UNIVERSITY OF CALIFORNIA SAN DIEGO

Development of a Novel Regulatable Dual AAV System for Therapeutic Delivery of BDNF

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by

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The Thesis of Yuhsiang Cheng is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATIONS

I dedicate my thesis to my family and friends for their love and support.

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I would like to thank Professor Mark Tuszynski for giving me the opportunity to work in a lab that both cultivates and challenges young scientists. I have learned so much in his lab, and I look forward to giving back to the scientific community.

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ABSTRACT OF THE THESIS

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by

Yuhsiang Cheng

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Professor Daniel Gibbs, Chair

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Alzheimer's disease (AD) is a neurodegenerative disease that causes a reduction in brain-derived neurotrophic factor (BDNF) in multiple animal models, and delivery of BDNF provides neuroprotective effects in those disease models. BDNF regulates a wide range of biological functions in the human body, and therapeutic delivery requires effective delivery routes and targeting specificity.

Adeno-associated viral (AAV) vector systems have recently become popularized for targeted gene delivery. AAVs are non-pathogenic in humans, provide long-term gene expression,

and do not integrate into the host genome. Despite the advancements in viral gene therapy over the past decades, AAV vector systems are limited by the lack of regulation.

Here, we developed a novel regulatable dual AAV vector system for therapeutic delivery of BDNF that can be turned off by oral administration of tamoxifen to adult mice. Our dual AAV vector system contains two co-dependent AAV vectors. One vector carries a pancellular promoter and the BDNF transgene. The second vector carries a drug-inducible Cre recombinase enzyme (Cre) that we hypothesize will result in degradation of the AAV genome and elimination of gene expression.

We found that our dual vector system provides a modest reduction in AAV gene expression following administration of tamoxifen. Further optimization of the system could lead to the first regulatable AAV system suitable for clinical applications. This regulation could act as an “off” switch for eliminating gene expression in the event of adverse side-effects. Moreover, this regulation could allow for transient gene delivery for treating medical conditions such as traumatic brain injury and spinal cord injury. The ability to tightly regulate AAV gene expression is potentially applicable to all 105 on-going clinical trials involving AAV gene therapy.

Introduction

Alzheimer's Disease

Alzheimer's disease is a neurodegenerative disease and the leading form of dementia[1], affecting approximately 47 million people worldwide in 2015[2]. AD is primarily a late-onset disease, although ~4-5% of patients are diagnosed with early-onset AD.[3] Despite decades of research, there has yet to be a unified hypothesis for the cause of the disease.[4] The amyloid cascade hypothesis remains one of the most popular and dominant hypotheses: familial mutations in amyloid precursor protein (APP), presenilin 1, and presenilin 2 genes cause increased beta-amyloid 42 ($A\beta_{42}$) production, resulting in beta-amyloid (Aβ) plaques and neurofibrillary tangles (NFT).[5] Approximately 70% of the risk of AD is attributed to genetic factors including the familial genes mentioned above and risk genes such as apolipoprotein E, glycogen synthase kinase-3 beta, and tau.[6]

AD pathology originates in the frontotemporal region before progressing throughout the rest of the cerebral cortex and hippocampus.[7] Neuropsychological assessments such as the Mini-Mental State Examination are used to confirm cognitive loss associated with AD in areas such as memory, language, calculation, orientation, and judgement.[8] Clinical symptoms of a classical patient with moderate dementia include impairment of memory[9], language and speech[10], writing[11], proprioception[12], and perception[13].

An AD diagnosis is commonly supported by the biomarkers Aβ, tau protein, and hyperphosphorylated tau protein.[14] Misfolded Aβ can aggregate as plaques[15], and hyperphosphorylated tau proteins, normally functioning in stabilizing microtubules[16], can result in NFTs.[17] Lastly, progression of the disease can be monitored through a variety of neuroimaging

techniques such as magnetic resonance imaging, computed tomographic imaging, and positron emission tomography.[18]

AD drug development has focused primarily on modulating Aβ levels, inhibiting protein aggregation, and reducing neuroinflammation.[19] Unfortunately, AD drug development over the past decades has yielded only a few agents with limited therapeutic capacities.

Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor was identified in 1982 by Yves Barde and Hans Thoenen.[20] BDNF binds to and activates tropomyosin-related kinase receptor type B (TrkB), a tyrosine kinase receptor widely expressed throughout the central nervous system in areas such as the cerebral cortex, hippocampus, dentate gyrus, striatum, brainstem, and spinal cord.[21] TrkB subsequently promotes cell survival through the following downstream pathways: the MEK-ERK pathway stimulates anti-apoptotic proteins, the PI3K-AKT pathway phosphorylates and inhibits apoptotic proteins, and the Gαq signaling cascade induces calcium mobility and modulates synaptic signaling and plasticity.[22]

BDNF, as an endogenous human protein, has the potential to provide therapeutic effects in neurodegenerative diseases including Alzheimer's disease[23], Parkinson's disease[24,25,26], Huntington's disease[27], amyotrophic lateral sclerosis[28,29,30], and spinal cord injury[31,32,33]. AD results in decreased levels of BDNF in the entorhinal cortex and hippocampus.[34,35] Moreover, Nagahara et al. showed that BDNF provides neuroprotective effects in multiple AD models such as APP transgenic mice, aged rats, and aged monkeys.[23] Beyond neuroprotection, BDNF also plays a role in a variety of additional trophic functions such as the regulation of metabolic processes.[36] Unfortunately, the broad range of BDNF effects also introduces the potential for

adverse, off-target effects when using BDNF as a therapeutic molecule. Furthermore, intrathecal and systemic infusions of BDNF into the CNS are insufficient to penetrate brain parenchyma.[37] Alternative methods are currently being developed for counteracting BDNF loss in AD, including viral gene therapy[23], nanoparticle delivery of BDNF[38], and modulation of endogenous BDNF production and signaling.[39] BDNF has great therapeutic potential, but treatment paradigms are currently limited by targeting specificity and delivery methods.

