore the cellular phenotype in A-T cells, implicating the therapeutic potential of AMOs. However, the clinical potential was greatly hampered by the low *in vivo* correction efficiency and systemic delivery of AMOs to the brain—the primary site of pathology in this disorder. The most debilitating feature of A-T is the progressive loss of Purkinje cells in the cerebellum and the accompanying progressive ataxia (11,12). Therefore, for any compound to be effective in treating A-T patients, it will most likely have to cross the blood brain barrier (BBB) and target brain cells, particularly Purkinje cells (3,5,12).

Cell-penetrating peptides (CPPs) are a class of small cationic peptides of approximately 10 to 30 amino acids that have shown great potential as transmembrane delivery agents for macromolecule compounds such as oligonucleotides (13,14). Recently, arginine-rich CPP-conjugated AMOs have been developed to improve splicing correction efficiency and systemic delivery ability (15–17). CPPs with repeated RXR have been shown to enhance nuclear delivery of AMOs in cell cultures (18) and correct splicing in *mdx* mice (19–22). However, there are few reported applications of CPP-AMOs in other genetic disorders besides Duchenne muscular dystrophy (DMD). Moreover, reported *in vivo* brain deposition of arginine-rich CPP-AMOs was not significant (19,20). Herein, we tested the activity of (RXRRBR)₂-XB-conjugated AMOs on two *ATM* splicing mutations, using lymphoblastoid cell lines (LCLs) derived from A-T patients. We found that (RXRRBR)₂-XB-AMOs almost fully corrected aberrant splicing. The systemic delivery of the (RXRRBR)₂-XB for AMOs was also investigated in mice. Fluorescently labeled (RXRRBR)₂-XB-AMO crossed the BBB and targeted Purkinje cells and other areas. These findings highlight the therapeutic potential of optimized arginine-rich CPP-tagged AMOs in A-T and other genetic disorders with similar types of splicing mutations.

RESULTS

(RXRRBR)₂-XB-AMOs dramatically enhance *ATM* splicing correction efficiency

We first compared two types of AMOs, (RXRRBR)₂-XB-AMOs and neutral AMOs, for splicing correction efficiency, using A-T cells carrying different *ATM* splicing mutations. The first cell line (TATC) was homozygous for c.7865C>T, which causes deletion of the last 64 nt of exon 55 (23). The second cell line (AT203LA) was heterozygous for IVS28-159A>G (24). This mutation results into a pseudo-exon insertion and was selected to evaluate gene-dose effects of AMOs. In TATC cells, an (RXRRBR)₂-XB-AMO fully converted mutant transcripts to wild-type (WT) transcripts at a concentration of 10 μ M, whereas only a small proportion of WT transcripts was induced by the same concentration of neutral AMOs (Fig. 1A and B). The dose–response data showed that the (RXRRBR)₂-XB-AMO was

effective at concentrations >0.5 μ M (Fig. 1B), and no mutant transcripts were detected by reverse-transcription polymerase chain reaction (RT-PCR) after treatment at concentration >2.5 μ M. In order to assess the correction efficiency, real-time RT-PCR was performed. As shown in Figure 1D, ≥ 2 μ M concentrations of (RXRRBR)₂-XB-AMOs corrected >80% of mutant transcripts in TATC cells, whereas the neutral AMOs showed much less activity. Similar results were seen when another customized (RXRRBR)₂-XB-AMO was used to treat AT203LA cells (Fig. 1D, right panel). The specificity of such AMOs has already been established (9).

