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Neuronal activation stimulates cytomegalovirus promoter-driven transgene expression

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Short title: Neuronal activators stimulate the CMV promoter

Abstract

The cytomegalovirus (CMV) immediate early promoter has been extensively developed and exploited for transgene expression *in vitro* and *in vivo*, including human clinical trials. The CMV promoter has long been considered a stable, constitutive and ubiquitous promoter for transgene expression. Using two different CMV-based promoters, we found an increase in CMV-driven transgene expression in the rodent brain and in primary neuronal cultures in response to methamphetamine, glutamate, kainic acid, and activation of G-protein coupled receptor signaling using designer receptors exclusively activated by designer drugs (DREADDs). In contrast, promoters derived from human synapsin 1 (hSyn1) gene or elongation factor 1a (EF1a) did not exhibit altered transgene expression in response to the same neuronal stimulation. Overall, our results suggest that the long standing assertion that the CMV promoter confers constitutive expression in neurons should be reevaluated and future studies should evaluate the activity of the CMV promoter in a given application.

Introduction

Due to strong expression and compatibility with numerous cell types, promoters derived from the enhancer region of the cytomegalovirus (CMV) immediate early (IE) promoter are among the most common for general expression vectors.[1,2] Its reputation as a source of stable, long-term expression has even allowed the CMV promoter to be used in clinical trials for gene therapy in the central nervous system.[3,4] The CMV promoter/enhancer contains an array of regulatory elements including multiple binding sites for cellular transcription factors, such as ATF/CREB (activating transcription factor/cAMP response element binding protein), NF-κB/Rel, (nuclear factor κB/Rel), SP-1 (specificity protein 1), ELK-1 (ETS transcription factor), RAR-RXR (retinoic acid receptor family), and AP-1 (activator protein 1).[5] The activation of these transcription factors is known to be rapid and affected by a wide range of

acute stimuli, including neuronal transmission, growth factors, cell stress and inflammation. Indeed, CMV reactivation as a consequence of wild-type CMV-IE promoter activation has been suggested in response to increased cAMP levels, activation of protein kinase C, proinflammatory cytokines or lipopolysaccharide, and heat shock.[6-10]

Despite the above-mentioned possible confounding effects of transcription factor activity regulating CMV-driven transgene expression, the CMV promoter continues to be widely used to drive transient or long-term expression of transgenes in vitro and in vivo without addressing potential effects of experimental treatments on transgene expression. During our studies on neurotoxic effects of the widely abused stimulant methamphetamine (Meth), we found indications of a treatment-induced upregulation of CMV-driven transgene expression, leading to strong false-positive results in our model system. Using different *in vivo* and *in vitro* models for neuronal activation, we observed a strong interaction between neuronal activation and CMV promoter-controlled transgene expression, creating a bias that may influence fundamental pre-clinical, as well as clinical, research findings.

Results

Subcutaneous methamphetamine affects CMV promoter activity in the rat striatum

To study changes in brain-derived transgene expression, we set up a method for detection of a secreted, luminescent reporter protein in the rat brain by repeated cerebral spinal fluid (CSF) sampling from rat cisterna magna (Figure S1A). Intrastriatal injections of increasing titers of an adeno-associated virus serotype 1 (AAV1) vector encoding the constitutively secreted *Gaussia* Luciferase (GLuc) led to corresponding increases in rat CSF GLuc activity, thereby establishing a method to longitudinally monitor transgene expression in the brain (Figure S1B).

A group of rats received bilateral injections of the AAV1-CMV-GLuc vector and, following collection of three baseline CSF samples, rats were injected with four subcutaneous (s.c.) injections of saline or Meth 2.5 mg/kg given with 2-h intervals (Figure 1A) following a neurotoxic model of Meth exposure.[11]. Meth-injected rats had a robust 5.4-fold increase in GLuc activity in their CSF at 8 h after the first Meth injection (Figure 1B). A subset of rats were sampled 16 h and 40 h later, indicating that the levels of GLuc protein had returned to baseline levels within the first two days after the Meth challenge (Figure 1B). Using qPCR, we detected significantly higher levels of GLuc mRNA in the striatum of Meth-treated rats at 8 h after the first Meth injection compared to vehicle control group (Figure 1C). The observed increase in mRNA significantly correlated to the increase in CSF GLuc activity (Figure S1C), indicating that Meth altered expression of the transgene. Meth and saline groups had similar levels of transgene DNA in their striatum at the end of the study (Figure S1D) and similar CSF GLuc activity at baseline (Figure S1E), excluding basal inter-group variations as possible explanation for the observed differences in GLuc protein and mRNA from Meth and saline groups.

The observed effect of Meth on transgene expression could be due to inherent characteristics of a Meth-induced stimulatory effect on overall transcriptional activity. Therefore, we constructed AAV1 vectors with GLuc expression under the control of the human eukaryotic translation elongation factor 1α (EF1α) or synapsin 1 (Syn1) promoter and injected them bilaterally into the rat striatum. After verification of similar baseline GLuc CSF levels between treatment groups (Figure S1F-G), rats were injected with saline or Meth. In contrast to the AAV1-CMV-GLuc vector, Meth did not affect the GLuc protein levels in CSF nor GLuc mRNA levels in the striatum for vectors with the Syn1 (Figure 1D-E) or EF1α promoter

(Figure 1F-G), indicating that the Meth effect on the AAV1-CMV-GLuc vector is specific to the vector promoter. A lack of Meth-induced increase in GLuc activity in the CSF of rats injected with AAV1-Syn1-GLuc or AAV1-EF1α-GLuc also excluded a drug-related increase in secretion as the cause of increased extracellular GLuc activity in the AAV1-CMV-GLuc-injected rats.

In addition to the CMV-IE promoter, the promoter/enhancer region in our AAV1-CMV-GLuc vector contains a chimeric intron with sequences from the first intron of the human β-globin gene and the intron of an immunoglobulin gene heavy chain variable region (see Table S1 for promoter sequence). We will hereby refer to this promoter as CMV', and the vector as AAV1-CMV'-GLuc. To test if the intron proportion of the CMV' promoter contributed to the Meth-induced increase in transgene expression, rats were intrastriatally injected with AAV1 vectors carrying the GFP transgene under the control of the CMV' promoter or the CMV-IE promoter (CMV). Four s.c. injections of 2.5 mg/kg Meth (two hours apart) resulted in similar approximately 5- to 7-fold increases in striatal levels of GFP mRNA at four weeks post-transduction (Figure 2A), supporting that the CMV-IE portion of the promoter/enhancer is responsible for the Meth-induced upregulation of transgene expression.