Advances in Gene Therapy

In the 1990s, viral gene therapy trials failed with serious adverse effects including death.[40] Following the recommendations from an NIH advisory committee, the basic biology and intricacies of gene therapy approaches were further elucidated, and several successful clinical trials were conducted thereafter.[41] Decades of development culminated in the translational potential of gene therapy today, and popular contemporary approaches include viral vector gene delivery, engineered T cells, and hematopoietic stem cells[42]. As a recently validated vehicle for gene delivery, engineered recombinant adeno-associated viral (rAAV) vectors provide unique benefits such as lack of pathogenicity[43,44], detectable gene expression without integration[45], and the ability to provide long-term gene expression[46,47]. Recently, Nagahara et al. were able to perform an MRI-guided delivery of AAV2-BDNF into the entorhinal cortex of non-human primates (NHP), which subsequently resulted in anterograde supply of BDNF to the hippocampus.[48]

Adeno-associated Virus

Adeno-associated virus, part of the *Parvoviridae* family, was discovered in 1965 as a contaminant in adenovirus preparations, and Medawar et al. described AAV as an "incomplete or defective virus" that remained dormant until presented with helper virus machinery.[49,50] This helper-dependent wild-type AAV (wtAAV) consists of a non-enveloped capsid that is used to package a piece of single-stranded DNA (ssDNA) that contains three protein-encoding open reading frames (orf) flanked by 145 nucleotide-long inverted terminal repeats (ITRs).[50] The wtAAV orfs encode for Rep protein 78 (Rep78), Rep68, Rep50, Rep42, capsid proteins (VP1, VP2, VP3), and an assembly-activating protein (AAP).[51] AAP promotes transportation of the capsid proteins to the nucleolus for capsid assembly[51], and the Rep proteins play a role in wtAAV DNA replication and packaging of that DNA into the empty capsid.[52,53,54] Different AAV serotypes have been discovered, each differentiated by unique capsid structures and tissue specificity.[55]

After wtAAV enters a cell, the viral ssDNA must synthesize its own complementary DNA strand in a process known as second strand synthesis.[56] Second strand synthesis requires the viral ITRs and Rep proteins, helper virus machinery, as well as cellular factors polymerase delta, replication factor C, proliferating cell nuclear antigen, and minichromosome maintenance complex proteins.[56] Infection is complete following replication, and the viral double-stranded DNA (dsDNA) produces new viruses primed for endosomal escape from the host cell.[57] A latent infection takes place in the absence of helper virus machinery, in which the wtAAV ssDNA integrates into a site-specific sequence known as AAVS1 located on the long arm of chromosome 19 with the help of its ITRs and Rep proteins.[58] If a helper virus subsequently infects the cell, the integrated wtAAV DNA can be "rescued" and undergoes DNA replication.[58]

Recombinant Adeno-associated Virus

Recombinant AAV used for human gene therapy is primarily produced through the triple-plasmid transfection technique, although additional methods have been developed to further reduce contamination for large-scale productions. Adenovirus contamination of rAAV preparations were eliminated in the triple-plasmid transfection method, in which human embryonic kidney 293 cells are simultaneously transfected with a plasmid expressing rAAV-transgene, a plasmid expressing the AAV2 *rep* orf and the *cap* orf of the desired AAV serotype, and a plasmid expressing adenovirus replication machinery.[59,60] For the purpose of large-scale rAAV production, Allen et al. were able to further eliminate wtAAV contamination by using separate heterologous promoters to transcribe AAV2 *rep* and AAV8 *cap* orfs positioned in opposite directions.[61]

In recent years, the development of dual AAV vector systems have allowed functional expansion of AAV packaging capacity from ~4.7kb to ~9.4kb, in which two independent AAV vectors undergo directed homologous recombination within a transduced cell to form a complete episome.[62] Moreover, capsid engineering[63] and cell-specific mini-promoters[64] have been used to provide increasing levels of targeting specificity and transduction efficiency in next generation AAV systems. As viral gene therapy transitions once again towards translational and clinical projects, AAV treatment models are limited by a lack of proper regulation.

Regulatable Gene Therapy Systems

Tight regulation is potentially applicable to all on-going clinical trials involving AAV gene therapy. A regulatable AAV system could be used to eliminate AAV gene expression in the

event of adverse side effects or for transient gene delivery for treating conditions such as traumatic brain injury and spinal cord injury.

Regulation of gene therapy has been studied extensively, and current regulation candidates for targeting the central nervous system include tetracycline, mifepristone, rapamycin, ecdysone, and cumate-regulated systems. Unfortunately, none of these systems can currently be used for clinical applications.

The tetracycline (Tet) system is one of the most popular methods for regulating gene expression. The system uses a transactivator, a transcription-activating unit, that contains a Tet repressor DNA-binding protein (TetR) fused to a herpes simplex virus transcriptional regulatory protein vmw65 (VP16).[65] The TetR sequence serves as a DNA recognition domain, much like zinc finger domains found in nuclear receptors, and the VP16 moiety functions to promote transcription through recruitment of transcription factors.[65] The Tet system is further divided into TetOff and TetOn systems. The TetOff system uses a Tet-controlled transactivator protein (tTA) that binds to DNA and promotes transcription in the presence of Tet.[65] The TetOn system uses a reverse tTA that binds to DNA and promotes transcription in the absence of Tet.[66] The Tet systems serve as a model for a number of other regulatable systems.

In medicine, mifepristone (RU-486) is a progesterone receptor antagonist that is used as an abortion drug.[67] The mifepristone inducible system uses a transactivator that contains a fusion of GAL4, VP16, and a mutant progesterone receptor ligand-binding domain (PR-LBD).[68] GAL4 acts as a DNA recognition domain, VP16 promotes transcription, and PR-LBD selectively binds RU-486.[68] When RU-486 binds to PR-LBD, the transactivator complex is translocated into the nucleus to promote transcription.[68]

The rapamycin system consists of two major components: a zinc finger homeodomain-1 (ZFHD1) fused to FK-binding proteins (FKBP) and an FKBP-rapamycin-associated protein 1 (FRAP) fused to a nuclear factor kappa-light-chain-enhancer of activated B cells p65 (NFκB p65).[69] Rapamycin simultaneously binds to both FKBP and FRAP to induce heterodimerization of the two complexes. ZFHD1 serves as the DNA recognition domain, and NFκB p65 promotes transcription.[69]

The ecdysone system uses a transactivator complex that contains the ecdysone receptor fused to VP16.[70] In the presence of ecdysone, the transactivator complex heterodimerizes with the retinoid X receptor before binding to and promoting transcription.[70]

