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Increased activity of cyclin-dependent kinase 5 leads to attenuation of cocaine-mediated dopamine signaling

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Abbreviations: Cdk5, cyclin-dependent kinase 5; DARPP-32, dopamine and cAMP-regulated phosphoprotein, molecular mass 32 kDa; PKA, cAMP-dependent kinase; MEK, extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; CREB, cAMP-response element-binding protein

Abstract

Cocaine, a drug of abuse, increases synaptic dopamine levels in the striatum by blocking dopamine re-uptake at axon terminals. Cyclin-dependent kinase 5 (Cdk5) and its activator p35, proteins involved in phosphorylation of substrates in postmitotic neurons, have been found to be upregulated following chronic exposure to cocaine. To further examine the effects of Cdk5 and p35 induction on striatal dopamine signaling, we generated two independent transgenic mouse lines in which Cdk5 or p35 was overexpressed specifically in neurons. We report here that increased Cdk5 activity, as a result of p35 but not of Cdk5 overexpression, leads to attenuation of cocaine-mediated dopamine signaling. Increased Cdk5-mediated phosphorylation of DARPP-32 (dopamine and cAMP-regulated phosphoprotein, molecular mass 32 kDa) at Thr 75, was accompanied by decreased phosphorylation of DARPP-32 at Thr 34. Increased Cdk5-mediated phosphorylation of extracellular signal-regulated kinase kinase 1 (MEK1) at Thr 286 was accompanied by decreased activation of extracellular signal-regulated kinase 1/2 (ERK1/2). These effects contributed to attenuation of cocaine-induced phosphorylation of cAMP-response element-binding protein (CREB) as well as to a lesser induction of c-fos in the striatum. These results support the idea that Cdk5 activity is involved in altered gene expression following chronic exposure to cocaine, and hence impacts the long-lasting changes in neuronal function underlying cocaine addiction.

Introduction

Cocaine increases synaptic dopamine levels in the striatum and alters gene expression in the dopaminoceptive neurons by activating intracellular pathways that propagate the initial signal from the dopamine D1 receptor to the nucleus (1). Chronic exposure to cocaine upregulates several transcription factors, resulting in the long-lasting changes in gene expression that are thought to underlie neuronal adaptations in cocaine addiction (2). ΔFosB, identified as such a transcription factor (3), has been shown to enhance the behavioral responsiveness of animals to cocaine (4, 5). Therefore, identification of the target genes that are regulated by ΔFosB induction is expected to contribute to a greater understanding of the molecular mechanism underlying cocaine addiction. Recently, chronic treatment of animals with cocaine has been shown to upregulate the expression of cyclin-dependent kinase 5 (Cdk5) and its activator p35 in the striatum through the induction of ΔFosB (6, 7).

Cdk5 is a member of the Cdk family of serine/threonine kinases. Unlike other Cdks that are major regulators of cell-cycle progression, Cdk5 is mainly involved in phosphorylation of substrates in postmitotic neurons (8). The neuronal specificity of Cdk5 activity is achieved through the association with its activators, either p35 or p39, which are predominantly expressed in postmitotic neurons (8). In addition to the essential role of Cdk5 in brain development (9, 10), it has also been implicated in dopaminergic transmission in postnatal brain (11, 12). Inhibition of Cdk5 activity results in increased dopamine release in the striatum, indicating a presynaptic function of Cdk5 as a negative regulator of dopamine release (11). Furthermore, Cdk5 modulates the efficacy of postsynaptic dopamine signaling by phosphorylating DARPP-32 (dopamine-

and cAMP-regulated phosphoprotein, molecular mass 32 kDa) at Thr 75, which converts DARPP-32 into an inhibitor of cAMP-dependent kinase (PKA) (12).

These observations suggest that Cdk5 and p35 are downstream regulators of the prolonged activation of dopamine signaling following chronic exposure to cocaine, and hence in cocaine addiction. To further address the role of Cdk5 on striatal dopamine signaling, we generated two transgenic mouse lines in which either Cdk5 or p35 was overexpressed specifically in neurons under the control of the p35 promoter. Our findings indicated that Cdk5 activity was upregulated with the increased levels of p35 protein, but not with the increased levels of Cdk5 protein, suggesting that the level of p35 protein is rate-limiting for Cdk5 activity. We provide here *in vivo* evidence that increased Cdk5 activity, as a result of p35 overexpression, leads to attenuation of cocaine-mediated dopamine signaling to the nucleus through an inhibition of the PKA- and extracellular signal-regulated kinase (ERK)-cascades.

Materials and Methods

Antibodies. Polyclonal antibodies to Cdk5 (C-8) and p35 (C-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The phosphorylation-dependent and -independent antibodies to ERK kinase (MEK)1/2, ERK1/2 and cAMP-response element-binding protein (CREB) were obtained from Cell Signaling Technology, Inc. (Beverly, MA). The antibodies to phospho-Thr 34 DARPP 32 (13), phospho-Thr 75 DARPP-32 (12), total DARPP-32 (12) and c-fos (14) were used as described. An antibody to actin was purchased from Sigma (St. Louis, MO).

Experimental Animals. We have earlier cloned the mouse p35 gene with its promoter region, and characterized its genomic structure (15). To generate the transgenic mouse with neuronal overexpression of p35 (Tgp35), the 6 kb *EcoR*I-*EcoR*I fragment containing the 1.2 kb promoter region was subcloned into a pGEM9Z(-) plasmid, and a 45 bp tag derived from SV40 was inserted into the *Kpn*I site downstream of the poly(A^+) signal (Fig. 1A). The tag contained a *Spe*I site for genotyping of the animals. The 6 kb fragment was excised from the plasmid and purified, followed by pronuclear injection of the transgene to generate the transgenic mice. To examine the expression profile of the transgene under the regulatory control of the 1.2 kb p35 promoter *in vivo*, a double transgenic mouse (Tgp35;p35-/-) was further generated using a two-step breeding strategy by which the Tgp35 mouse was regenerated in an endogenous p35-null background. The other mouse models used in this study included p35+/-, p35-/-, Cdk5+/- and a transgenic mouse with neuronal overexpression of Cdk5 (TgCdk5) (9, 16, 17). Genotypes of these mice were determined by performing either Southern blot analysis or PCR on genomic DNA isolated from the tail biopsies. Mice were housed under a 12 h light/ 12 h dark cycle. All care was given in compliance with National Institutes of Health guidelines on the care and use of laboratory and experimental animals.

Southern blot analysis. Genomic DNA extracted from tail biopsies was digested with *EcoR*I and *Spe*I, electrophoresed on a 0.9% agarose gel and transferred onto a nylon membrane. The membrane was hybridized with a random-primed ^{32}P-labeled probe at 42°C overnight. The 485 bp probe for genotyping of p35 knockout (p35-/-) and Tgp35 mice was generated by PCR using the following primers: 5'-

ACATCCTGCTGCCACGGTGAC-3' and 5'-CCACTGTAAAAGCAACAAGA-3'. The hybridized membrane was washed 2 times in 2X SSC, 0.1% SDS at 42°C for 10 min, and 2 times in 0.1X SSC, 0.1% SDS at 65°C for 20 min, and exposed to X-ray film.

Drug treatment. Cocaine (Sigma, St. Louis, MO) was dissolved in sterile saline. Animals were intraperitoneally injected with cocaine (15 mg/kg) or an equal volume of saline at the age of 3 months, and sacrificed by decapitation at different time points (15, 30, 60 and 120 min) following the injection. Brains were rapidly removed, and chilled in ice-cold PBS. The striata were then dissected out and subjected to Northern or Western blot analysis. For immunohistochemical analysis, striatal sections were obtained from mice 2 hrs after the injection.

Northern blot analysis. Total RNA was extracted from the striata with TRIzol reagent (Invitrogen Life Technogies, Carlsbad, CA) and subjected to Northern blot analysis as described (19). For detection of c-fos mRNA, a 189 bp fragment of mouse c-fos cDNA was used as a probe as described (18). The levels of c-fos mRNA were quantified by measuring the optical density of the specific band using an image analysis system with NIH Image software, version 1.62.

Western blot analysis. Striatal tissues were sonicated in 1% SDS and boiled for 10 min. The protein concentration in each sample was determined by BCA protein assay (Pierce, Rockford, IL). Equal amounts of protein were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) before being transferred onto a

nitrocellulose membrane. The membranes were blocked in 1× PBS containing 5% skim milk and 0.05% Tween 20, and incubated with primary antibodies overnight at 4°C. Incubation with peroxidase-conjugated anti-mouse or rabbit IgG (Sigma, St. Louis, MO) was performed at room temperature for 60 min. A signal was detected by enhanced chemiluminescence (Pierce, Rockford, IL) and the optical densities of the bands were quantified as described above.

Cdk5 kinase assay. Striatal lysates were prepared with a lysis buffer consisting of 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and phosphatase inhibitors (phosphatase inhibitor cocktail I and II, Sigma, St. Louis, MO). The lysates were immunoprecipitated with either anti-Cdk5 (C-8) or anti-p35 (C-19) antibodies. The Cdk5 immunoprecipitates were prepared by incubation of 300 μl of the lysate (corresponding to 300 μg of protein) with anti-Cdk5 antibody (3 μg) overnight at 4°C followed by further incubation with 25 μl of Protein A-agarose beads (50% slurry in the lysis buffer; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 3 hrs at 4°C. For the preparation of p35 immunoprecipitates, 500 μl of the lysate (corresponding to 1 mg of protein) was incubated with anti-p35 antibody (3 μg) as described above. The immunoprecipitates were washed twice with the lysis buffer and twice with a kinase buffer consisting of 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol, and resuspended in 60 μl of the kinase buffer. Kinase activity was measured using histone H1 as substrate (19).