Amphetamines have long been used in pre-clinical Parkinson's disease research to evaluate the function of the nigrostriatal dopaminergic pathway and the therapeutic potential of experimental treatments, including overexpression of therapeutic proteins.[12,13] To test if a protocol widely used to detect amphetamine-induced asymmetric behavior in rodent models of Parkinson's disease will alter transgene expression, we injected unilaterally 6-hydroxydopamine-lesioned rats overexpressing GFP under the CMV' promoter in the striatum[14] with a single s.c. dose of Meth (2.5 mg/kg). Even after a single Meth injection 34

weeks after striatal transduction with AAV-CMV'-GFP, we detected a significant effect of Meth on GFP mRNA levels in the lesioned tissue (two-way ANOVA, Meth versus no Meth (0 h time point in Figure 2B) between effect, $F_{3,16} = 14.8$, $p < 0.001$). In addition, we detected a time-dependent increase in GFP mRNA levels in the striatum, with an approximate maximum 5.6-fold increase four hours post-injection that returned to baseline levels within eight hours (Figure 2B).

Activation of excitatory pathways induces CMV-dependent transgene expression in primary cortical neurons

Meth has been reported to alter the activation and binding of several transcription factors, including AP-1,[15,16] CREB,[16,17] and NF-κB.[18] These transcription factors are also important in the activation of CMV-driven expression,[5] and an increase in their activity could be driving the observed Meth-induced increase in transgene expression. Thus, we hypothesized that other substances known to indirectly or directly modify the activity of these transcription factors could also alter transgene expression under the control of the CMV promoter. Indeed, a 24-h treatment with 100 μM kainic acid or glutamate[19,20] increased both secreted (Figure 3A) and intracellular (Figure 3D) GLuc activity in rat primary cortical neurons transduced with AAV1-CMV'-GLuc. On the contrary, application of excitatory amino acids to cells transduced with AAV1-Syn1-GLuc or AAV1- EF1α-GLuc caused a significant decrease in extracellular (Figure 3B, C) and intracellular (Figure 3E, F) GLuc levels, likely due to treatment-induced cell toxicity (Figure 3G).

Stimulation of neuronal activity by the introduction of designer receptors or channels that can be activated chemically or optically has been extensively exploited over the past few years to dissect neuronal pathways in mammals. Considering the effects of excitatory amino acids on

CMV-driven transcription, we introduced Cre-dependent expression of the DREADDs (designer receptors exclusively activated by designer drugs) hM3D (Gq) or hM4D (Gi)[21] into rat primary cortical neurons together with our CMV'-GLuc reporter. A 24-h incubation in the DREADD ligand, clozapine N-oxide (CNO; 0.03-3 μM), resulted in a significant dose-dependent increase in both secreted GLuc protein (Figure 4A, left side) and GLuc mRNA (Figure 4B, left side) in cells transduced with hM3D, indicating that Gq-activating signaling pathways or compounds can affect CMV-driven transgene expression. In cells overexpressing hM4D, the transgene expression was unchanged following exposure to CNO (Figure 4A-B, right sides). The overall expression of hM3D (Figure S2A) and hM4D (Figure S2B) was comparable in the different treatment groups. Again, expression of GLuc under the control of the Syn1 (Figure S2C-D) or EF1α promoter (Figure S2E-F) did not change in response to CNO treatment in cells overexpressing hM3D or hM4D, although we did detect a small, but significant, increase in Syn1-GLuc mRNA levels following activation of hM3D receptors with 3 μM CNO (Figure S2D), indicating that an activation of Gq-dependent pathways may also have an effect on Syn1 promoter activity. However, this small increase was not reflected at the protein level (Figure S2C).

The observations that the CMV promoter can be induced by a variety of neuronal stimulants creates a new caveat when selecting promoters for neuroscience applications. This knowledge also provides opportunity to exploit this property to provide transgene expression in response to neuronal stimulation. Previous work has shown that expression of an anti-Meth antibody can reduce Meth exposure in the brain and serum.[22] Based on our observations that Meth induced CMV promoter, we tested whether Meth could induce expression of MethAb when put under control of the CMV promoter. HEK293 cells were transfected with pAAV-MethAb, in which the MethAb gene was expressed by the CMV promoter. At two hours post-

transfection, cells were treated with Meth (100 μM) for 20 hours, and, after that, the MethAb mRNA levels in the cell lysates were examined by qRT-PCR analysis (Figure 5). A significant difference in MethAb mRNA production was found after Meth treatment (ß-actin as the reference gene: $F_{2,15} = 71.65$, $p < 0.0001$, one-way ANOVA; GAPDH as the reference gene: $F_{2,15} = 78.89$, $p < 0.0001$, one-way ANOVA). Post hoc Dunnett's comparison test indicated that treatment with 100 μM Meth significantly enhanced MethAb mRNA production (ß-actin as the reference gene: 2.59 ± 0.217 vs 1 ± 0.16, $p < 0.0001$; GAPDH as the reference gene: 2.35 ± 0.18 vs 1 ± 0.15, $p < 0.0001$), as compared to the control group (pAAVMeth + 0 μM Meth). These data show that Meth induced expression of the MethAb transgene driven by the CMV promoter.

Discussion

Our results suggest that a general increase in neuronal activity, whether through excess of excitatory amino acids, activation of G protein-coupled signaling, or pharmacological stimulation of dopaminergic signaling, can have a strong activating effect on the CMV promoter. Although we cannot exclude that some of the observed activation of the CMV promoter may be a secondary outcome of neuronal activation with downstream neurotoxic pathways serving as the primary activator of transcription, we do show that CMV activation does not generalize to other commonly used transgene promoters. Given these findings, caution should be applied when using the CMV promoter to drive expression of effectors of neuronal activation. Our results identify a new caveat for designing transgenic studies and suggest a need to re-evaluate results generated in studies with concomitant use of CMV-driven transgene expression and stimulatory treatments. These studies range from research on addiction and effects of stimulatory substances on neurotransmission and neurotoxicity, to studies involving, e.g., kainic acid-induced seizures or using Meth as a tool to manipulate

dopaminergic signaling in animal models. Our observation that a single dose of Meth can induce a transient increase in transgene expression in the rat brain when administered eight months after transduction suggests a potential confounding effect on treatment outcome and emphasizes the need for evaluating transgene expression levels proximal to manipulations done months after transgene delivery. Although the effect on transcription was transient, the resulting change in transgene-mediated protein expression will depend on the magnitude of gene expression induction as well as the half-life and biology of the protein (e.g. neurotrophic factors, such as glial cell line-derived neurotrophic factor, GDNF, have long-lasting effects following a single intracranial bolus injection[23]). Most notably, several clinical trials for human gene therapy of CNS disease have used AAV vectors with CMV promoters,[3,4] the use of which may have altered the expected outcomes based on the preclinical model data where amphetamines are often used to probe behavioral phenotypes at various times following gene delivery.