(RXRRBR)₂-XB-AMOs efficiently restore *ATM* protein expression in A-T cells

We next compared *ATM* protein restoration induced by the two customized AMOs, each in a tagged and untagged form. Western blotting showed that (RXRRBR)₂-XB-AMO at ≥ 2.5 μ M dramatically induced *ATM* protein expression in TATC cells, with much higher efficiency than a neutral untagged AMO (Fig. 2A). Notably, the (RXRRBR)₂-XB-AMO was effective at concentrations as low as 0.5 μ M (Fig. 2B). The maximum protein levels restored were comparable to that of WT cells (Fig. 2A and B). Strong restoration of the *ATM* protein was also observed in AT203LA cells (heterozygous mutation), despite the reduced gene dosage (Fig. 2C). Quantitative *ATM*-enzyme-linked immunosorbent assay (ELISA) demonstrated that the *ATM* protein levels in treated TATC cells was $\sim 80\%$ of WT cells, whereas the *ATM* level in AT203LA was $\sim 50\%$ of WT cells (Fig. 2D). This has significant translational relevance since most American A-T patients are compound heterozygotes rather than homozygotes (25).

(RXRRBR)₂-XB-AMOs restore *ATM* kinase activity in A-T cells

We next assessed the enzymatic kinase activity of the restored *ATM* protein. We measured the phosphorylation of two well-established *ATM* substrates, SMC1 and KAP1, following ionizing radiation (IR)-induced double-strand breaks (26,27). As seen in Figure 3A and B, no IR-induced phosphorylation of SMC1 and KAP1 was observed in untreated A-T cells (both TATC and AT203LA), whereas phosphorylation of both proteins was comparable with WT levels in all of the treated cells (1, 2 and 5 μ M). Notably, it appears that only small amounts of the functional *ATM* protein were able to fully phosphorylate these substrates. For example, whereas 1 μ M of (RXRRBR)₂-XB-AMOs only partially restored the *ATM* protein levels of both cell lines, these amounts sufficed for full restoration of SMC1 and KAP1 phosphorylation. Next, we demonstrated the restored *ATM* autophosphorylation of serine residue 1981 level (*ATM*-ps1981), using fluorescence-activated cell sorting (FACS) analysis of A-T cells (28). (RXRRBR)₂-XB-AMOs significantly restored IR-induced *ATM* Ser1981 autophosphorylation in both cell lines (Fig. 3C). Notably, when compared with unmodified AMOs, CPP-AMOs were much more efficient in restoring *ATM* kinase activity (Supplementary Material, Fig. S1). At low concentrations (1–5 μ M), regular unmodified AMO was not able to significantly restore *ATM* kinase activity as demonstrated

Figure 1. (RXRRBR)₂XB-AMOs dramatically improved ATM splicing correction efficiency at the mRNA level. A-T cells were treated with two types of AMOs for 48 h. After that, RNAs were isolated, and RT-PCR and RT-qPCR were performed. (A) Splicing correction efficiency comparison of two types of AMOs in TATC cells with homozygous mutation. (B) Dose-response of (RXRRBR)₂XB in TATC cells. (C) Efficiency comparison of two types of AMOs in AT203LA cells with heterozygous mutation. (D) Quantitation of correction efficiency of AMOs by RT-qPCR (left panel, TATC cells; right panel, AT203LA cells). Note that in all figures, WT refers to wild-type cells.

by phosphorylation of KAP1 and SMC1, whereas the CPP-AMO fully restored ATM cellular kinase activity (Supplementary Material, Fig. S1).

(RXRRBR)₂XB-AMOs abrogate radiosensitivity of A-T cells

One of the hallmarks of A-T cells is their highly decreased clonogenic survival following IR damage. We tested whether (RXRRBR)₂XB-AMOs can correct this phenotype. Cells were treated with (RXRRBR)₂XB-AMOs for 4 days and evaluated by a clonogenic survival assay (CSA). As shown in Figure 4, the two untreated A-T LCLs showed 7 and 12% survival fractions (SF), falling into a 'radiosensitive range' (<21% SF) (the first bars of Figure 4A and B) (29). (RXRRBR)₂XB-AMOs restored SF to a 'normal range' (>37% SF) even at concentrations as low as 1–2 μM.