Immunohistochemistry. Mice were anesthetized by intraperitoneal injections of avertin (250 mg/kg, Fluka, Milwaukee, WI), and perfused transcardially with 0.1 M sodium phosphate buffer, pH 7.4, followed by Streck Tissue Fixative (Streck Laboratories, Inc., Omaha, NE), a non-crosslinking fixative. Dissected brains were further fixed in the same fixative overnight at 37°C. Then, brains were embedded in paraffin, cut into 5 μm-thick coronal sections and subjected to immunohistochemistry using avidin-biotin-peroxidase complex technique (Vector Laboratories, Burlingame, CA) with diaminobenzidine as a substrate. The sections were incubated with an affinity-purified polyclonal antibody against c-fos overnight at 4°C. The staining specificity was assessed by omission of the primary antibody.

Results

Generation of transgenic mice with neuronal overexpression of p35. The transgene used to achieve increased neuronal expression of p35 comprised the 6 kb fragment of the cloned mouse p35 gene containing the 1.2 kb promoter and the entire coding sequence of p35 (Fig.1*A*). The genotypes of mice were determined by Southern blot analysis using a probe that was designed to distinguish p35 -/- and Tgp35 mice from wild-type mice (Fig. 1*A* and *B*). To examine the transgene expression under the control of the 1.2 kb p35 promoter, we generated double transgenic mice (Tgp35;p35-/-) in which p35 expression was driven only from the transgene. The p35 expression in Tgp35;p35-/- mice was observed only in the brain (Fig. 1*C*), where the spatial expression pattern was similar to that of wild-type mice (Fig. 1*D*). Lack of p35 has been shown to result in abnormal layering structure in the cerebral cortex and hippocampus of mice (10). However, the

Tgp35;p35-/- mice showed a complete rescue of the p35-/- brain phenotype (Fig. 1*E*). These data indicated that the 1.2 kb p35 promoter controlled the expression of the transgene with a similar expression profile to that of p35 from the endogenous p35 gene.

p35 protein level is rate-limiting for the upregulation of Cdk5 activity. We examined the gene-dosage effects of the genes encoding p35 and Cdk5 on protein expression in striatal extracts from p35-/-, p35+/-, wild-type, Tgp35, Cdk5+/- and TgCdk5 mice at the age of 3 months. The levels of p35 and Cdk5 protein correlated well with the gene-dosages, respectively (Fig. 2*A* and *B*). Tgp35 mice showed an approximately 1.6-fold increase in p35 protein level as compared to wild-type mice, while Cdk5 protein levels were unaffected by the different levels of p35 protein. TgCdk5 mice showed an approximately 1.9-fold increase in Cdk5 protein level as compared to wild-type mice, while p35 protein levels were unaffected by the different levels of Cdk5 protein. To examine the effects of different amounts of p35 protein on Cdk5 activity, Cdk5 was immunoprecipitated from striatal extracts with anti-Cdk5 antibody and kinase activity was measured. Likewise, to examine the effects of different amounts of Cdk5 protein on kinase activity, p35 was immunoprecipitated from striatal extracts with anti-p35 antibody and kinase activity was measured. Cdk5 activity correlated well with the level of p35 protein, but not with the level of Cdk5 protein (Fig. 2*C* and *D*). These results indicated that the amount of p35 protein is a rate-limiting factor for Cdk5 activity. We therefore used Tgp35 mice to investigate the effects of increased Cdk5 activity on striatal dopamine signaling.

Cocaine-induced phosphorylation of DARPP-32 at Thr 34 is attenuated in Tgp35 mice. The function of DARPP-32 is dependent on its phosphorylation state at multiple sites (20). PKA phosphorylates DARPP-32 at Thr 34 while Cdk5 phosphorylates DARPP-32 at Thr 75. Thus, we examined the phosphorylation state of DARPP-32 in striatal extracts from wild-type and Tgp35 mice. The level of phospho-Thr 75 DARPP-32 was higher in Tgp35 mice (Fig. 3*A*; 1.6 ± 0.2-fold above the value of wild-type mice). We next assessed the effects of increased Cdk5 activity on striatal dopamine signaling. We examined the cocaine-induced PKA activation in Tgp35 mice by analyzing the phosphorylation state of DARPP-32 at Thr 34. The level of phospho-Thr 34 DARPP-32 was increased in wild-type mice 15 min after the cocaine injection (Fig. 3*B*; 1.8 ± 0.2-fold above the basal level). However, the effect of cocaine on Thr 34 phosphorylation of DARPP-32 was attenuated in Tgp35 mice (1.2 ± 0.3-fold above the basal level). These results indicated that an increase of Cdk5 activity attenuated the cocaine-induced PKA activation most probably through the DARPP-32 phosphorylation at Thr 75 (6, 12). It is also possible that an increase in presynaptic Cdk5 activity leads to decreased dopamine release, and that this contributes to the reduced effect of cocaine. Notably, a single injection of cocaine did not affect the levels of p35 and Cdk5 protein as well as the kinase activity (Fig. 3*C* and *D*). This is in contrast to a previous study in which chronic exposure to cocaine has been shown to upregulate the expression of p35 and Cdk5 (6).

Upregulation of Cdk5 activity attenuates cocaine-induced activation of ERK1/2. Recent evidence indicates that dopamine receptor activation in the striatum also activates other signaling cascades, including the ERK pathway (21, 22), which has an important

role in the behavioral response to cocaine (23). We therefore examined whether Cdk5 activity might affect the cocaine-induced activation of the ERK pathway. Activation of the ERK pathway was observed following cocaine injection in striatal extracts from wild-type mice as evident by increased phosphorylation of MEK1/2 at Ser 217 and Ser 221 (1.5 ± 0.2-fold above the basal level) and of ERK1/2 at Thr 202 and Tyr 204 (ERK2 phosphorylation: 1.5 ± 0.2-fold above the basal level) (Fig. 4*A* and *B*). However, the cocaine-induced activation of MEK1/2 (1.2 ± 0.2-fold above the basal level) and of ERK1/2 (ERK2 phosphorylation: 1.2 ± 0.2-fold above the basal level) was attenuated in Tgp35 mice (Fig.4*A* and *B*). Moreover, the basal levels of phospho-ERK1/2 were lower in Tgp35 mice (0.8 ± 0.2-fold below the value of wild-type mice) while this trend was not statistically significant. This latter result might be attributed to Cdk5-dependent phosphorylation of MEK1 at Thr 286, resulting in a decrease of the catalytic activity (24). To assess this possibility, we examined the phosphorylation state of MEK1 at Thr 286, and found that higher levels of phospho-Thr 286 MEK1 were present in striatal extracts from Tgp35 mice (Fig. 4*C*; 1.3 ± 0.1-fold above the value of wild-type mice). Furthermore, the phosphorylation state of MEK1 at Thr 286 was not altered by a single injection of cocaine, consistent with the findings that Cdk5 activity was not affected by the treatment (Fig. 3*D*).

Propagation of dopamine signaling to the nucleus is attenuated by increased Cdk5 activity. Cocaine-induced activation of multiple signaling cascades involving PKA and ERK leads to subsequent activation of the transcription factor CREB in the nucleus through its phosphorylation at Ser 133 (22, 25). To investigate whether the Cdk5-

mediated inhibitory effects on PKA and ERK activation cascades may converge on CREB phosphorylation in the nucleus, we examined the phosphorylation state of CREB at Ser 133 in striatal extracts from wild-type and Tgp35 mice. The basal level of phospho-CREB was lower in Tgp35 mice (0.7 ± 0.1-fold of the value of wild-type mice) (Fig. 5). In response to injection of cocaine, the level of phospho-CREB was increased in the striatum of wild-type mice (1.5 ± 0.1-fold above the basal level), but this response to cocaine was attenuated in Tgp35 mice (1.2 ± 0.1-fold above the basal level) (Fig. 5).

Phosphorylation of CREB at Ser 133 augments its transcriptional activity via a cyclic AMP response element in the promoter region of certain genes, including the c-fos gene (26). We therefore examined the induction of c-fos in the striatum of wild-type and Tgp35 mice following cocaine injection. In wild-type mice, the level of c-fos mRNA increased to a peak value (1.8 ± 0.2-fold above the basal level) 30 min after injection of cocaine, and subsequently returned to the basal level by 120 min after the injection (Fig. 6*A* and *B*). However, the levels of c-fos mRNA were approximately 30% lower in Tgp35 mice than in wild-type mice until 30 min after the injection (Fig. 6*A* and *B*). The lesser induction of c-fos in Tgp35 mice was further corroborated by immunohistochemistry (Fig. 6*C*-*F*). Cocaine administration increased c-fos immunoreactivity, strongly in the dorsomedial-dorsocentral parts of the striatum and weakly in the lateral parts, in both wild-type and Tgp35 mice. However, the cocaine-induced increase in number of c-fos-immunopositive cells was notably attenuated in the striatum of Tgp35 mice (Fig. 6*G*). Together, these results indicated that cocaine-mediated enhancement of striatal dopamine signaling to the nucleus was inhibited in Tgp35 mice, a likely result of increased Cdk5 activity.

Discussion

Cdk5 and its activator p35 have been identified as target genes that are upregulated by chronic exposure to cocaine (6). We report here the evidence that increased Cdk5 activity, as a result of p35 upregulation rather than Cdk5 upregulation, leads to attenuation of cocaine-mediated dopamine signaling in striatal neurons. To examine the consequences of upregulated expression of either Cdk5 or p35 on striatal dopamine signaling, two transgenic mouse lines, TgCdk5 and Tgp35 mice, were analyzed. We found that Cdk5 activity was upregulated in proportion to an increased level of p35 protein, but was not affected by an increased level of Cdk5 protein. Our previous report has also demonstrated that Cdk5 activity in TgCdk5 mouse brain was lower than that in wild-type mouse brain when the activity was measured using Cdk5 immunoprecipitates (17), suggesting that Cdk5 overexpression results in an increased level of monomeric Cdk5 if p35 level is not increased. These results indicated that the level of p35 protein is a rate-limiting factor for Cdk5 activity.