Conversely, our findings could also be exploited for experimental or therapeutic purposes. For example, amphetamine-derivatives approved for clinical use could be used to increase the expression of transgenes in the brain, thereby decreasing the virus titers needed for efficient transgene expression. Our results suggest that the activation of transgene expression is transient and a cessation of drug delivery would quickly bring down the levels of exogenous protein production. We previously demonstrated that systematic administration of virus AAV-MethAb induced persistent MethAb expression in the periphery, lowered the plasma Meth level, and attenuated Meth-induced hyperactivity and stereotyped behavior in adult mice.[22] In the current study, we found that Meth could stimulate the production of MethAb mRNA through a CMV promoter, which was likely contributing to our positive findings in our previously published work. Here, we used non-dopaminergic cells, HEK293 cells to show

altered CMV transcription by Meth. Non-dopaminergic response of Meth has been previously reported in HEK293 cells. Meth alters Ca^{2+}-activated potassium channels (KCa1.1, BK) in HEK293 cells expressing GFP-α subunits of the BK channel; this effect was blocked by protein kinase C inhibitor BIM-I.[24] The new data presented herein further support the use of CMV promoter-driven MethAb expression as a therapeutic intervention for the treatment of Meth dependence and intoxication.

In addition to the implications of our findings for CMV-mediated transgene expression, our data provide additional context for studies of the human CMV. For example, CMV shedding in HIV-infected Meth users on antiretroviral therapy suggest Meth induces reactivation of latent CMV infection.[25] Although this could be an indirect result of Meth's effects on the immune system with loss of control of the latent virus,[25] based on our results it is reasonable to hypothesize that Meth impacts the transcription of CMV-IE genes. Importantly, increased shedding of CMV in HIV-patients has been linked to an increase in T cell activation and proliferation, as well as higher levels of HIV DNA in blood cells,[26] possibly affecting HIV outcome and morbidity.

Lastly, in addition to the models used in the current study, our results suggest that other stimulatory manipulations may act on the CMV promoter. We therefore strongly recommend researchers working with CMV-related promoters to assess the transgene expression in their model systems. Although we focused solely on the effects in central nervous system, we also predict CMV-driven transgene expression will have activating effects in peripheral tissue in response to events previously reported to increase the expression of CMV-IE genes, such as inflammation and treatments affecting intracellular signaling pathways involved in transcription factor activation.[6-9]

Material and methods

Viral vectors

The AAV vectors were produced using serotype 1 capsid proteins as previously described[27] and titered by droplet digital PCR. The following plasmids were used for AAV vector production (promoter sequences described in Table S1; plasmids available upon request from corresponding authors):

pAAV CMV' GLuc: The vector has been previously described.[28] The promoter region is similar to what is used in the pCI-Neo mammalian expression vector (# E1841 Promega, Madison, WI).

pAAV CMV' GFP and pAAV CMV GFP: The pAAV EF1a DIO iRFP (Addgene #47626) was edited to replace the region beginning at the trs element in the left ITR and ending with the human growth hormone polyadenylation element with a GFP expression cassette driven by the CMV' promoter/enhancer (from AAV CMV' GLuc) to create pscAAV CMV' eGFP bGHpA (pOTTC552). The CMV' promoter was then replaced with the CMV promoter amplified from pEGFP-C1 (Clontech) to produce pscAAV CMV eGFP (pOTTC730).

pAAV EF1α GLuc and pAAV Syn1 GLuc: The GLuc coding region was amplified from pLenti6.3 CMV Manf-sigpep-GLuc-MCS (pOTTC7001) and used to replace the fluorescent protein coding region in pAAV EF1α iRFP-FLAG (pOTTC1464) and pAAV SYN1 Nuc-EGFP-Myc (pOTTC1532) to produce pAAV EF1α GLuc (pOTTC1544) and pAAV Syn1 GLuc (pOTTC1547), respectively.

pAAV hSyn DIO hM3D(Gq)-mCherry (Addgene #44361) and pAAV hSyn DIO hM4D(Gi)-mCherry (Addgene #44362) were a gift from Bryan Roth (UNC School of Medicine, Chapel Hill, NC).

pAAV1 EF1a iCre: The improved Cre coding region[29] was amplified from pFos iCre (pOTTC161) and used to replace the lox sites and fluorescent protein coding region in pAAV EF1a DIO iRFP (Addgene #60057) to produce pAAV1 EF1a iCre (Addgene #89760).

Animals and animal procedures

All animal protocols were reviewed and approved by the Animal Care and Use Committee (ACUC) at the NIDA IRP using the National Institutes of Health guidelines, or by the national Animal Experiment Board of Finland. The animals were group-housed in a 12-h light/dark cycle, with ad lib excess to rodent chow and water.

Adult male Long-Evans rats (250-290 g, Charles River Laboratories, Wilmington, MA) received intracranial AAV1 injections into the striatum in a stereotaxic surgery under isoflurane anesthesia. Using a 10-µl Nanofil syringe with a 33-gauge needle coupled to a UMP4 microinjector pump (World Precision Instruments, Sarasota, FL), 2 µl of $0.5x10^{12}$ vg/ml were injected 0.0 mm anterior, 3.0 mm lateral, and 5.0 mm ventral from bregma with the injection speed 0.5 µl/min, leaving the needle in place 2 min before retraction. Four weeks after the intracranial AAV injections, rats received four subcutaneous injections of Meth 2.5 mg/kg ((+)-Methamphetamine hydrochloride, Sigma-Aldrich, St Louis, MO) or saline given with 2-h intervals, adding to a total Meth dose of 10 mg/kg.

For the initial virus titer study, ten rats were injected bilaterally with 2 µl AAV1-CMV'-GLuc $0.5x10^{10}$, $0.5x10^{11}$, or $0.5x10^{12}$ vg/ml.