The cumate system is divided into the repressor, activator, and reverse activator systems. The repressor system uses a cumate-dependent repressor (CymR) to inhibit transcription in the presence of cumate, the activator system uses a transactivator containing CymR fused to VP16 to promote transcription in the absence of cumate, and the reverse activator system uses a transactivator containing reverse CymR fused to VP16 to promote transcription in the presence of cumate.[71]

The primary concern for most of these systems is the inclusion of a VP16 moiety. VP16 has been shown to cause immunogenic responses in NHPs, which would prevent any system using VP16 from reaching clinical trials.[72,73] Aside from the immunogenicity of VP16, each system has additional concerns that must be addressed. Beyond the political controversy surrounding mifepristone as an abortion-inducing drug, there have been conflicting results about the "leakiness" of this system.[72,74] If the transactivator "leaks", or translocates independently, the entire system becomes compromised. Moreover, clinical use of the rapamycin system is limited by the drug's immunosuppressant effects.[75] Lee et al. have developed an ecdysone regulated

system involving minimal VP16 sequences[70], but a lack of immunogenicity within NHPs has not been shown.

The above systems all depend on a transactivator complex that is constitutively expressed, whether the system is turned on or off. This characteristic could further exacerbate any potential issues with immunogenicity or leakiness of those systems. Lastly, all discussed regulatable systems also require continuous drug treatment for regulation, which could potentiate undesired side-effects and also relies on patient compliance. In conclusion, there is a need for a regulatable AAV gene delivery system that is suitable for translational and clinical endeavors.

Tamoxifen Regulated System

In 1997, Feil et al. developed a tamoxifen inducible-Cre recombinase system that uses a Cre recombinase enzyme fused to a triple mutant estrogen receptor (ER^{T2}).[76] First, Cre induces recombination events at a repeat known as locus of X-over P1 (loxP) sites that flank sequences of DNA.[77] Direct loxP repeats induce excision of a flanked DNA segment, and inverted loxP repeats induce inversion of a flanked DNA segment.[78] For example, Orban et al. were able to excise a transgene flanked by direct loxP repeats in a specific and Cre-inducible manner.[79] Secondly, wild-type estrogen receptors (ER), localized in the cytoplasm, will homodimerize upon binding estrogen translocate to the nucleus to regulate transcription.[80] ER^{T2} is a mutant estrogen receptor developed by Feil et al. that is insensitive to the natural ligand estrogen and can only be selectively activated by the synthetic ligand tamoxifen.[76] The ER^{T2}-Cre fusion protein is sequestered in the cytoplasm by the ER^{T2} moiety in the absence of tamoxifen, and the Cre moiety can interact with DNA after the fusion protein is translocated into the nucleus in the presence of tamoxifen.[76] Unfortunately, the Cre-ER^{T2} system was shown to have background activity even in

the absence of tamoxifen.[81] Casanova et al. improved the inducible-Cre system by using an ER^{T2}-Cre-ER^{T2} fusion protein that eliminated the leakiness by adding a second mutant estrogen receptor.[82] In theory, the inducible-Cre system could be used to regulate AAV expression by flanking the gene expression cassette with loxP sites, and Cre could be activated when elimination of gene expression is desired.

Novel Regulatable Dual AAV Vector System

In this study, we tested a novel regulatable dual AAV vector system that can be turned off by oral administration of tamoxifen to adult mice. The dual AAV vector system consists of two co-dependent vectors. One vector carries a pancellular promoter and the BDNF transgene. The second vector carries ER^{T2}-Cre-ER^{T2} which, following administration of tamoxifen, we hypothesize will result in excision of the AAV genome and elimination of AAV gene expression. If successful, this dual vector system could be used as a regulatable vehicle for therapeutic delivery of BDNF, and this system would be the first clinically relevant regulatable system for AAV gene therapy.

Results

We tested a novel regulatable dual AAV vector system that consisted of two co-dependent AAV vectors: AAV9-CAG-loxP-Venus-P2A-BDNF-IRES-SD-AP (5' vector) and AAV9-AP-SA-ER^{T2}-Cre-ER^{T2}-loxP-polyA (3' vector). Co-dependency was achieved through the absence of a polyA signal in the 5' vector and the absence of a promoter in the 3' vector. When co-transfected, the 5' vector and 3' vector should recombine to form a complete episome (Fig. 1). In the absence of proper recombination, the 5' vector mRNA should be degraded without a polyA signal, and the 3' vector should not be transcribed without a promoter. A dual vector system was the vehicle of choice, as opposed to a single vector system, in order to package all components of the regulatable gene delivery system. The recombined episome should generate comparable levels of Venus and BDNF, which are driven by a pancellular CMV enhancer/chicken beta-actin (CAG) promoter and a porcine teschovirus-1 (P2A) skip sequence respectively. The internal ribosomal entry site (IRES) should allow for a lower expression of ER^{T2}-Cre-ER^{T2} relative to Venus and BDNF. Venus and BDNF should be released through endosomal escape from the transfected cells, and ER^{T2}-Cre-ER^{T2} should be sequestered within the cytoplasm. Upon administration of tamoxifen, ER^{T2}-Cre-ER^{T2} should translocate into the nucleus, excise the AAV gene expression cassette, and eliminate AAV gene expression (Fig. 2). This system provides unique strengths by addressing the flaws of previously developed regulatable systems. First, all AAV machinery should be eliminated following administration of tamoxifen. Next, IRES allows the potentially immunogenic Cre moiety to be produced at a low level. Lastly, this regulatable mechanism would provide a one-time treatment for temporally regulating AAV gene therapy. As a one-time treatment, risk of unwanted side-effects from using tamoxifen are decreased substantially.

We performed bilateral intraparenchymal injections of the hippocampus were performed at -1.7mm anteroposterior (A/P), ±1.6mm mediolateral (M/L), and -1.7mm dorsoventral (D/V) relative to the bregma. Ai9(RCL-tdT) Cre reporter mice were used to detect for Cre activity. We injected 10 animals with the dual vectors (1E10 vg in 0.5µL per site), 2 animals with sterile phosphate buffered saline (0.5µL per site), and 2 animals with AAV9-CaMK2a-Cre (1E10 vg in 0.5µL per site). 6 animals injected with the dual vectors were fed a tamoxifen diet for 6 weeks after surgery, and the other 4 animals injected with the dual vectors were fed a standard mouse diet for the duration of the experiment. 8 weeks after surgery, all animals were euthanized, perfused, and dissected. The animal brains were removed, fixed, and coronally sectioned at 30 microns on a microtome.