Tgp35 mice exhibited a lesser induction of both CREB phosphorylation and c-fos in the striatum following acute injection of cocaine, suggesting that the striatal response to cocaine was inhibited by increased Cdk5 activity. The attenuation of the cocaine-mediated dopamine signaling in Tgp35 mice was likely achieved through the Cdk5-mediated inhibition of multiple signaling cascades involving DARPP-32, PKA and ERK. Cocaine administration increased PKA phosphorylation of DARPP-32 at Thr 34 in wild-type mice while this response was attenuated in Tgp35 mice. PKA phosphorylation of DARPP-32 at Thr 34 has been shown to inhibit the activity of protein phosphatase 1

(PP1), the enzyme responsible for dephosphorylation of Ser 133 of CREB (27). Thus, PP1 activity would not be antagonized via the DARPP-32/PP1 pathway in Tgp35 mice.

Cocaine-induced activation of ERK1/2 was also attenuated in Tgp35 mice. There are several distinct mechanisms by which Cdk5 could inhibit the cocaine-induced activation of ERK1/2. First, Cdk5-dependent phosphorylation of DARPP-32 at Thr 75 could inhibit PKA, leading to subsequent inhibition of any PKA-mediated MEK1/2 activation that is required for ERK1/2 activation. A recent study has also found that phosphorylation of DARPP-32 at Thr 34 is required for cocaine-mediated activation of ERK1/2 by multiple pathways involving indirect regulation of MEK activation as well as involving the regulation of striatal-enriched phosphatase (STEP), a tyrosine phosphatase that acts directly on ERK1/2 (28). Support for this possibility is suggested by the finding that cocaine-induced phosphorylation of MEK1/2 at Ser 217 and Ser 221 was abolished in Tgp35 mice. Another likely pathway is via Cdk5-dependent phosphorylation of MEK1 at Thr 286, which would result in a decrease of its catalytic activity and would lead to inhibition of ERK1/2 activity (24).

Inhibition of Cdk5 activity in the striatum has been shown to potentiate behavioral effects of chronic cocaine treatment in animals (6). Consistent with the hypothesis that upregulation of Cdk5 activity may contribute to neuronal adaptation for counteracting the effects of repeated cocaine administration (6), we found that Cdk5-mediated phosphorylation of DARPP-32 and MEK1 contributed to the attenuation of cocaine-induced activation of ERK1/2, resulting in the lesser induction of CREB phosphorylation and c-fos in the striatum. Our findings support the idea that increased Cdk5 activity, as a result of p35 upregulation, may alter gene expression in the striatum

following chronic exposure to cocaine. This may occur through changes in the activities of the transcription factors such as CREB and c-fos. Thus, the Cdk5 activator p35, by virtue of its rate-limiting effects on Cdk5 activity, may contribute to long-lasting changes in neuronal function underlying cocaine addiction.

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Figure legend

Fig. 1. Generation of transgenic mouse with neuronal overexpression of p35 directed by the p35 promoter (Tgp35). (*A*) The transgene construct is shown with the schematic structures of wild-type and targeted p35 alleles. Red-colored bars indicate the probe used for genotyping. The open reading frame (ORF) of the p35 gene is indicated by the closed box with restriction enzyme sites: E, *EcoR*I; N, *Not*I; S, *Spe*I and K, *Kpn*I. (*B*) Southern blot analysis of *EcoR*I-and *Spe*I-digested genomic DNA of progenies obtained from crosses between Tgp35;p35+/- and p35+/- mice. (*C*) Brain-specific expression of p35 protein in wild-type (WT) as well as in Tgp35;p35-/- (TgKO) mice in which p35 is expressed by the transgene. (*D*) Expression profiles of the transgene product, p35 protein, and endogenous p35 gene in the brain. Higher expression of p35 was found in cerebral cortex and striatum as compared to that in brain stem and spinal cord. (*E*) Cresyl violet-stained sections showing a complete rescue of the p35-/- brain phenotype in Tgp35;p35-/- mice. The six distinct laminae are marked as I-VI in the wild-type cerebral cortex and the large pyramidal neurons characteristic of layer V are indicated by an asterisk. p35-/- mice exhibit abnormal layering structure in the cerebral cortex and in the CA1, CA3, and dentate gyrus (DG) of the hippocampus. Scale = 200 μm.

Fig. 2. Upregulation of Cdk5 activity is rate-limited by the p35 protein level. (*A* and *B*) Western blots showing that protein levels of p35 and Cdk5 correlate with the gene-dosages of the p35 and Cdk5 genes, respectively (*A*). Relative levels of p35 or Cdk5 protein to those of wild-type mice are shown as means ± s.e.m. (n=4) (*B*). (*C* and *D*) Cdk5 activity is upregulated with the increased level of p35 protein, but not with the

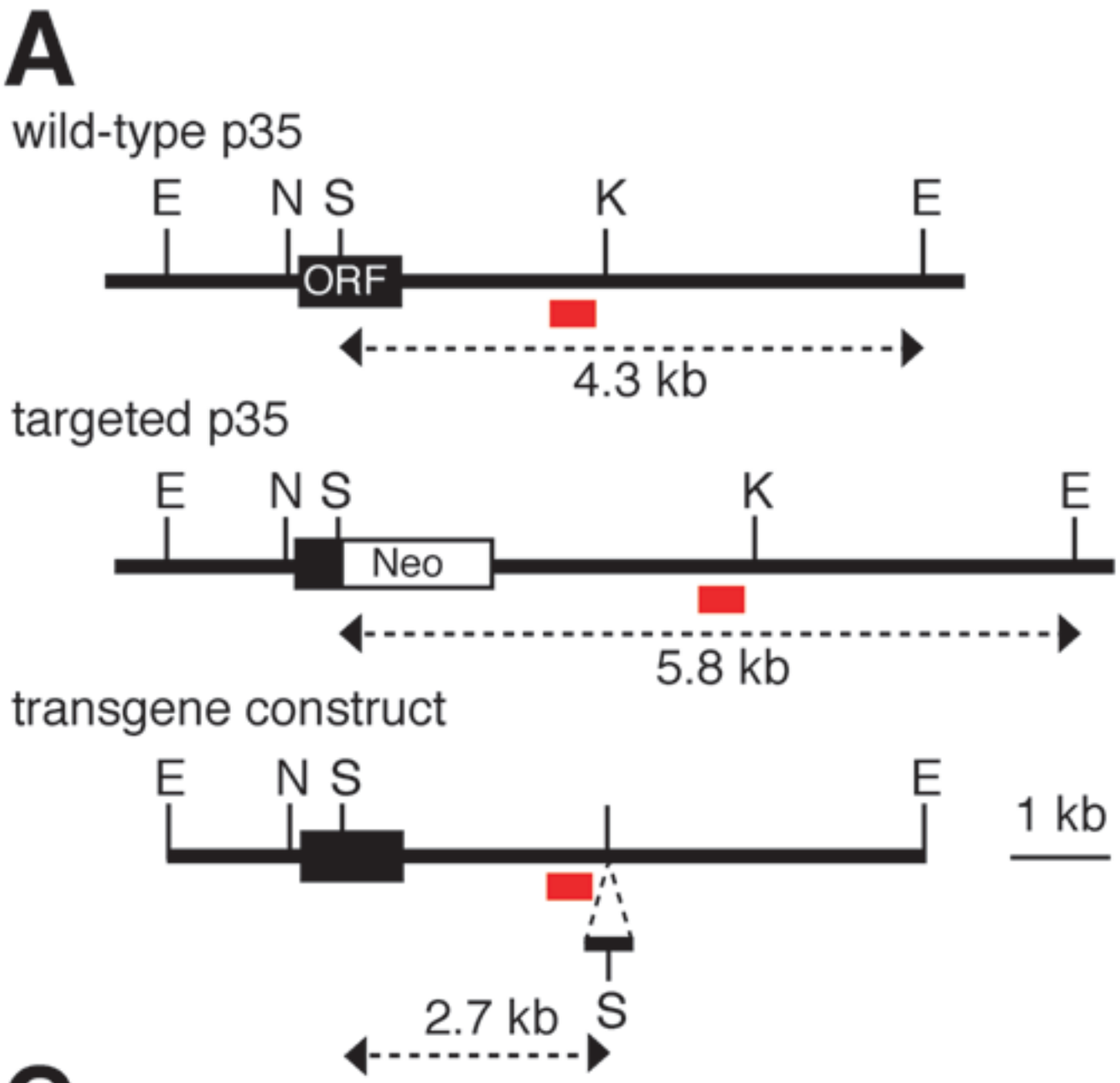
increased level of Cdk5 protein. The quantitative results, obtained by measuring the optical density of the autoradiograms, indicate the activities relative to those of wild-type mice and are shown as means ± s.e.m. (n =4) (*D*). Data in the panels of (*B*) and (*D*) are expressed in arbitrary units.

Fig. 3. Upregulation of Cdk5 activity increases the level of phospho-Thr 75 DARPP-32 and attenuates cocaine-induced PKA activation. (*A*) Immunoblot showing increased phosphorylation of DARPP-32 at Thr 75 (P-D32 Thr 75) in striatal extracts from Tgp35 mice. In the bar graph, the results indicate the levels of P-D32 Thr75 in Tgp35 mice relative to those of wild-type mice and are shown as means ± s.e.m. (n = 4). Asterisk indicates $P < 0.05$, compared with wild-type mice (Mann-Whitney test). (*B*) Cocaine-induced phosphorylation of DARPP-32 at Thr34 (P-D32 Thr 34), the site phosphorylated by PKA, is attenuated in Tgp35 mice. Striatal extracts were prepared from wild-type (WT) and Tgp35 mice 15 min after the injection of either cocaine or saline, and subjected to immunoblotting with anti-P-D32 Thr34 and total-DARPP 32 (Total D32) antibodies. In the bar graph, the results indicate the levels of P-D32 Thr34 relative to those of wild-type mice injected with saline, and are shown as means ± s.e.m. (n = 4). Asterisk indicates $P < 0.05$, compared with saline-injected wild-type mice (Mann-Whitney test). (*C* and *D*) A single injection of cocaine had no impact on the levels of p35 and Cdk5 protein (*C*) as well as Cdk5 activity (*D*) in striatal extracts from wild-type (WT) and Tgp35 mice. The quantitative results indicate the levels of Cdk5 activity relative to those of saline-injected wild-type mice, and are shown as means ± s.e.m. (n = 4) (*D*). Data in the panels of (*A*), (*B*) and (*D*) are expressed in arbitrary units.