Wistar rats (Harlan/Envigo, Horst, The Netherlands) injected intrastriatally with 6-hydroxydopamine and AAV1-CMV'-GFP have been previously described.[14] Briefly, three weeks following intrastriatal delivery of AAV1-CMV'-GFP, the nigrostriatal pathway was partially lesioned with 3 x 2 µg 6-hydroxydopamine.[14] To study whether the effect of CMV induction can still be seen after long-term transduction, we let the expression continue for several months. A single s.c. injection of Meth 2.5 mg/kg was administered to 10.5-months old rats, i.e. 31 weeks post-lesion and 34 weeks post-AAV.

Repeated collection of CSF was done from rat cisterna magna under isoflurane anesthesia (method adapted from[30]). The rat head was fixed in a stereotaxic apparatus, with the head bent down in an approximately 45-degree angle (Figure S1A). Using a 23-gauge needle attached to a 1-ml syringe with PE-50 tubing (Scientific Commodities Inc., Lake Havasu City, AZ), 50 µl CSF was withdrawn and stored in -80°C until analysis.

Cell culture

Rat primary cortical neurons were prepared from Sprague-Dawley embryos as previously described[31] and in accordance with approved procedures by the National Institutes of Health Animal Care and Use Committee. Isolated cells were plated at $6x10^4$ cells/well in 96-well polyethyleneimine-coated plates. Fifty percent media exchanges were performed on day in vitro (DIV) 4, 6, 8, 11, and 13. On DIV6 transductions were performed with the following viruses: AAV1-CMV'-GLuc ($5.8x10^9$ vg/ml), AAV1-Syn1-GLuc ($5.8x10^9$ vg/ml), AAV1-EF1α-GLuc ($5.8x10^9$ vg/ml), AAV1-EF1α-iCre ($1.0x10^{11}$ vg/ml), AAV1-Syn1-Dio-

hM3D(Gq)-mCherry ($1.0x10^{11}$ vg/ml), AAV1-Syn1-DIO-hM4D(Gi)-mCherry ($1.0x10^{11}$ vg/ml). On DIV13, cells were treated with kainic acid (100 μM, Cayman Chemical Company, Ann Arbor, MI), glutamate (100 μM, Sigma Aldrich), or clozapine N-oxide (CNO, 0.03, 0.3, 3 μM, Enzo Life Sciences, Farmingdale, NY) via 50% media exchange. Extracellular media samples were collected 24 h post-treatment. Subsequently, a subset of cells were rinsed twice with phosphate buffered saline and incubated with lysis buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% NP-40, and protease inhibitors (Sigma Aldrich)) for 20 minutes at 4°C. Cell viability was assessed 24 h post-treatment via CellTiter96 AQueous One Solution Cell Proliferation Assay (MTS assay, Promega).

Detection of *Gaussia* Luciferase

For *in vivo* studies, CSF (5 μl) was transferred to white 96-well plates (Corning, Corning, NY) as technical triplicates. Using Biotek Synergy II plate reader (Winooski, VT), the luminescence was detected following injection of 100 μl of 100 μM coelenterazine (Regis Technologies, Morton Grove, IL). The plate reader parameters were set to an integration time of 5 s and a sensitivity of 100. For determination of GLuc activity *in vitro*, luminescence was detected in 5 μl extracellular media or cell lysate following injection of 8 μM coelenterazine.

Real-time quantitative reverse transcription PCR and droplet-digital PCR

For quantitative PCR, anesthetized rats were decapitated eight hours after the first Meth injection and their brains snap-frozen in isopentane. The rat striata were dissected in a freezing microtome and processed using Qiagen RNeasy Lipid Tissue Mini Kit (Germantown, MD). After the initial lysis in Qiazol buffer, the aqueous fraction was used for RNA isolation according to the supplied protocol, including an on-column DNA digestion (RNase-free DNase set, Qiagen). Collection of RNA from cell culture was performed using a Nucleospin

RNA mini kit (Takara, Kusatsu, Japan). The RNA concentration was determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and 0.2 μg (cell culture) or 0.5 μg (rat tissue) RNA underwent reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Five microliters of 1:20 dilution of cDNA was applied in technical duplicates to white PCR plates (Bio-Rad Laboratories) and incubated in a mix of appropriate primers and hydrolysis probes (450 nM and 100 nM, respectively) and Universal TaqMan Master Mix (Thermo Fisher Scientific) for a final volume of 20 μl. Target sequences, which included GLuc, GFP,RNA polymerase II (PRNAII), and ubiquitin-conjugating enzyme E2I (Ube2i), were amplified using BioRad CFX96 with 50 cycles of 20 s at 94°C and 1 min at 60°C. The transgene cycle threshold (Ct) values were estimated with the Bio-Rad CFX Manager software using single threshold mode, and normalized to the geometric mean of the Ct values for the reference genes. Fold change in gene expression was calculated using $2^{-\Delta\Delta Ct}$ and the results are expressed as fold change with upper and lower limits determined using the standard deviation (SD) for ΔCt. The following sequences were used for target amplification and detection: GLuc, cacgcccaagatgaagaagt (forward), gaacccaggaatctcaggaatg (reverse), tacgaaggcgacaaagagtccgc (FAM/BHQ-labelled probe); eGFP, agcaaagacccccaacgagaa (forward), ggcggcggtcacgaact (reverse), cgcgatcacatggtcctgctgg (FAM/BHQ-labelled probe); hM3Dq, tctggcaagtggtcttcatc (forward), ttaccaggatgttgccgatg (reverse), ctttcttaacgggcatcctggcc (FAM/BHQ-labelled probe); hM4Di, catcccacaatcgctatgag (forward), acagtcaccaggctcagg (reverse), cctgtcactgtggcaatgaagacc (FAM/BHQ-labelled probe); PRNAII, tagtcctacctactccccaacttc (forward), agtagccaggagaagtgggag (reverse), actcgcccaccagtcccacctact (HEX/BHQ-labelled probe); Ube2i, gccaccactgtttcatccaaa (forward), gccgccagtccttgtcttc (reverse), cgtgtatccttctggcacagtgtgc (HEX/BHQ-labelled probe).