Effect of (RXRRBR)₂XB-AMO is prolonged in A-T cells

In order to evaluate the cellular uptake of CPP-AMOs, an aminomethylcoumarin acetate (AMCA)-labeled (RXRRBR)₂XB

was used to tag TATC-specific AMOs. Both WT and A-T LCL cells were treated at 0.5 μM (see Materials and Methods). The cellular fluorescence was monitored after exposure. Blue fluorescence was clearly observed at 1 day after treatment and remained strong for 7 days. The intensity of the fluorescence was reduced after 12 days but still remained visible (Fig. 5A). We interpreted these data to indicate slow cellular degradation of the CPP-AMO. To study how long the effect of a single dose of (RXRRBR)₂XB-AMOs could be documented, we followed ATM protein expression by immunoblot in TATC cells after a single 5 μM treatment with (RXRRBR)₂XB-AMO. As shown in Figure 5B, the restored ATM was detected as early as 1 day after treatment, reached a high level (close to WT) at ~4–5 days and was still clearly measurable until 20 days.

(RXRRBR)₂XB-AMOs are deliverable to brain and Purkinje cells in mice

Finally, we investigated the ability of (RXRRBR)₂XB to deliver AMOs across the BBB after systemic administration. Mice received either a single tail injection or four

Figure 2. (RXRRBR)₂XB-AMOs efficiently restored ATM protein expression in A-T cells. A-T cells were treated with AMO for 4 days and then the nuclear protein was extracted for western blotting. (A) ATM protein level in TATC cells treated with (RXRRBR)₂XB-AMOs or neutral AMOs. (B) Dose–response effect of (RXRRBR)₂XB in TATC cells. (C) ATM protein level in TATC cells treated with (RXRRBR)₂XB-AMOs and neutral AMOs. (D) Quantitation of the ATM protein level in A-T cells treated with (RXRRBR)₂XB-AMOs by ATM-ELISA.

consecutive intravenous injections of a 22mer fluorescein isothiocyanate (FITC)-labeled random scrambled sequence (5'-TGCTCTGTCTACAGTAGTGCA-3), conjugated with (RXRRBR)₂XB, at a dose of 60 mg/kg. Green (FITC) fluorescence was widely detected throughout the brain, including the cerebellum, although no fluorescence was observed over background levels in the brains of mice injected with PBS. Moreover, fluorescence intensity was significantly increased in brains isolated from mice that received multiple tail vein injections of CPP-AMO, compared with brains from mice that received a single injection (Fig. 6). Strikingly, the Purkinje cell layer was remarkably stained (first column of Fig. 6, arrows). As an alternative way to confirm the distribution of the AMO in the brain, anti-FITC immunostaining was performed to further localize the FITC-labeled (RXRRBR)₂XB-AMOs, using Texas Red-labeled anti-FITC antibody. Consistent with the results obtained after fluorescence analysis of unstained tissues, red immunofluorescence was detected throughout the brain, including the cerebellum, indicating the distribution of the AMO in the brain. Figure 7 shows the anti-FITC (red) staining of the hippocampus (Fig. 7A) and cerebellum (Fig. 7B) areas, including the Purkinje cell layer. In order to confirm that the AMO was

delivered to Purkinje cells, calbindin immunostaining was performed specifically to visualize the Purkinje cell layer; Purkinje cells are calbindin-D28k-positive (30). As seen in Figure 8A, calbindin-positive staining localized with anti-FITC staining of FITC-(RXRRBR)₂XB-AMO. We also did confocal imaging to sub-localize the AMO in Purkinje cells. As shown in Figure 8B, red fluorescence staining was predominant in the cytoplasm and around the cell nuclei, whereas nuclear localization of the (RXRRBR)₂XB-AMO was clearly detected in all Purkinje cells analyzed (see enlarged Purkinje cells in right column). Additionally, mice treated with the (RXRRBR)₂XB-tagged AMO did not demonstrate significant phenotypical, behavioral and motility changes, compared with PBS-injected mice (sham). Hematoxylin and eosin staining of brain sections did not identify morphological changes in brains of the mice treated by the (RXRRBR)₂XB-AMOs (Fig. 8A, last column).