Hippocampal sections closest to the injection sites were immunostained against BDNF (Fig. 3A). Hippocampal neurons expressing BDNF were counted. In order to correct for endogenous BDNF in the untreated and tamoxifen-treated cohorts, the average cell count of the saline control was subtracted from each cell count. There was a 44% reduction in the number of neurons expressing exogenous BDNF following administration of tamoxifen (Fig. 3B). To further investigate the pattern of AAV gene expression, hippocampal sections closest to the injection sites were immunostained against Venus and tdTomato. There was a reduction in the intensity of Venus in the tamoxifen-treated cohort compared to the untreated cohort (Fig. 4). The untreated cohort expresses only Venus, and the tamoxifen-treated cohort expresses both Venus and tdTomato (Fig. 5).

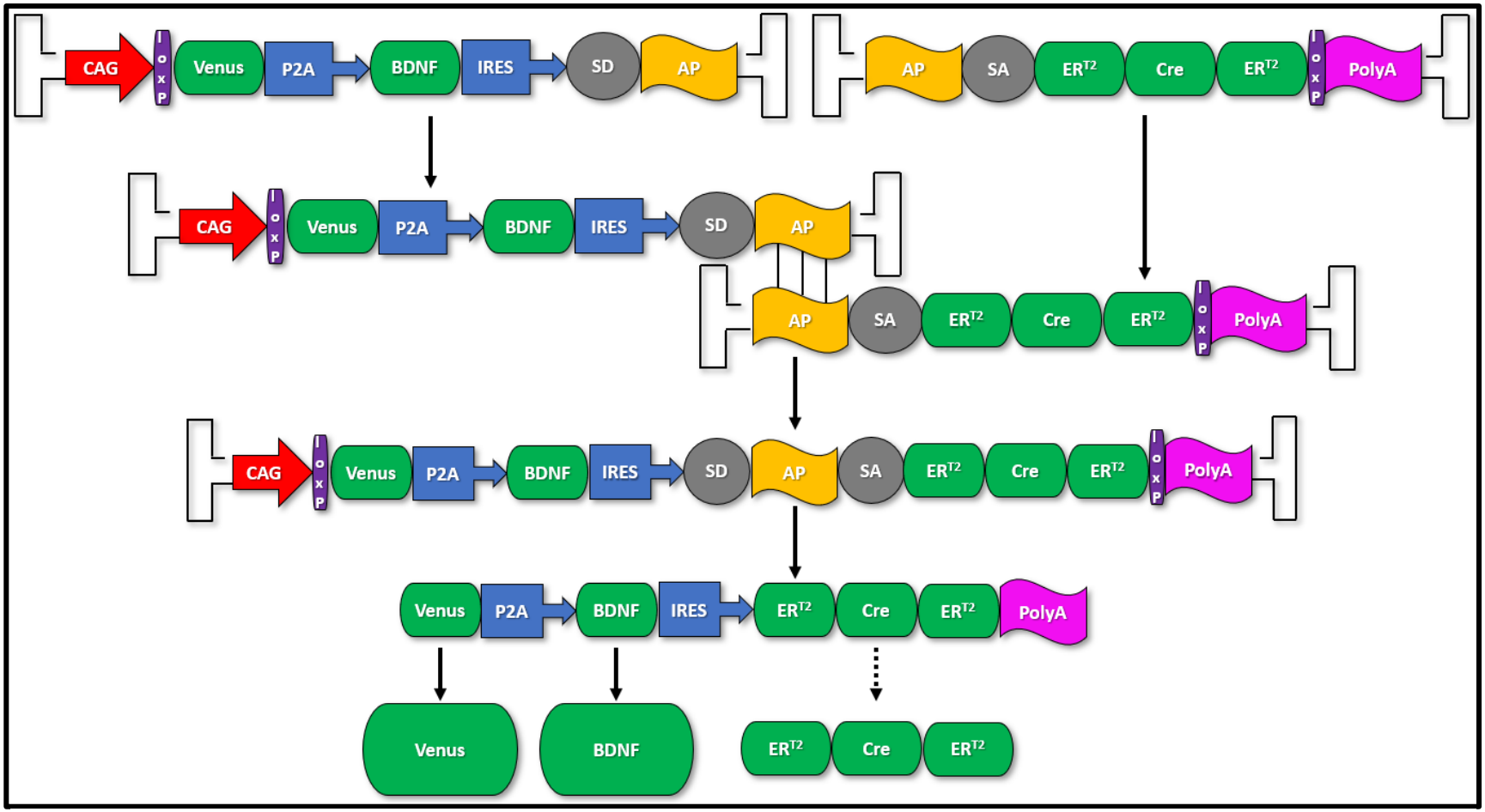


Figure 1. Schematic of the recombination of the dual AAV vector system following co-injection of the 5' and 3' vectors. The 5' vector (top left) contains the pancellular CAG promoter, the first loxP site, the reporter gene Venus, the porcine teschovirus-1 (P2A) skip sequence, the BDNF transgene, the internal ribosomal entry site (IRES), the splicing donor (SD), and the alkaline phosphatase (AP) hybridization sequence. The 3' vector (top right) contains the AP hybridization sequence, the splicing acceptor (SA), the ER^{T2}-Cre-ER^{T2} transgene, the second loxP site, and the polyadenylation signal (PolyA). Upon co-transfection, the 5' and 3' vectors recombine to form a complete episome, which can produce the Venus, BDNF, and ER^{T2}-Cre-ER^{T2} proteins. ER^{T2}-Cre-ER^{T2} is translated at a lower level (dotted arrow) than Venus and BDNF translational levels.