For isolation of DNA, the interphase and organic phase from the initial lysis with Qiazol were used. DNA was precipitated with 100% ethanol, and the DNA pellet washed three times in 0.1 M sodium citrate in 10% ethanol. After a final wash in 75% ethanol, the DNA pellet was air-dried and then resuspended in 8 mM NaOH, with subsequent adjustment of pH with 100 mM HEPES and 100 mM EDTA. After determination of DNA concentration, the samples were diluted to 1000 rat genomes / 5 µl (635.7 pg DNA /µl). The DNA was applied as technical triplicates to a semi-skirted 96-well PCR plate (Eppendorf AG, Hamburg, Germany) along with ddPCR Supermix for Probes (Bio-Rad Laboratories) and GLuc and Ggt1 primers (450 nM) and hydrolysis probes (100 nM) in a 25 µl reaction mix. After droplet generation (Automated Droplet Generator, Bio-Rad Laboratories), the target genes were amplified using 40 cycles of 20 s at 94°C and 1 min at 60°C (T100 Thermal Cycler, Bio-Rad Laboratories). The number of positive and negative droplets was estimated with the QX200 Droplet Reader and QuantaSoft software (Bio-Rad Laboratories) using absolute quantification mode. The oligonucleotides used for Ggt1 amplification and quantification were ccacccctttccctactcctac (forward), ggccacagagctggttgtc (reverse), ccgagaagcagccacagccatacct (HEX/IBFQ-labelled probe).

MethAb expression

The expression plasmid pAAV-MethAb, encoding both heavy- and light-chains of the Meth-specific monoclonal antibody (MethAb), was constructed as described in a previous study (Figure 5).[22] HEK293 cells were seeded (5×10^4 cells/cm^2) in 24-well plates and grown as monolayers in the growth medium (DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin) overnight at 37°C in a humidified incubator with 5% CO_2. Cells were then transfected with pAAV-MethAb (0.5 µg/well) using jetPEITM transfection reagent (Cat. No. 101-10N, Polyplus-transfection, France) as directed by the

manufacturer. Two hours later, cells were incubated with fresh growth medium supplemented without or with Meth (100 μM) for 20 hours. After that, the total RNA was purified by Trizol reagents (#1559608, Invitrogen) and then subjected to reverse transcription for cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (#K1622, Thermo Fisher Scientific). The synthesized cDNA were used as templates in the quantitative polymerase chain reaction (qPCR) analysis, performed on the ABI StepOnePlus system. Briefly, 2 μl of diluted cDNA, 10 μl of 2× SYBR green PCR master mix (#K0371, Thermo Scientific), and 2 μl of each primer (5 μM) were mixed in distilled water to a final volume of 20 μl. The PCR cycling program was set as follows: 50˚C for 1 minute and 95˚C for 10 minutes, followed by 40 cycles of denaturation at 95˚C for 15 seconds and annealing/extension at 60˚C for 1 minute. The primers used for the qPCR were listed as the following: MethAb, 5'-GCCTCGTGAAACCTTCTCAG-3' (forward), 5'-CCGAATCCAGCTCCAGTAAC -3' (reverse); beta-actin, 5'-CATTGCTGACAGGATGCAGAAGG-3' (forward), 5'-TGCTGGAAGGTGGACAGTGAGG-3' (reverse); GADPH, 5'-CATCACTGCCACCCAGAAGACTG-3' (forward), 5'-ATGCCAGTGAGCTTCCCGTTCAG-3' (reverse). The mRNA levels of MethAb were normalized to that of beta-actin or GAPDH and presented as fold changes relative to control (pAAV-MethAb + 0 μM Meth).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 or IBM SPSS Statistics. Test details can be found in the main text or corresponding figure legend. Statistical significance was considered when the p-value was < 0.05.

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Author contributions

Conceptualization: S.B. and B.K.H.; formal analysis: S.B., A.D., M.A., and B.K.H.; investigation: S.B., A.D., I.P., Y.W. and P.K.; methodology: S.B., A.D., M.A., Y.H.C., Y.W., C.T.R., and B.K.H.; writing – original draft: S.B.; writing – editing and review: A.D., I.P., M.A., Y.W., C.T.R., and B.K.H.

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Figure legends

Figure 1. Subcutaneous methamphetamine activates the CMV promoter in rat brain. See also Figure S1. (A) Timeline for studying the effect of methamphetamine (Meth) on transgene expression and secretion. (B) Fluctuations in CSF GLuc activity following administration of Meth (2.5 mg/kg x 4) in rats with striatal overexpression of CMV-GLuc (n = 3-4). ****$p<0.0001$ compared to saline (treatment effect $F_{1,6} = 17.23$, $p = 0.0060$ with two-way ANOVA, and Sidak's test), and to all other time points within the group (one-way ANOVA

$F_{5,20}$ = 30.45, p < 0.0001, and Tukey's test). (C) CMV-GLuc expression levels determined with real-time RT-qPCR in the striatum 8 h after the first Meth/saline injection (n = 3-4, p = 0.0292, t(5) = 3.028 with unpaired t-test of ΔCt values). (D-G) Measurements of (D, F) CSF GLuc activity and (E, G) striatal GLuc mRNA in rats intrastriatally transduced with (D, E) AAV1-Syn1-GLuc or (F, G) AAV1-EF1α-GLuc. (D, F) Two-way ANOVA treatment effect, (D) n = 4, $F_{1, 6}$ = 0.0004065, p = 0.9846; (F) n = 3, $F_{1, 4}$ = 0.02145, p = 0.8906; (E, G) unpaired t-test, (E) n = 4, t(6) = 0.8211, p = 0.4430; (G) n = 4, t(6) = 0.9038, p = 0.4009. Results from GLuc activity are shown as mean ± SD. Expression results are shown as $2^{-\Delta\Delta Ct}$ ± upper and lower limits with individual ΔΔCt values plotted.

Figure 2. CMV-driven expression of striatal GFP reporter increases following multiple or single doses of methamphetamine. (A) Transgene mRNA levels in the striatum of rats overexpressing GFP under the CMV' or CMV promoter following s.c. injections of saline or methamphetamine (Meth 2.5 mg/kg x 4) (n = 4, *p<0.05, ***p<0.001, unpaired t-test of ΔCt values; CMV': t(6) = 8.744, p = 0.0001; CMV: t(6) = 3.538, p = 0.0122). (B) Transgene expression levels in the striatum of GFP-transduced, 6-OHDA-lesioned rats 0, 2, 4, and 8 h after a single injection of 2.5 mg/kg Meth (n = 4-5, one-way ANOVA, $F_{3, 13}$ = 2.608, p = 0.0960; unpaired t-test 0 h vs 4 h, t(7) = 2.357, p = 0.0505). Results are presented as s $2^{-\Delta\Delta Ct}$ ± upper and lower limits with individual ΔΔCt values plotted.