DISCUSSION

As a new generation of AOs, AMOs have been used to correct splicing mutations in various genes, such as *CFTR*, *beta-globin*, *LMNA*, *DMD*, *SMN2* and *ATM* (31–34). The

Figure 3. (RXRRBR)₂XB-AMOs fully restored ATM kinase activity in A-T cells. A-T cells were irradiated with 10 Gy after 4-day treatment of (RXRRBR)₂XB-AMOs. Nuclear protein was extracted for western blotting to assess phosphorylation of SMC1 and KAP1: (A) in TATC cells; (B) in AT203LA cells. (C) ATMs1981 autophosphorylation in TATC and AT203LA cells measured by FACS analysis, and the right shift of the fluorescence intensity indicates autophosphorylation of ATMs1981.

Figure 4. (RXRRBR)₂XB-AMOs abrogated radiosensitivity of A-T cells. Cells were treated with (RXRRBR)₂XB-AMOs for 4 days. Cells were then plated into a 96-well plate, and irradiated with 1 Gy, followed by the CSA assay. Cell SFs were calculated after 14 days: (A) in TATC cells; (B) in AT203LA cells.

application of AMOs has also been extended to correcting nonsense mutations by skipping exons that contain mutations in the *DMD* and *parkin* genes (2,35). Furthermore, AMOs can be used to modulate disease-related alternative splicing such as in spinal muscular atrophy (36). Together, these highlight the therapeutic potential of AMOs in human diseases, but the efficiency and systemic delivery of AMO require

additional study. Our laboratory has previously shown that neutral AMOs (untagged) can restore up to 20% of the ATM protein in A-T cells with splicing mutations when high concentrations (~50 μM) were used (9). Although it is difficult to predict exactly what level of reconstitution of the ATM protein will be therapeutic for A-T patients, new chemically modified AMOs with higher cell-penetrating efficiency

Figure 5. Cellular stability of (RXRRBR)₂XB-AMO and the effect on ATM expression. (A) Cellular fluorescence of AMCA-labeled (RXRRBR)₂XB-AMOs in TATC cells. Cells were treated with 0.5 μM of RXR-AMO and fluorescence was then monitored using microscope. (B) Western blot of ATM protein expression kinetics in TATC cells after a single 5 μM treatment with (RXRRBR)₂XB-TATC-AMO.

will greatly increase the chances of AMOs being used in the clinic. Furthermore, neutral AMOs cannot be used for disorders of the central nervous system, such as A-T, because they cross the BBB inefficiently.

In this study, we show that by attaching an arginine-rich CPP (RXRRBR)₂XB to AMOs, the AMO targeting efficiency is greatly improved. Compared with non-charged neutral AMOs, these CPP-AMO conjugates almost fully corrected aberrant splicing events in A-T cells, as evidenced by efficient correction of both mRNA and protein levels. ATM kinase activity was also fully restored, and A-T cellular radiosensitivity, as measured by a clonogenic assay, was abrogated. Impressively, (RXRRBR)₂XB-tagged AMOs were effective at concentrations as low as ~1 μM. We also found that the restored ATM protein remained detectable for ~3 weeks after a single exposure of 5 μM CPP-AMO, suggesting both the high stability of CPP-AMOs, in culture medium and in cells after uptake, as well as a slow turnover of the ATM protein. Thus, optimized (RXRRBR)₂XB-AMOs appear to be good candidates for treatment of certain types of splicing mutations.

With regard to the mechanisms whereby a modified CPP-AMO acts, we think that the (RXRRBR)₂XB improves cellular AMO uptake through endocytosis (17). It may also help the endocytosed AMOs to escape from the endosome and entering the cytosol and nuclear compartment where they modify pre-mRNA splicing (37). Moreover, the potential cellular trapping mechanism of AMO into the nuclear

compartment may also contribute to its efficacy. After the CPP-AMO is taken up by the cell, the peptide may be degraded, which could limit the cell's ability to expel the AMO portion from the nucleus and lead to longer retention of the AMO in the cell (11,38). All these may work together to enhance AMOs' cellular efficacy *in vitro* and *in vivo* (39).