Fig. 4. Cdk5-mediated inhibition of MEK1/2 leads to attenuation of cocaine-induced activation of ERK1/2. Striatal extracts were prepared from wild-type (WT) and Tgp35 mice 15 min after the injection of either cocaine or saline, and subjected to immunoblotting to examine the phosphorylation state of MEK1/2 and ERK1/2. In the bar graphs, the results indicate the levels of phospho-MEK1/2 or –ERK2 relative to those of wild-type mice injected with saline, and are shown as means ± s.e.m. (n = 4). Data are expressed in arbitrary units; asterisk indicates $P < 0.05$, compared with saline-injected wild-type mice (Mann-Whitney test). (A) Cocaine-induced phosphorylation of MEK1/2 at Ser 217 and Ser 221 is attenuated in Tgp35 mice. (B) Phosphorylation of ERK1/2 at Thr 202 and Tyr 204 following cocaine injection is also attenuated in Tgp35 mice. (C) Increased phosphorylation of MEK1 at Thr 286 in Tgp35 mice is not affected by the cocaine injection.

Fig. 5. Upregulation of Cdk5 activity results in decreased phosphorylation of CREB at Ser 133 in mice with injection of either saline or cocaine. Striatal extracts were prepared from wild-type (WT) and Tgp35 mice 30 min after injection and subjected to immunoblotting to examine the phosphorylation state of CREB. In the bar graph, the results indicate the levels of phospho-Ser 133 CREB relative to those of wild-type mice injected with saline, and are shown as means ± s.e.m. (n = 4). Data are expressed in arbitrary units; asterisk indicates $P < 0.05$, compared with saline-injected wild-type mice (Mann-Whitney test).