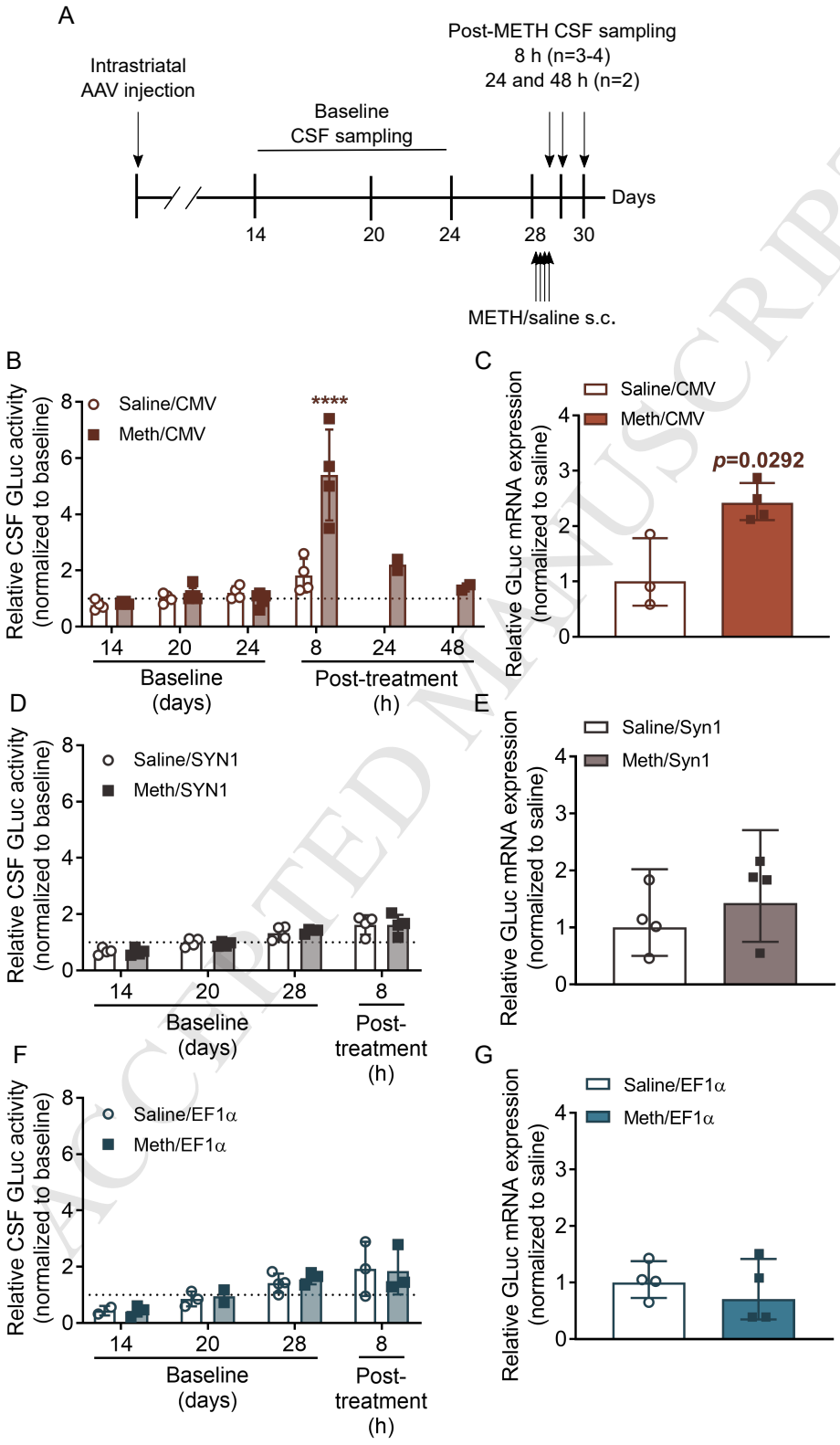
Figure 3. Excitatory amino acids activate the CMV promoter *in vitro*. (A-G) Primary cortical neurons transduced with AAV1 vectors coding for GLuc under the CMV, Syn1, or EF1α promoter were treated with 100 μM kainic acid (KA) or glutamate (Glut) for 24 h, with subsequent analysis of (A-C) extracellular and (D-F) intracellular GLuc activity, and (G) cell viability (MTS assay). n = 5-10, *p<0.05, ****p<0.0001 with one-way ANOVA; (A) $F_{2, 18}$ =

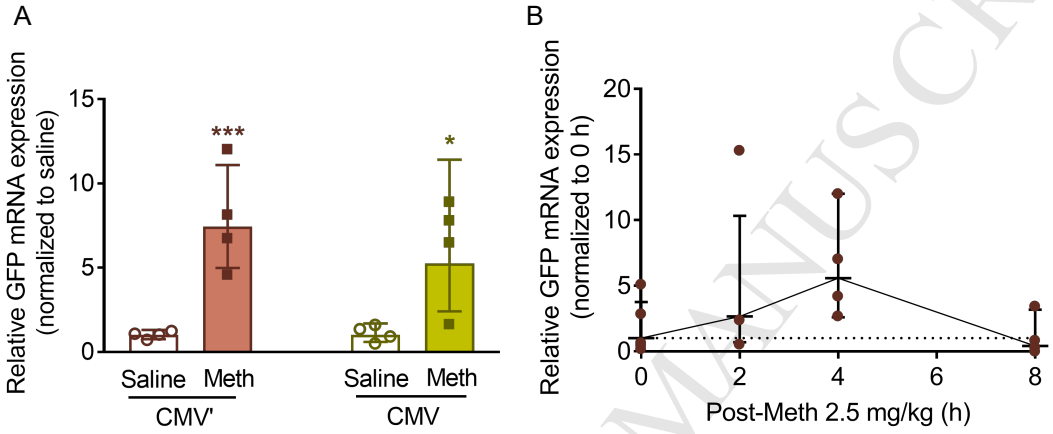
118.5, $p < 0.0001$; (B) $F_{2, 19} = 56.8$, $p < 0.0001$; (C) $F_{2, 20} = 5.381$, $p = 0.0135$; (D) $F_{2, 18} = 1083$, $p < 0.0001$; (E) $F_{2,20} = 121.8$, $p < 0.0001$; (F) $F_{2,20} = 50.79$, $p < 0.0001$; (G) $F_{2,21} = 54.12$, $p < 0.0001$, followed by Dunnett's multiple comparison test. Results are shown as mean ± SD.