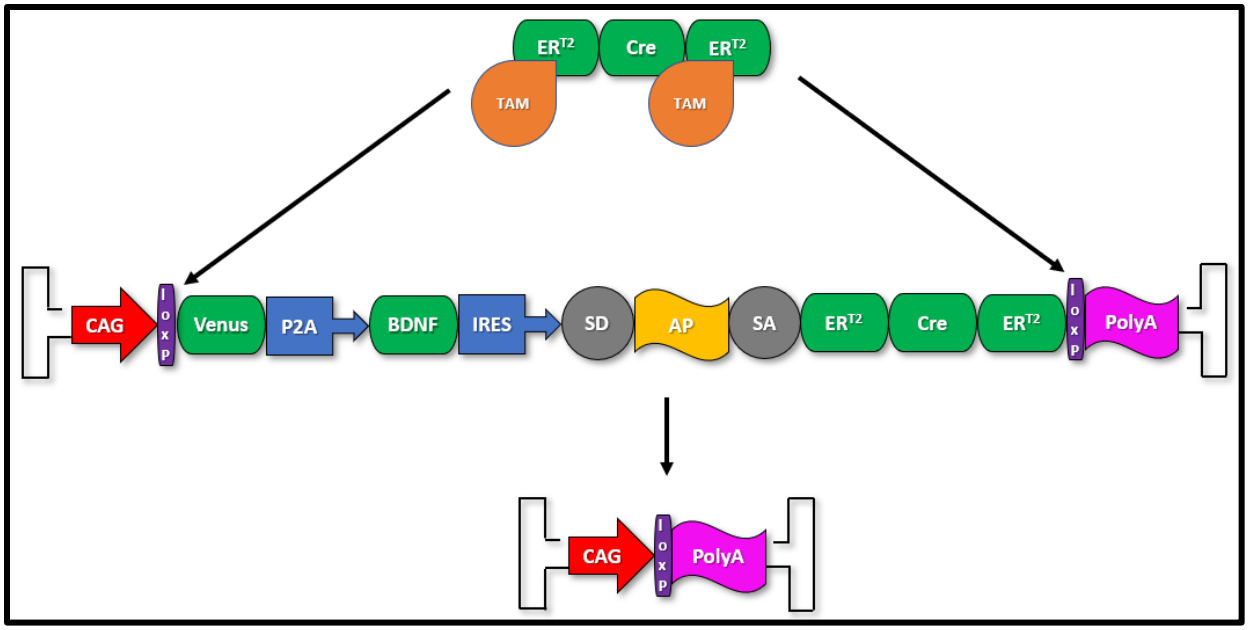


Figure 2. Schematic of ER^{T2}-Cre-ER^{T2} activity following administration of tamoxifen. Upon administration of tamoxifen, ER^{T2}-Cre-ER^{T2} translocates to the nucleus and excises the AAV gene expression cassette flanked by loxP sites. Subsequently, AAV machinery and associated transgenes are eliminated.

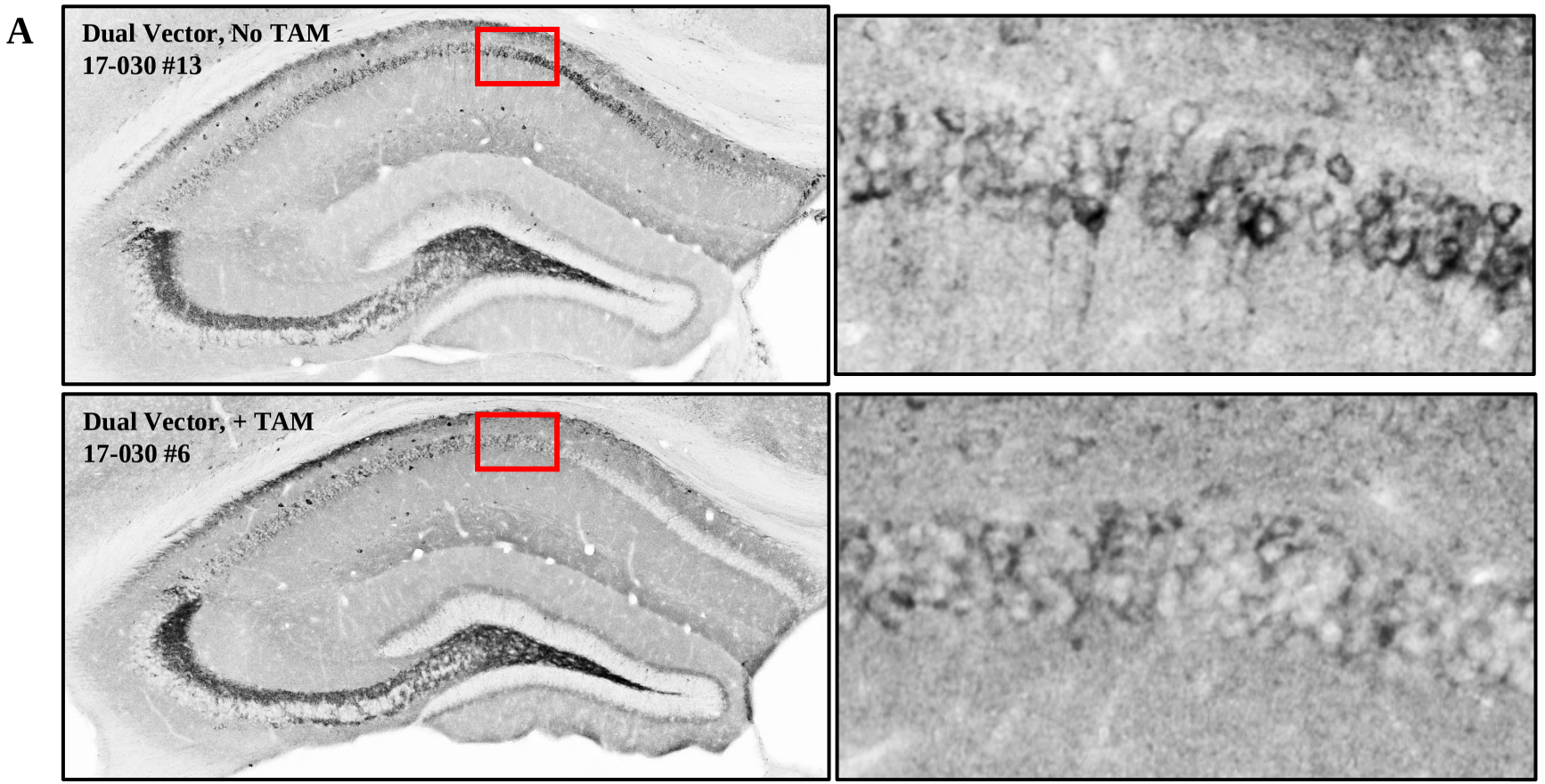
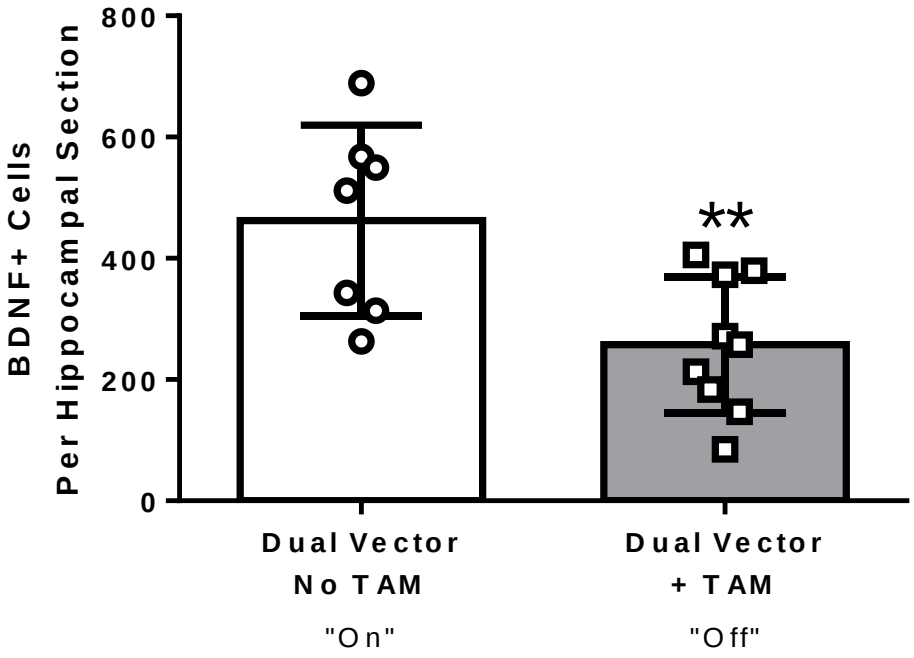