Fig. 6. Upregulation of Cdk5 activity results in a decrease of striatal c-fos expression and its lesser induction following cocaine administration. (*A*) Northern blot showing the time-course of c-fos induction in wild-type (WT) and Tgp35 (Tg) mice following cocaine injection. Equal application of total RNA was confirmed by ethidium bromide (EtBr) staining of the gels. (*B*) The quantitative results indicate the levels of c-fos mRNA relative to those of wild-type mice untreated with cocaine, and are shown as means ± s.e.m. (n = 3). Data are expressed in arbitrary units; asterisk indicates $P < 0.05$, compared with wild-type mice at the indicated time points (Mann-Whitney test). (*C*-*F*) Immunohistochemistry for c-fos was performed on coronal sections of the striatum obtained from wild-type and Tgp35 mice 2 hrs after the injection of either saline or cocaine. Scale = 200 μm. (*G*) The graph indicates quantitative data of the average number of c-fos-immunopositive cells in a 0.46 mm^2 area from the dorsomedial-dorsocentral striatum of three animals. Asterisk indicates $P < 0.05$, compared with saline-injected wild-type mice (Mann-Whitney test).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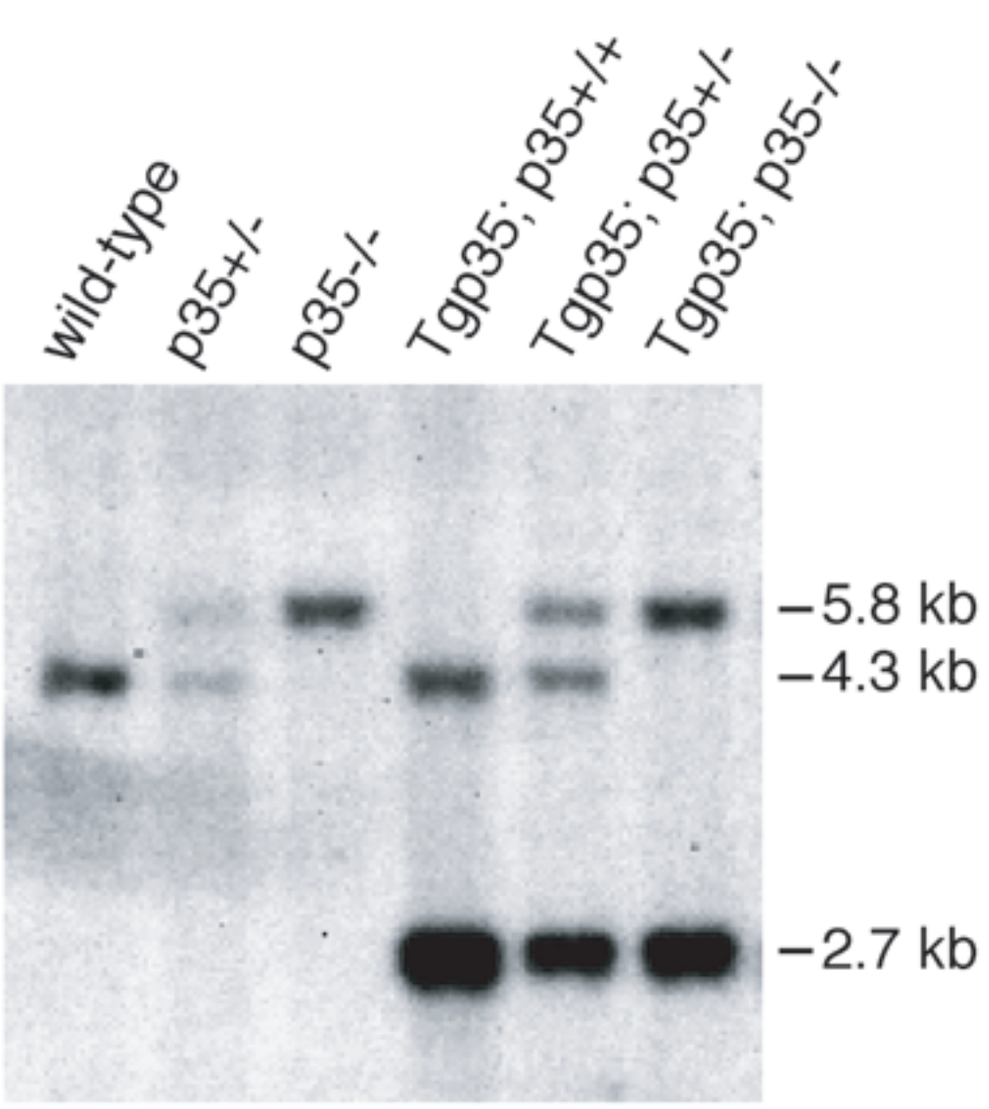
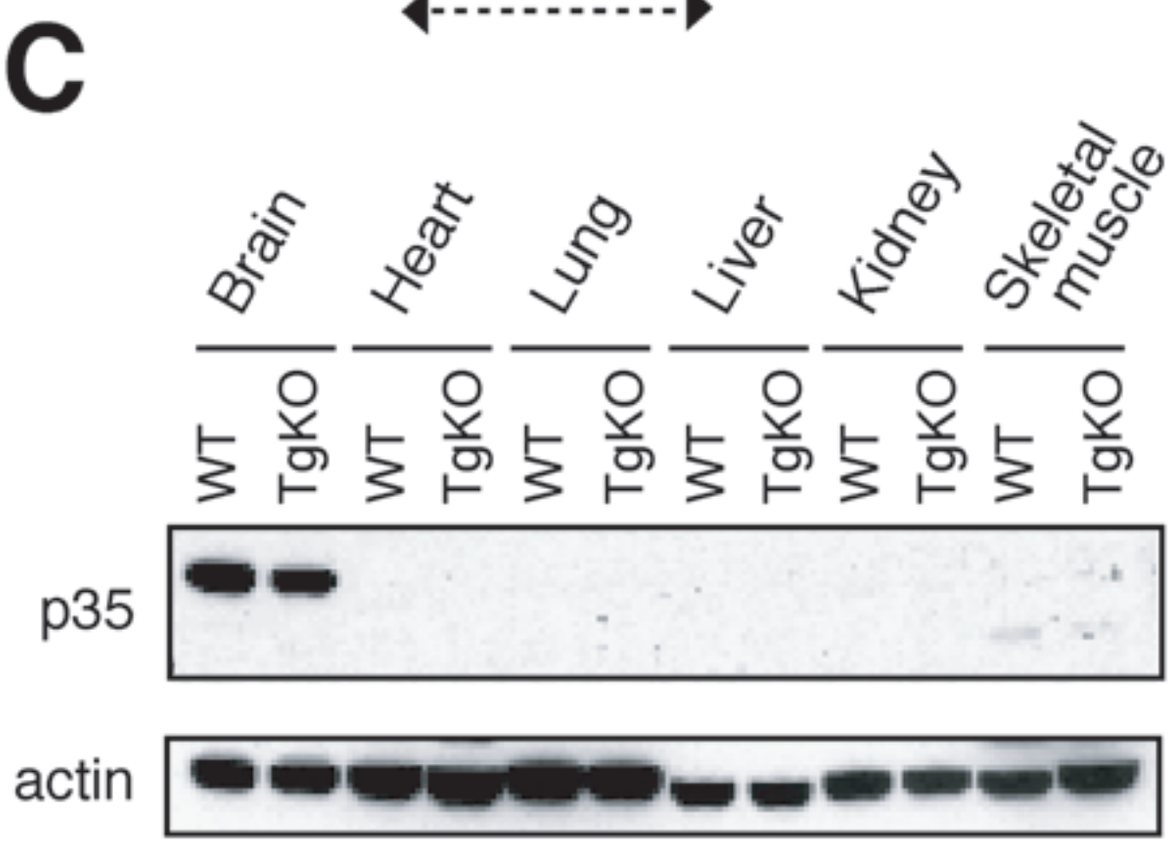
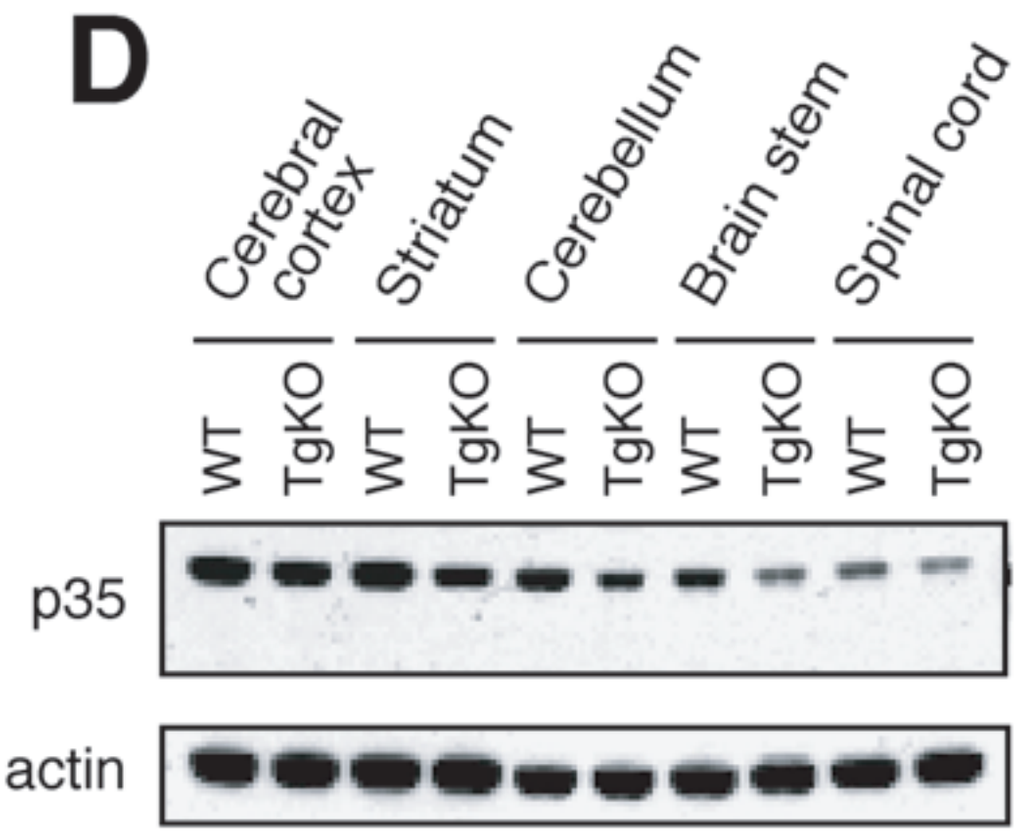
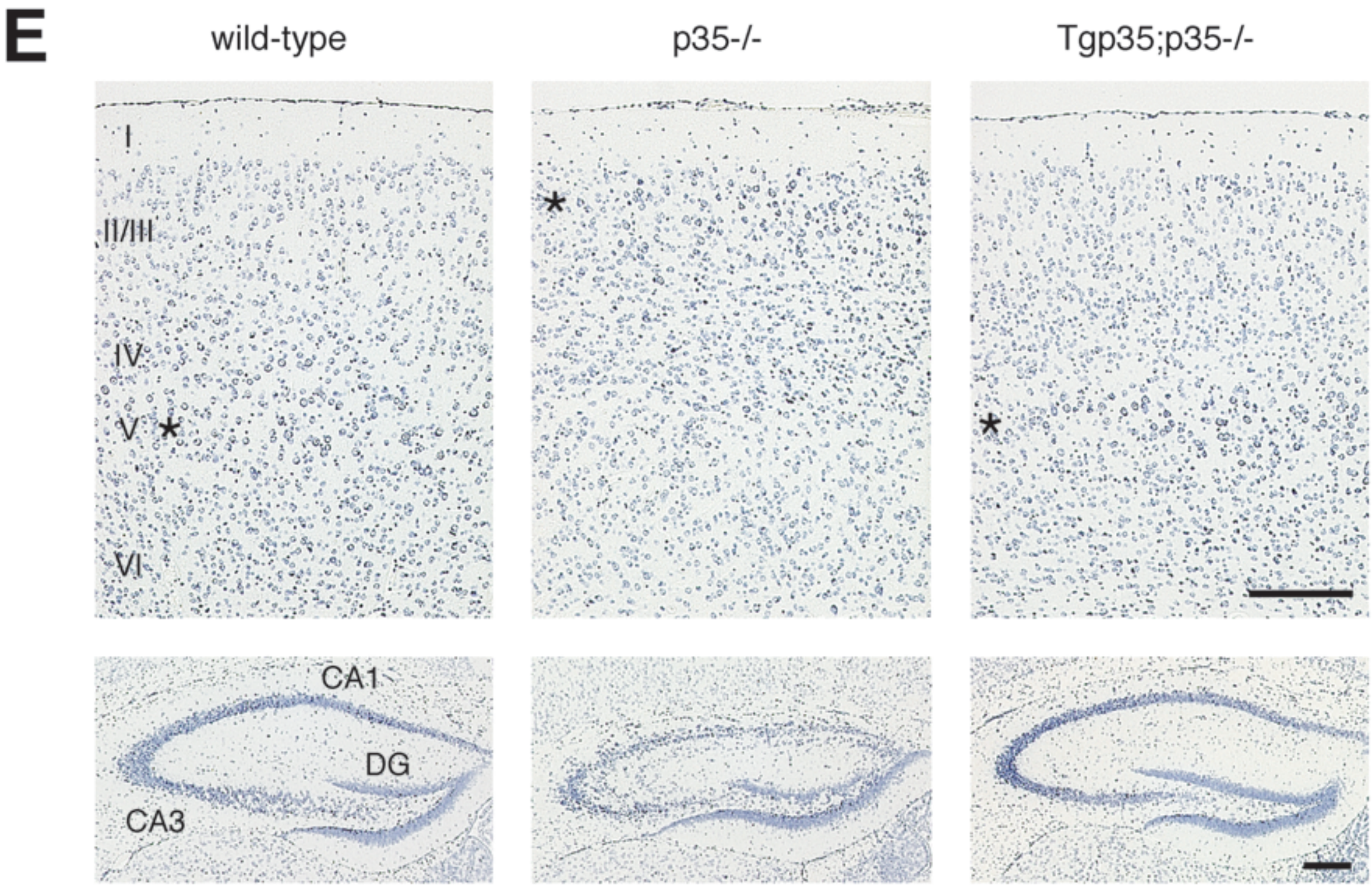
wild-type p35
E
N
S
K
E
ORF
4.3 kb
targeted p35
E
N
S
K
E
Neo
5.8 kb
transgene construct
E
N
S
E
1 kb
2.7 kb
S