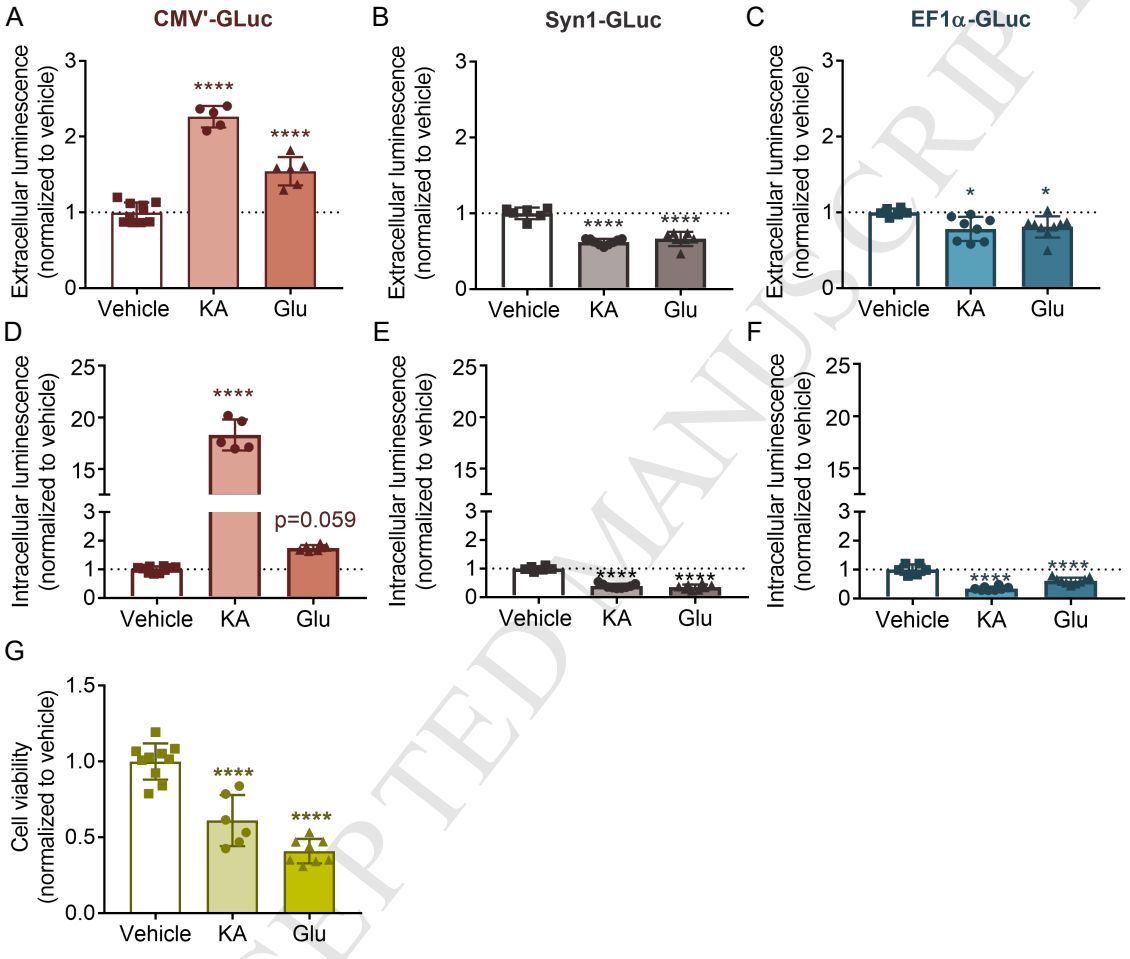
Figure 4. Induction of CMV-driven transgene expression following activation of Gq signaling. See also Figure S2. (A, B) Primary cortical neurons overexpressing hM3D (Gq) or hM4D (Gi) in combination with GLuc under the CMV' promoter were treated with CNO (0-3 μM) for 24 h, with subsequent analysis of (A) extracellular GLuc activity or (B) GLuc mRNA levels. Results are shown as (A) mean ± SD or (B) $2^{-\Delta\Delta Ct}$ ± upper and lower limits. (A, B) ***p<0.001 and ****p<0.0001 vs 0 μM CNO, ## p<0.01, ###p<0.001, ####p<0.0001 vs 0.03 μM CNO, one-way ANOVA [(A) n=3, hM3D: $F_{3,8} = 82.25$, $p<0.0001$; hM4D: $F_{3,8} = 0.952$, $p = 0.4604$; (B) n = 9, hM3D: $F_{3,32} = 63.95$, $p<0.0001$; hM4D: $F_{3,31} = 3.116$, $p = 0.0402$] and Dunnett's test.

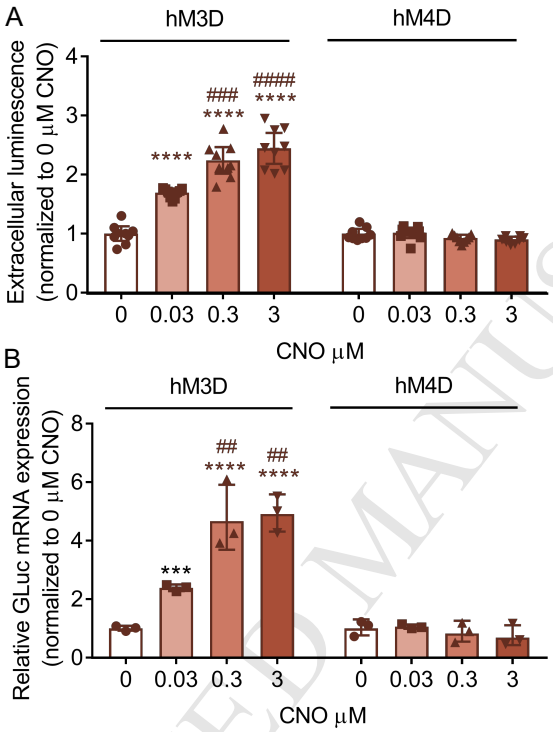
Figure 5. Methamphetamine enhances CMV-driven expression of MethAb. (A) Construct for methamphetamine (Meth)-specific monoclonal antibody (MethAb) expression. The heavy- and light-chain gene sequences of MethAb were cloned in the expression plasmid pAAV-MethAb, and their expression was driven by the cytomegalovirus promoter (CMV). Linker-2A = a sequence composed of a furin cleavage site (RKRR), a V5 epitope, a spacer peptide (SGSG), and a 2A self-cleavage sequence; SP = secretion signal peptide; WPRE = woodchuck hepatitis B virus post-transcriptional regulatory element; 6xHis = 6 x histidine tag; red line: the target region of the quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. (B, C) Gene expression of MethAb. HEK293 cells were transfected with or without pAAV-MethAb, and two hours later, cells were treated with or

without Meth 100 μM for 20 hours. The mRNA levels of MethAb were determined by qRT-PCR analysis and normalized to ß-actin (B) or GAPDH (C). The relative mRNA levels of MethAb are presented as folds of control (pAAV-MethAb + 0 μM Meth). **** $p < 0.0001$; one-way ANOVA followed by Dunnett's comparison test, data presented as mean ± standard error (SEM).