Figure 3. Tamoxifen treatment reduces the number of neurons expressing exogenous BDNF.
(A) Representative hippocampi from the untreated (top) and tamoxifen-treated (bottom) cohorts. The tissue was coronally sectioned at 30µm and immunostained against BDNF. BDNF expression in CA1 and CA2 is reduced following tamoxifen treatment. (B) The number of BDNF-positive neurons in each injected hippocampus was counted using the tissue section closest to the site of injection. To correct for endogenous BDNF, the number of cells counted in the sterile phosphate buffered saline controls were averaged, and the average was subtracted from each cell count. There were 44% fewer BDNF-positive neurons in the tamoxifen-treated cohort than in the untreated cohort (unpaired t-test: n = 7 untreated hippocampi and 9 tamoxifen-treated hippocampi, $^{**}P = 0.0086$). Error bars represent standard deviation.

Figure 3. continued.

B



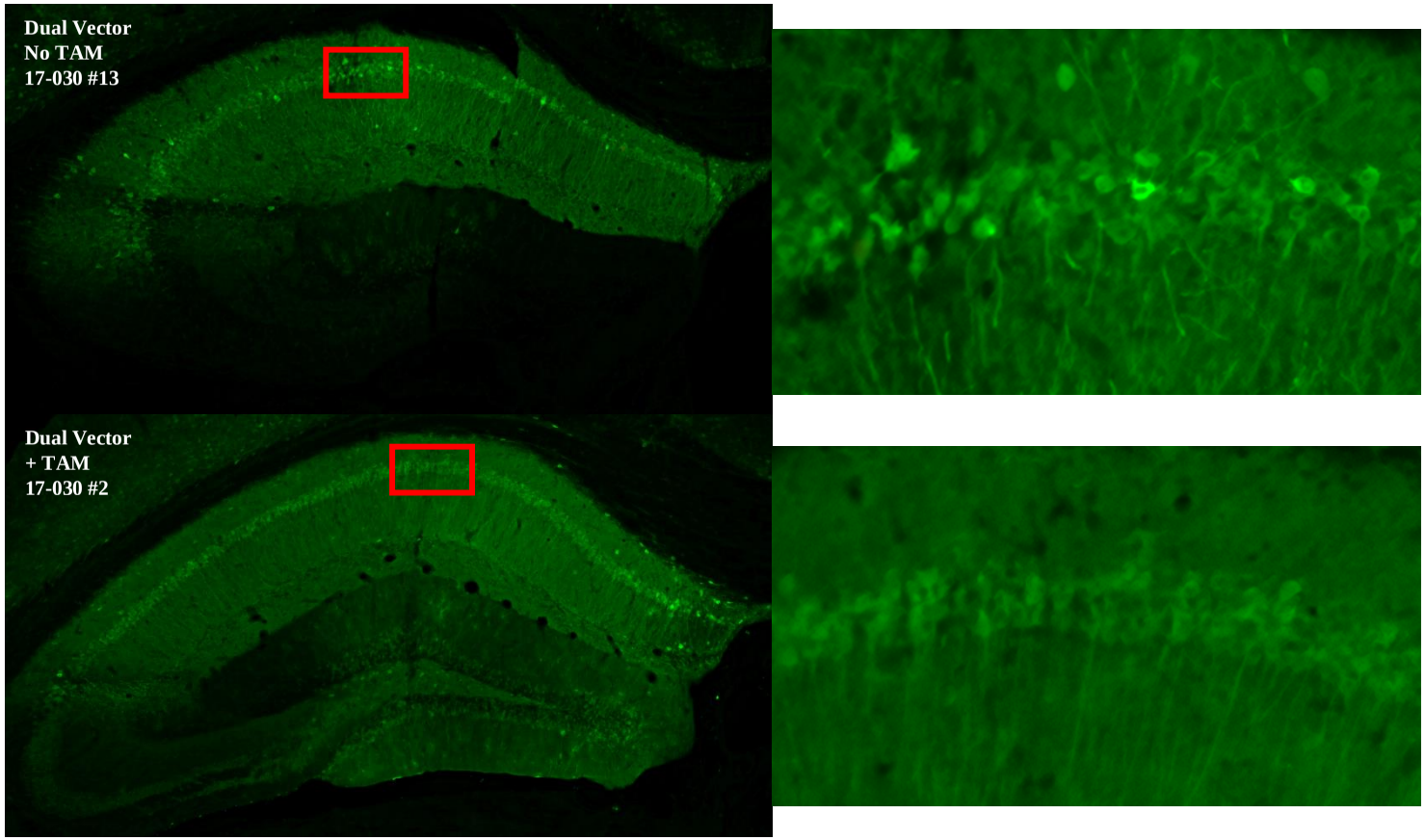


Figure 4. Tamoxifen treatment appears to reduce the intensity of Venus.
Representative hippocampus from the untreated (top) and tamoxifen-treated (bottom) cohorts. The tissue was coronally sectioned at 30µm and immunostained against Venus. Venus intensity in CA1 and CA2 appears to be reduced following tamoxifen treatment.