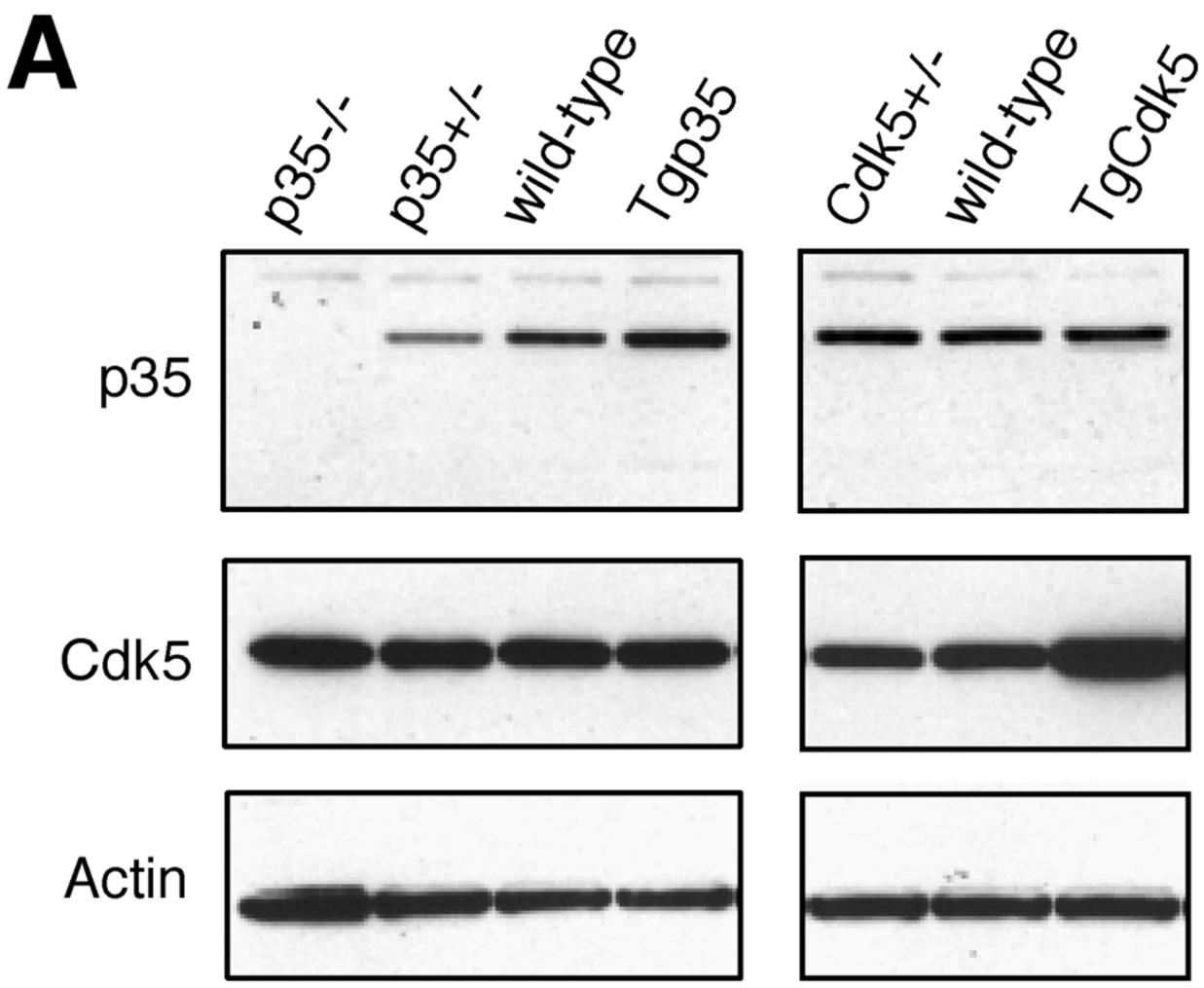

B
wild-type
p35+/-
p35-/-
Tgp35; p35+/+
Tgp35; p35+/-
Tgp35; p35-/-
5.8 kb
4.3 kb
2.7 kb


C
Brain
Heart
Lung
Liver
Kidney
Skeletal muscle
WT
TgKO
p35
actin

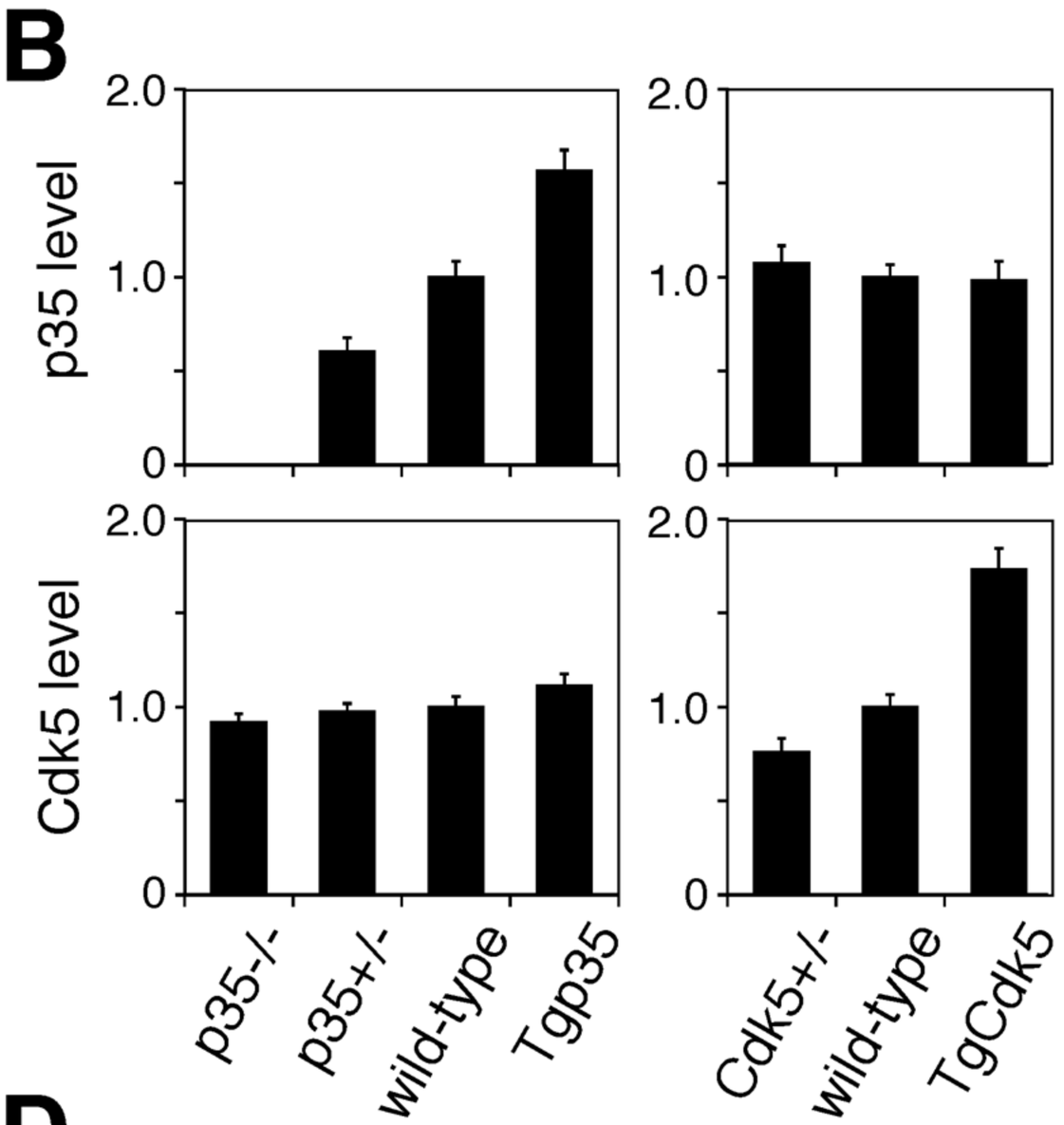

D
Cerebral cortex
Striatum
Cerebellum
Brain stem
Spinal cord
WT
TgKO
p35
actin


E
wild-type
p35-/-
Tgp35;p35-/-
I
II/III
IV
V
VI
*
CA1
DG
CA3

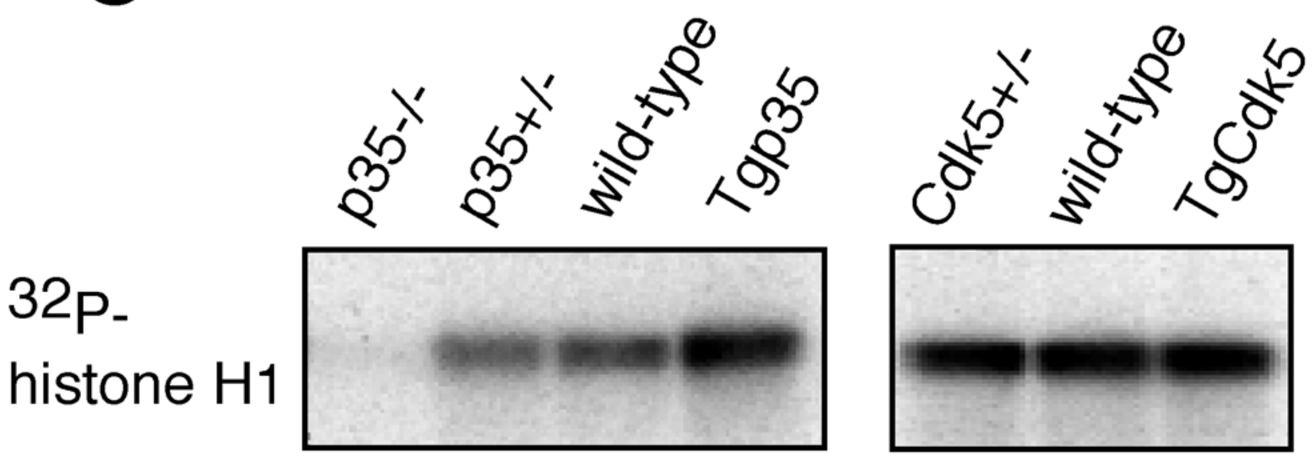
A


p35-/-
p35+/-
wild-type
Tgp35
Cdk5+/-
wild-type
TgCdk5
p35
Cdk5
Actin

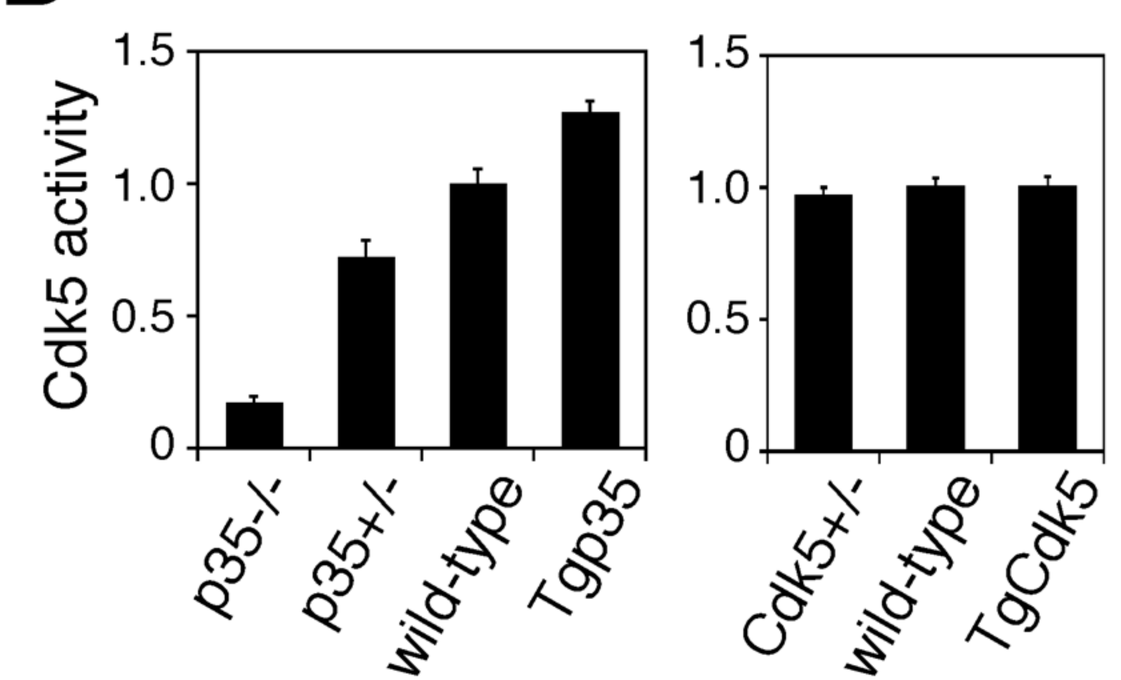
B


p35 level
Cdk5 level
2.0
1.0
0
p35-/-
p35+/-
wild-type
Tgp35
Cdk5+/-
wild-type
TgCdk5