With our focus on developing therapeutic options for A-T patients, we were especially encouraged by the distribution of (RXRRBR)₂XB-conjugated AMO in the Purkinje cell layer of mice following IV administration. Purkinje cells are large GABAergic neurons, which serve as the sole output of the cerebellar cortex. Purkinje cells typically form a single cell layer at the junction of the granular and molecular layers (30). This finding is significant because of the critical role that Purkinje cells play in A-T pathogenesis (11,12,40). We think that the apparent increase in fluorescent intensity detected in Purkinje cells is likely related to the size of those cells. CPP-AMO conjugates are believed to be taken up by endocytotic mechanisms (17). Purkinje cells are among the largest cells in the central nervous system, which may make them easier targets for the CPP-AMOs. Furthermore, degradation of the CPP portion may also limit the cellular efflux of the AMO once it is being taken into Purkinje cell nuclei (38).

Notwithstanding the above discussion, certain issues related to CPP-AMO delivery and efficiency still need to be

Figure 6. Fluorescence distributions of FITC-(RXRRBR)₂XB-AMO in the mouse brain. Mice were injected with a single dose and multiple doses of (RXRRBR)₂XB-AMO at 60 mg/kg/day. Images were taken from different areas of brain samples. Arrow indicates the Purkinje cell layer.

Figure 7. Texas red anti-FITC immunostaining of FITC-(RXRRBR)₂XB-AMO in the brain and cerebellum. Anti-FITC staining indicates the presence of (RXRRBR)₂XB-AMO. (A) Anti-FITC staining in the hippocampus area and (B) in the cerebellar area. Arrow indicates the Purkinje cell layer.

addressed. For example, AMO-mediated splicing modulation can only correct a small percentage of mutations. The potential off-target effects of AMOs will need to be defined for new CPP-AMO conjugates. Moreover, CPP-AMO conjugates may possibly trigger immune responses *in vivo* (41); hence, the potential immunogenicity of the CPP-AMOs must be monitored. However, taken together, further optimization of CPP-AMO-based approaches has the potential to eventually provide customized, mutation-targeted therapeutics for numerous genetic disorders.

MATERIALS AND METHODS

Neutral AMOs and (RXRRBR)₂XB-conjugated AMOs

The AMO sequences targeting TATC and AT203LA mutations were described previously (10). The neutral form of AMOs was synthesized by Gene-Tools (Philomath, OR, USA), and Endor-porter (Gene-Tools) was used to deliver neutral-form AMOs into cells. For the CPP-tagged AMOs, (RXRRBR)₂XB peptide was conjugated to relevant AMO sequence and used to treat A-T cells (R, L-arginine; X,

Figure 8. Localization of (RXRRBR)₂XB-AMO in Purkinje cell layer. **(A)** Localization of mouse anti-calbindin immunostaining and anti-FITC immunostaining of FITC-labeled-(RXRRBR)₂XB-AMO in Purkinje cell layer of the cerebellum. **(B)** Confocal imaging of the cellular distribution of (RXRRBR)₂XB-AMO to Purkinje cells (arrows). Image stocks were taken using a 63 objective at 1 μm intervals. The right column contains enlarged images of the squared areas of the left column.

6-aminohexanoic acid; B, β-alanine). For *in vivo* systemic delivery experiments, (RXRRBR)₂XB was conjugated to a random control AMO sequence of 5'-TGCTCTGTCTA CA GTAGTGTCA-3 and administrated into mice. (RXRRBR)₂XB-AMOs were provided by AVI Biopharma (Corvallis, OR, USA).

Reverse-transcriptase PCR and real-time PCR

Total RNA was extracted using RNAeasy Kit (Qiagen, Valencia, CA, USA). Reverse-transcription reactions were catalyzed by Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). After that, RT-PCR and real-time

RT-PCR were performed. Primer and PCR conditions were previously described (10).