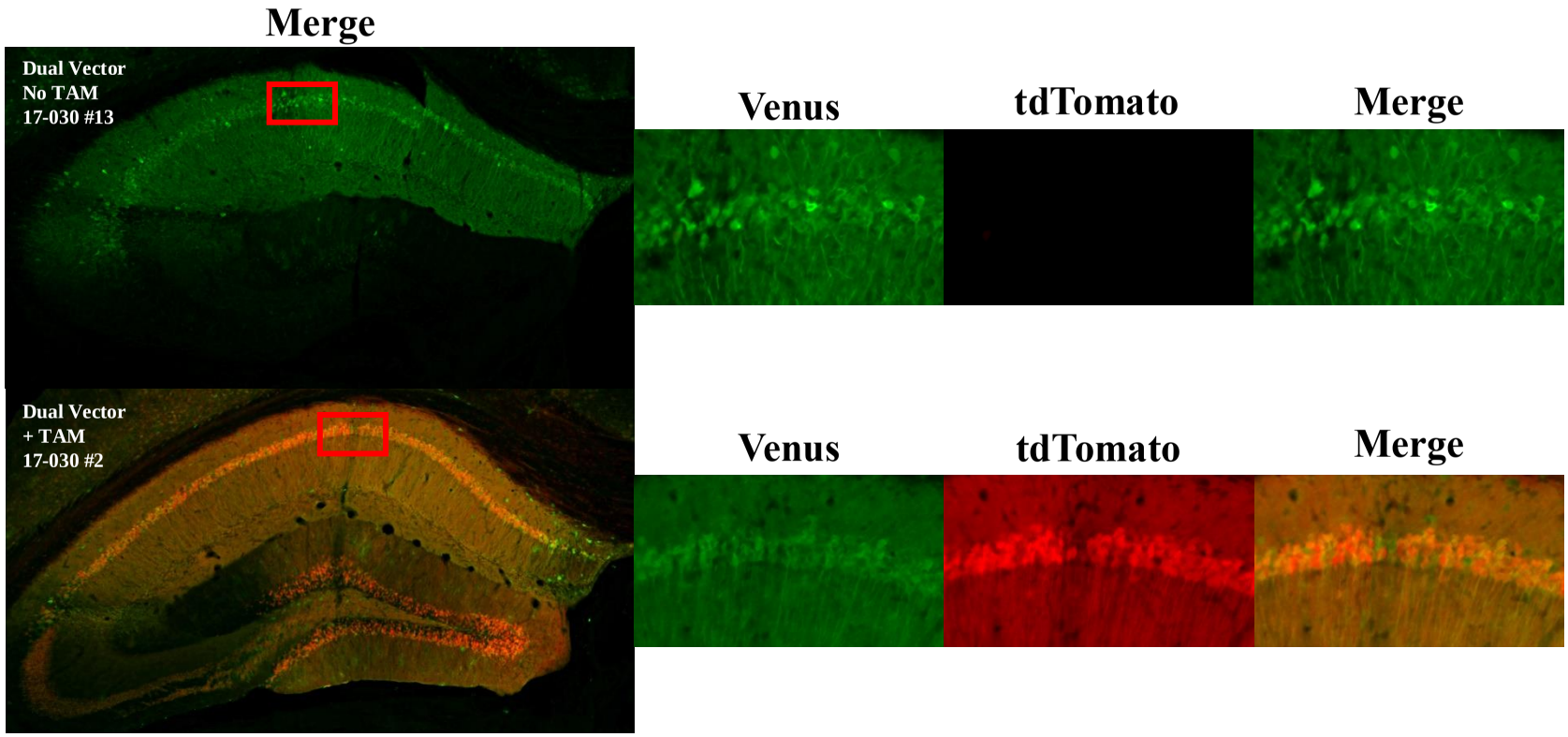


Figure 5. Tamoxifen translocates and activates ER^{T2}-Cre-ER^{T2}.
Representative hippocampus from the untreated (top) and tamoxifen-treated (bottom) cohorts. The tissue was coronally sectioned at 30µm and immunostained against Venus and tdTomato. The hippocampus of the untreated cohort expresses only Venus. The hippocampus of the tamoxifen-treated cohort expresses both Venus and tdTomato.

Discussion

AAV and BDNF therapies have great potential in treating neurological diseases. However, off-target effects remain a concern in both AAV and BDNF treatment paradigms, and no mechanisms exist for regulating AAV gene expression. Moreover, the current regulatable systems are not designed for translational research and are primarily limited by the immunogenicity of the regulatable machinery. Thus, there is a need for the development of a regulatable AAV system. A regulatable AAV system could be used as an "off" switch for AAV gene therapy in the event of adverse side-effects and when transient gene delivery is desired.

We observed a reduction in exogenous BDNF levels following tamoxifen treatment, which shows that our regulatable system was able to reduce but not completely eliminate AAV gene expression. To further investigate the mechanisms behind the incomplete effect of our system, we analyzed the fluorescent markers Venus and tdTomato. Venus expression represents successful co-transfection and recombination of the dual vectors. tdTomato expression, detected by the Cre reporter mouse line, represents successful translocation and activation of ER^{T2}-Cre-ER^{T2}. If tamoxifen-induced excision of the AAV genome was complete, we would expect to see tdTomato expression without Venus expression. The co-localization of tdTomato and Venus seen in the tamoxifen-treated cohort represents the successful translocation and activation of ER^{T2}-Cre-ER^{T2} and the incomplete elimination of AAV gene expression.