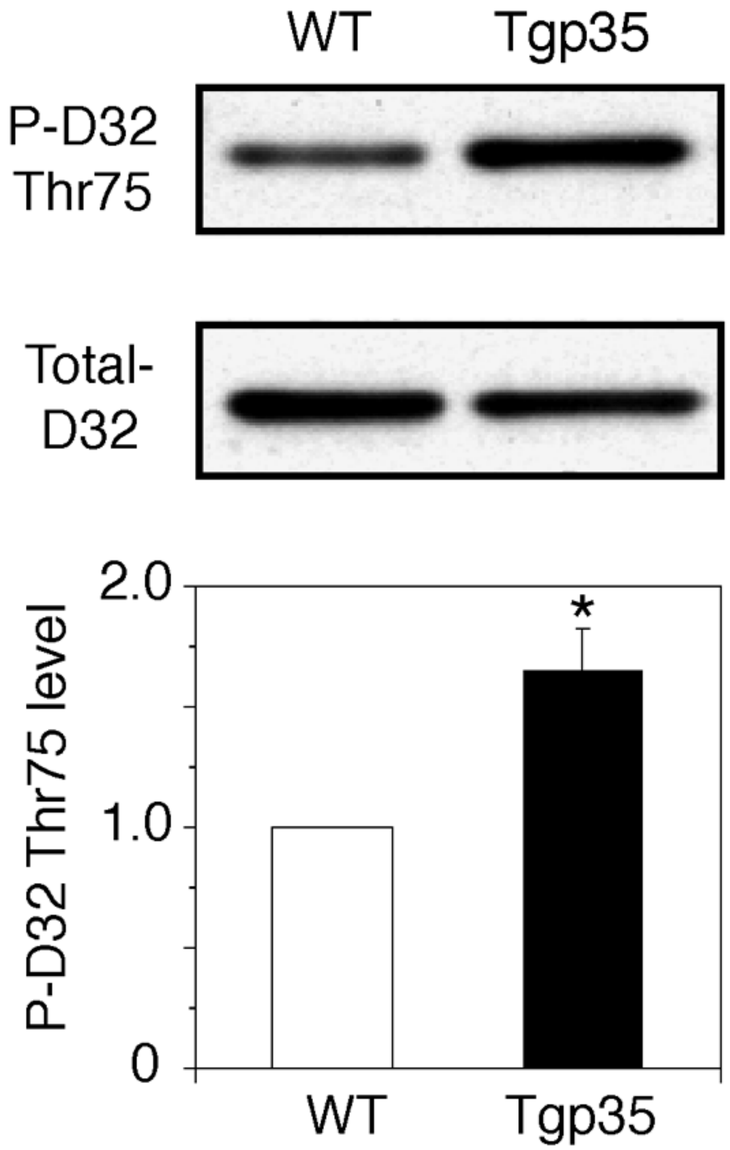
C


p35-/-
p35+/-
wild-type
Tgp35
Cdk5+/-
wild-type
TgCdk5
32P-
histone H1

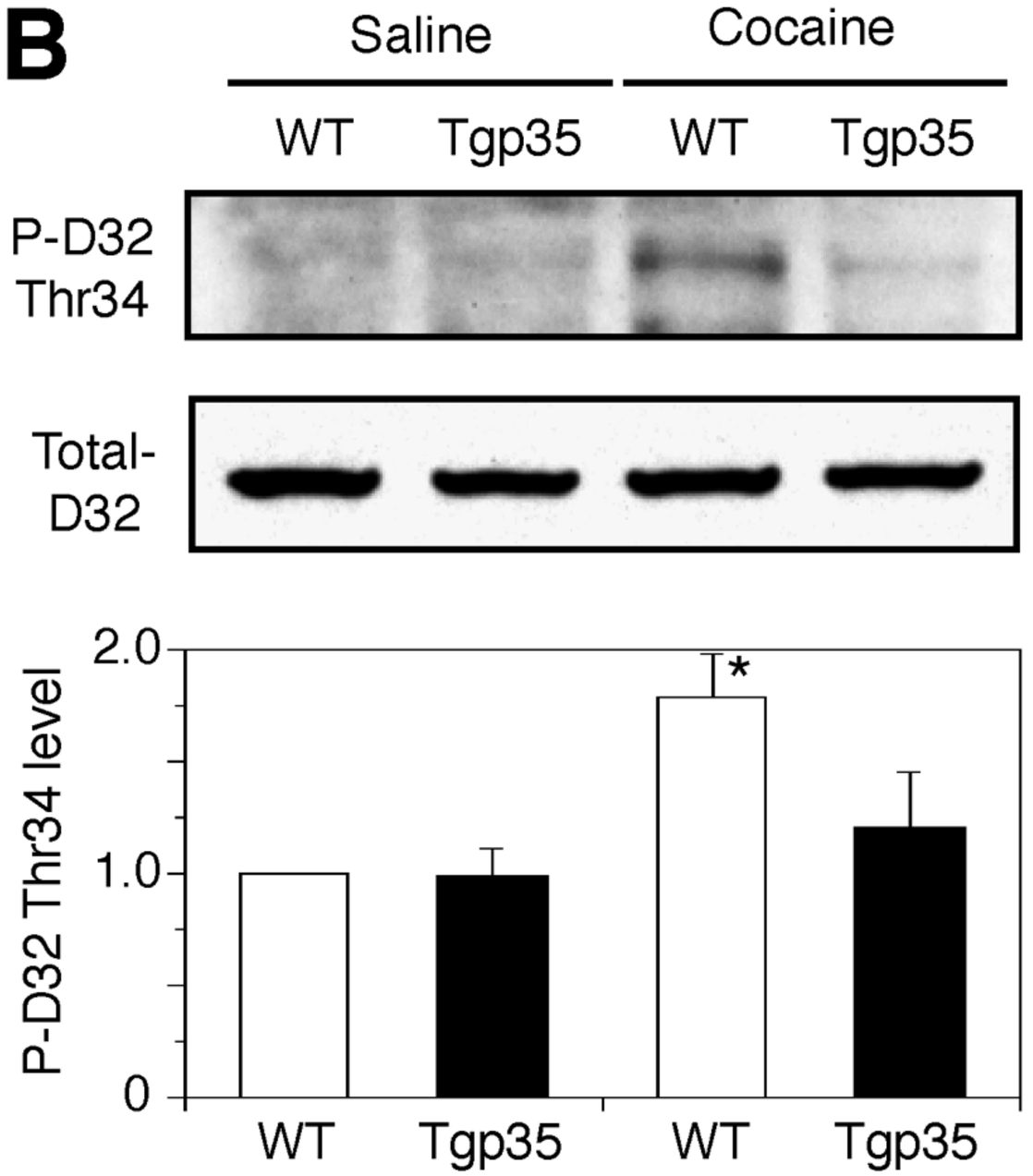
D


Cdk5 activity
1.5
1.0
0.5
0
p35-/-
p35+/-
wild-type
Tgp35
Cdk5+/-
wild-type
TgCdk5