Western blots

LCLs were treated with AMOs and nuclear extracts were prepared using NE-PER Kit (Pierce, Rockford, IL, USA). The proteins were analyzed by regular western blotting. Antibodies used were rabbit anti-SMC166 (Novus), rabbit anti-KAP1-s824 (Novus), rabbit anti-ATM, anti-SMC1 and anti-NB (Novus). Antibodies were used at dilutions of 1:2000 for overnight incubation at 4°C.

ATM-ELISA

The ATM protein was measured by ATM-ELISA immunoassay as previously described (42). Cell nuclear extracts were prepared using NE-PER Kit (Pierce). ATM concentrations of tested samples were calculated from a standard calibration curve, using the purified ATM protein kindly supplied by Dr Kotoka Nakamura in our laboratory (43).

FACS analysis of ATM-Ser1981 phosphorylation

FACS-ATM Ser1981 was performed using a mouse anti-ATM Ser1981 (Cell Signaling) as previously described (28). Cells were irradiated with 10 Gy and collected for assaying 1 h later.

Clonogenic survival assay

CSA was performed as previously described (29). After 3 days of treatment with CPP-AMOs, LCLs were plated; one plate was exposed to 1.0 Gy radiation, and the other was not. The cells were incubated for 10–14 days and then stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and SF were calculated.

Systemic delivery of (RXRRBR)₂XB-AMO in mice

The (RXRRBR)₂XB-AMO was re-suspended in sterile, purified water at a concentration of 5 mg/ml and stored at 4°C until required. The (RXRRBR)₂XB-AMO was delivered to WT mice by tail vein injection, at a dosage of 60 mg/kg. Multiple injections of (RXRRBR)₂XB-AMO were administered at a dosage of 60 mg/kg/day for four consecutive days at 24 h intervals. Sham-treated animals were injected with an equivalent volume of PBS. Mice were decapitated 24 h after the last injection and brains were frozen in isopentane and stored at –80°C. WT mice (C57BL/6) were purchased from The Jackson Laboratory. All experimental protocols involving the use of mice were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of California, Los Angeles Institutional Animal Care and Use Committee.

Tissue immunostaining

For each brain, 30 µm sections were immunostained for (RXRRBR)₂XB-AMO, using Texas red-conjugated monoclonal mouse anti-fluorescein antibody (Jackson

ImmunoResearch, PA, USA). Sections were incubated in blocking solution (5% normal goat serum in PBS) for 30 min at room temperature to minimize non-specific binding. Sections were then incubated with Texas Red mouse anti-fluorescein 1:100 and Hoechst 1:10 000 (Fisher Scientific, USA) in blocking solution for 2 h 45 min at room temperature, washed in PBS, mounted on coverslips and analyzed by fluorescence microscopy. Confocal images were acquired on a Leica laser scanning confocal microscope (Leica Microsystems, Germany) at the thickness of 1 µm. Calbindin staining of Purkinje cells was performed in brains perfused with 4% paraformaldehyde in PBS, post-fixed in the same fixative overnight and cryoprotected by infiltration with 30% sucrose in PBS for 48 h. For calbindin immunostaining, sections (30 µm) were blocked with 10% normal donkey serum in PBS containing 0.25% Triton for 1 h at room temperature, and incubated with a mouse monoclonal anti-calbindin D28k antibody (Swant Laboratories, Bellinzona, Switzerland) at 1:2000 overnight at 4°C. Sections were then incubated with 1:200 Cy2-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, PA, USA) and Hoechst 1:10 000 for 2 h at room temperature. Sections were washed in PBS containing 0.25% Triton, coverslipped and analyzed by fluorescence microscopy.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

Conflict of Interest statement. P.L.I. is currently employed by AVI BioPharma, Inc. and holds its stock and is currently conducting research sponsored by this company.

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