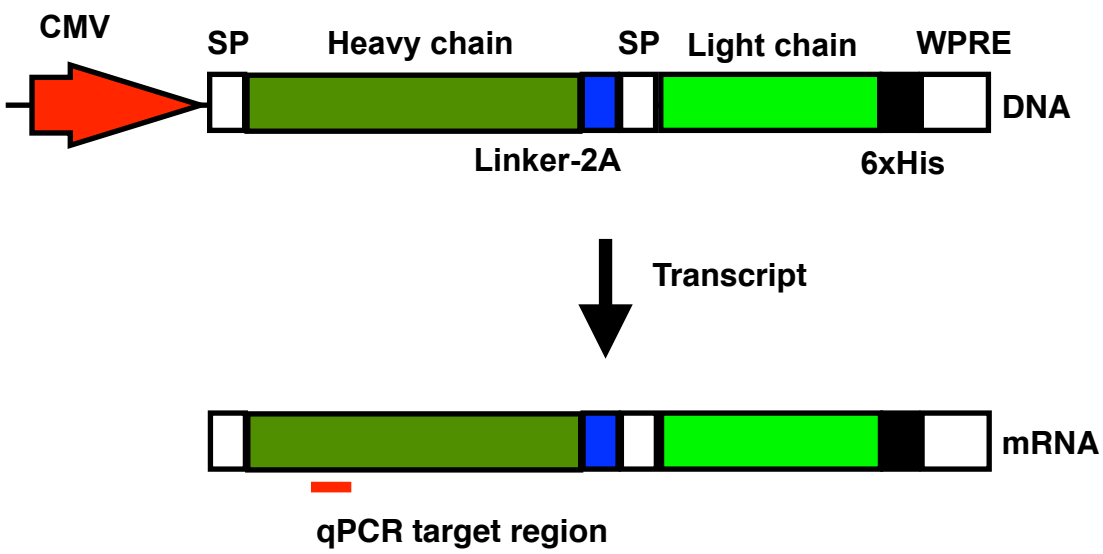

A
Intrastriatal
AAV injection
Baseline
CSF sampling
Post-METH CSF sampling
8 h (n=3-4)
24 and 48 h (n=2)
Days
14
20
24
28
30
METH/saline s.c.
B
Relative CSF GLuc activity
(normalized to baseline)
Saline/CMV
Meth/CMV
****
14
20
24
8
24
48
Baseline
(days)
Post-treatment
(h)
C
Relative GLuc mRNA expression
(normalized to saline)
Saline/CMV
Meth/CMV
p=0.0292
D
Relative CSF GLuc activity
(normalized to baseline)
Saline/SYN1
Meth/SYN1
14
20
28
8
Baseline
(days)
Post-
treatment
(h)
E
Relative GLuc mRNA expression
(normalized to saline)
Saline/Syn1
Meth/Syn1
F
Relative CSF GLuc activity
(normalized to baseline)
Saline/EF1α
Meth/EF1α
14
20
28
8
Baseline
(days)
Post-
treatment
(h)
G
Relative GLuc mRNA expression
(normalized to saline)
Saline/EF1α
Meth/EF1α



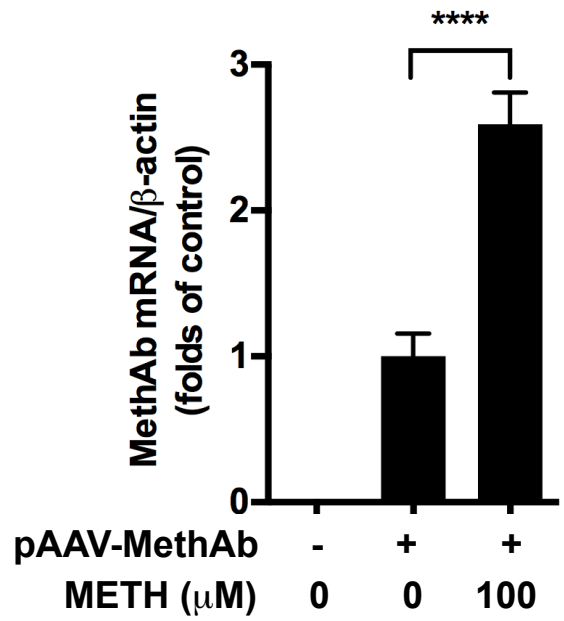




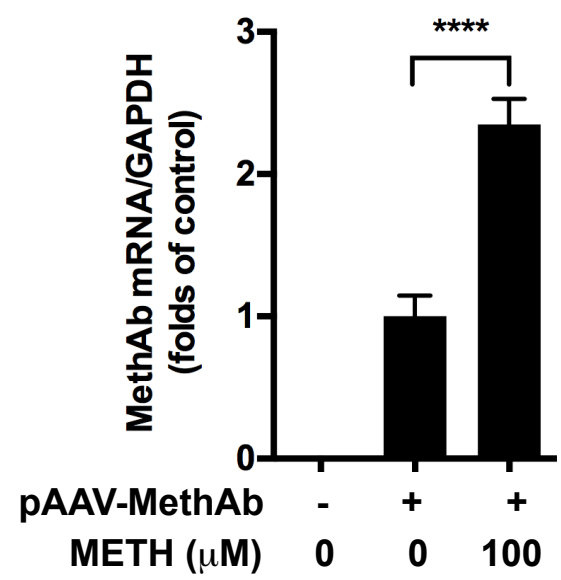
A


CMV
SP
Heavy chain
SP
Light chain
WPRE
DNA
Linker-2A
6xHis
Transcript
mRNA
qPCR target region

B


****
MethAb mRNA/β-actin
(folds of control)
3
2
1
0
pAAV-MethAb - + +
METH (μM) 0 0 100

C


****
MethAb mRNA/GAPDH
(folds of control)
3
2
1
0
pAAV-MethAb - + +
METH (μM) 0 0 100