There are several potential strategies for optimizing our regulatable system. First, alternative methods for administering tamoxifen, such as intraperitoneal injections, would allow for more controlled and higher dosages. Moreover, a prolonged tamoxifen treatment would give Cre more time to excise the AAV genome. Next, the dual vectors could be injected at a lower dosage to decrease the number of AAV genomes that Cre has to cleave. However, lowering the

dual vectors dosage would also result in lower ER^{T2}-Cre-ER^{T2} levels. We could combine a low dose dual AAV injection with the replacement of IRES with a strong promoter to maintain elevated levels of ER^{T2}-Cre-ER^{T2}.

Even partial reduction of AAV gene expression could be relevant to the clinical setting, and the optimization of our regulatable system could provide an additional layer of safety not only for AAV delivery of BDNF but also for delivery of other therapeutic genes.

Materials and Methods

Animals and Surgery Ai9(RCL-tdT) is a Cre reporter homozygous for Rosa-CAG-LSL-tdTomato-WPRE. The lox-STOP-lox (LSL) sequence allows for conditional transcription of red fluorescent protein tdTomato in the presence of Cre recombinase.[83] We performed bilateral intraparenchymal injections of the hippocampus of 14 adult Ai9 mice within the following cohorts: 5' vector + 3' vector (n = 10), AAV9-CaMKIIα-Cre (n = 2), and sterile phosphate buffered saline (n = 2). Injections were performed as described.[84] Equal numbers of adult male (~18-20 grams) and adult female (~16-18 grams) mice were used.

Viral Vector Our dual vector system consisted of two co-dependent AAV vectors: AAV9-CAG-loxP-Venus-P2A-BDNF-IRES-SD-AP (5' vector) and AAV9-AP-SA-ER^{T2}-Cre-ER^{T2}-loxP-polyA (3' vector). The vectors were generated by the Viral Vector Core at Salk Institute.[85] When co-transfected, the two vectors recombined to form a complete episome. The 5' vector and 3' vector were each injected at 1E10 vg/site. AAV9-CaMKIIα-Cre (AAV9-Cre) used to validate our transgenic Ai9 mouse line was injected at 1E10 vg/site. All dilutions were prepared in 0.5mL LoBind Eppendorf tubes.

Stereotaxic Coordinates We established stereotaxic coordinates through bilateral intraparenchymal dye injections (1µL/side) of the hippocampus in adult C57BL/6J mice.[86] Our bilateral targets of the central hippocampus were identified as -2.0mm anterioposterior (A/P), ±1.5mm mediolateral (M/L), and -1.7mm dorsoventral (D/V) relative to the bregma. The coordinates were adjusted based on our definition of bregma and the dye injection results. The

finalized injection coordinates were -1.7mm A/P, ±1.6mm M/L, and -1.7mm D/V relative to the bregma.

Diet 6 animals injected with the dual vectors were placed on a tamoxifen diet[87] from 2 weeks after surgery until 8 weeks after surgery. All other animals were placed on a traditional rodent diet (Teklad 4% fat mouse/rat diet)[88] for 8 weeks after surgery.

Perfusion-fixation, Dissection, and Tissue Sectioning 8 weeks after surgery, all animals were euthanized through 0.05mL intraperitoneal (IP) injections of ketamine/xylazine/acepromazine (KXA) in a 1:5 dilution in sterile phosphate buffered saline. Euthanized animals were perfused with 50mL of phosphate buffered saline, followed by 50mL of 4% paraformaldehyde (PFA). The animal brains were dissected and placed in 4% PFA. After one overnight in 4% PFA, the brains were washed with phosphate buffered saline before transfer to scintillation vials filled with 30% sucrose solution. Once the brains dropped to the bottom of the scintillation vials, the brains were coronally sectioned at 30 microns with a microtome. Brain sections were stored in 24-well plates in tissue cryoprotectant solution at -20ºC.

Immunofluorescent Staining & Imaging Brain sections from each animal were blocked in 5% heat inactivated donkey serum + 0.25% Triton X-100 in TBS for 1 hour at room temperature. Then, sections were incubated rabbit polyclonal anti-mCherry (1:500, EncorBio #RPCA-mCherry) and chicken polyclonal anti-GFP (1:1000, Aves Labs #GFP-1020) primary antibodies for 1 overnight at 4°C. Next, the sections were incubated in donkey anti-rabbit (1:500, Invitrogen Thermo Fisher #A10042) and donkey anti-chicken (1:500, Jackson ImmunoResearch #703-605-

155) secondary antibodies. After drying in the dark for 1 overnight, sections were mounted onto slides and coverslipped with mowiol. Immunostained sections were imaged at 10X on the Keyence BZ-X710.

BDNF Immunohistochemistry and Imaging Brain sections from each animal were mounted onto gelatin subbed slides and dried at room temperature. Two days later, sections from each animal were blocked in 5% heat inactivated donkey serum + 0.25% Triton X-100 in TBS for 1 hour at room temperature. Next, sections were incubated in primary antibody (anti-BDNF, rabbit IgG, 1:5000, #091207 Chicago Proteintech) for 4 overnights at 4°C. Then, sections were incubated in secondary antibody (anti-Rabbit Biotin, donkey IgG, 1:400, #711-065-152 Jackson ImmunoResearch Laboratories) for 2 hours at room temperature, followed by incubation in the Avidin/Biotinylated Enzyme Complex (ABC, VECTASTAIN #PK-6100) for 2 hours at room temperature. Lastly, sections were developed in 3,3-diamino-benzidine-HCl (DAB, Sigma #5637) for 4 minutes at room temperature. Sections were dried for 2 overnights at room temperature before coverslipping with DPX. BDNF-stained slides were imaged at 10X on the Keyence BZ-X710.

BDNF Quantification and Statistical Analysis BDNF images were quantified by using ImageJ to count the number of BDNF-positive neurons within CA1, CA2, and CA3, and the dentate gyrus of each hippocampi. Individual hippocampi were counted due to the variability of stereotaxic injections. The number of BDNF-positive neurons for each region were added up for each phosphate buffered saline control. In order to correct for endogenous BDNF, the average of the phosphate buffered saline controls were subtracted from each cell count of the untreated and

tamoxifen-treated cohorts. The two cohorts were compared in an unpaired t-test, which reported a p-value of 0.0086 (GraphPad Prism). Failed injections were determined based on the absence of tdTomato, Venus, and BDNF, and these samples were not included in quantification or statistical analysis.

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