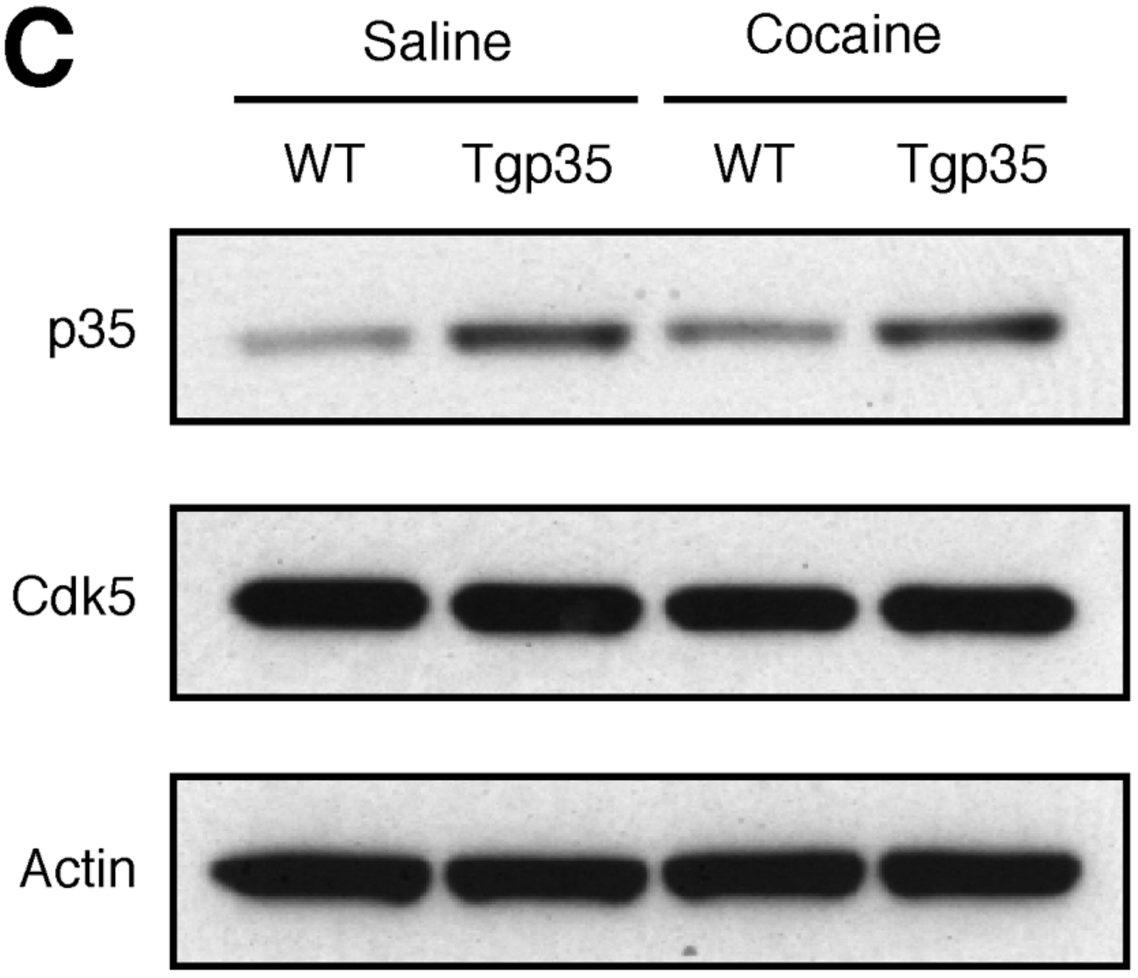
A


WT
Tgp35
P-D32 Thr75
Total-D32
P-D32 Thr75 level
2.0
1.0
0
*
WT
Tgp35

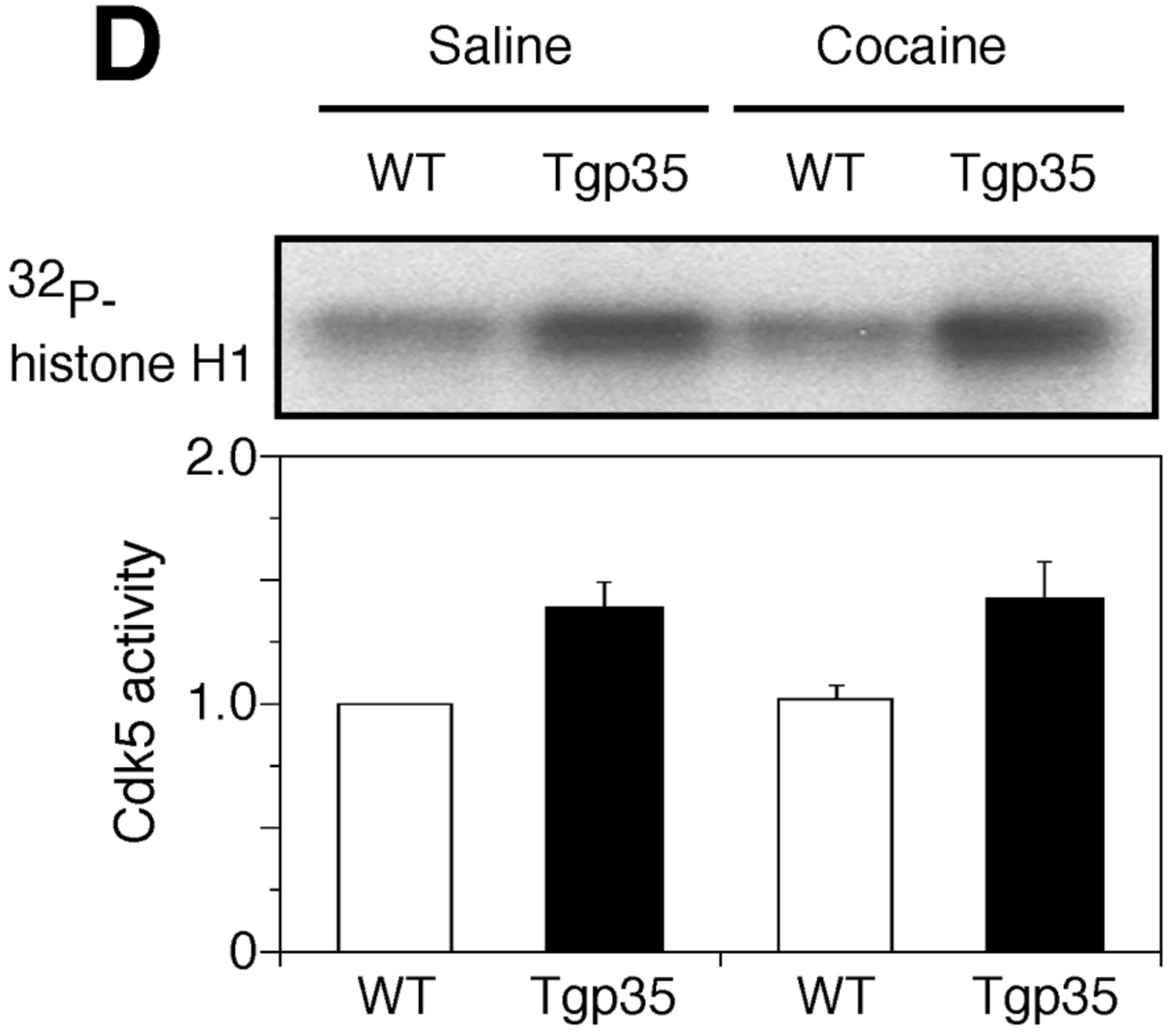
B

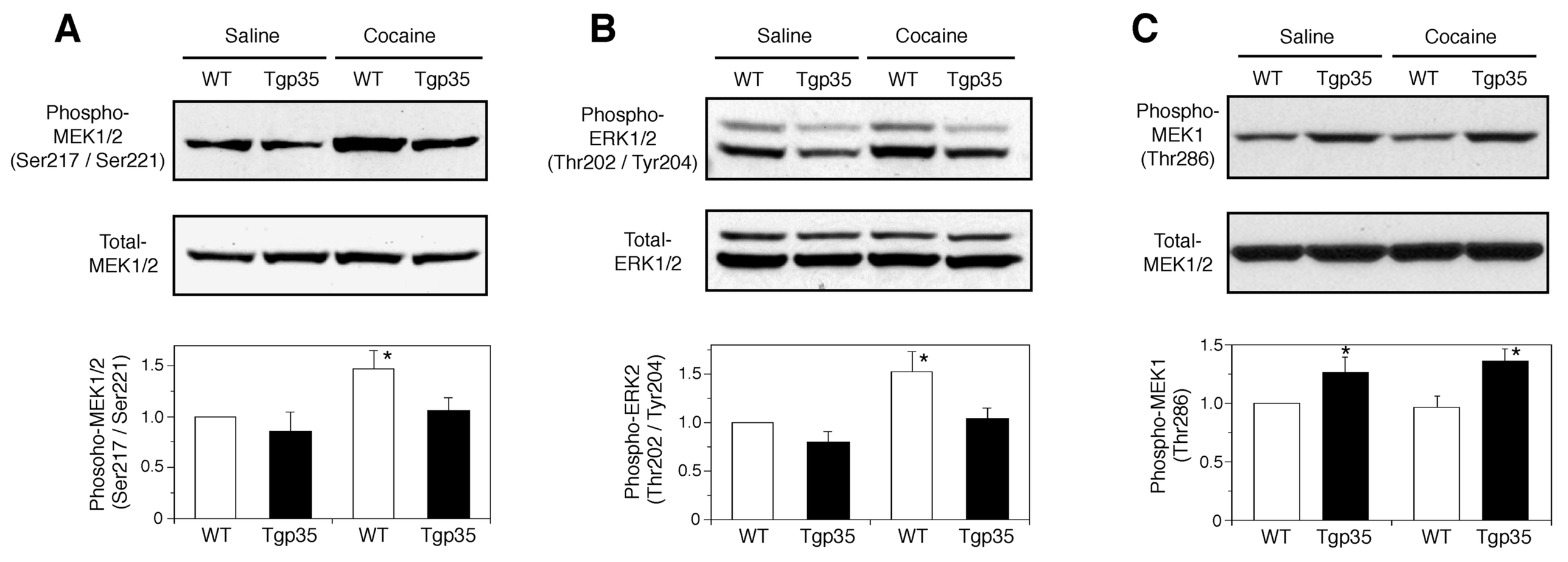

Saline
Cocaine
WT
Tgp35
WT
Tgp35
P-D32 Thr34
Total-D32
P-D32 Thr34 level
2.0
1.0
0
*
WT
Tgp35
WT
Tgp35

C


Saline
Cocaine
WT
Tgp35
WT
Tgp35
p35
Cdk5
Actin

D


Saline
Cocaine
WT
Tgp35
WT
Tgp35
32P-histone H1
Cdk5 activity
2.0
1.0
0
WT
Tgp35
WT
Tgp35


A
Saline
Cocaine
WT
Tgp35
WT
Tgp35
Phospho-
MEK1/2
(Ser217 / Ser221)
Total-
MEK1/2
Phosoho-MEK1/2
(Ser217 / Ser221)
1.5
1.0
0.5
0
*
WT
Tgp35
WT
Tgp35
B
Saline
Cocaine
WT
Tgp35
WT
Tgp35
Phospho-
ERK1/2
(Thr202 / Tyr204)
Total-
ERK1/2
Phospho-ERK2
(Thr202 / Tyr204)
1.5
1.0
0.5
0
*
WT
Tgp35
WT
Tgp35
C
Saline
Cocaine
WT
Tgp35
WT
Tgp35
Phospho-
MEK1
(Thr286)
Total-
MEK1/2
Phospho-MEK1
(Thr286)
1.5
1.0
0.5
0
*
*
WT
Tgp35
WT
Tgp35

Saline

Cocaine

WT

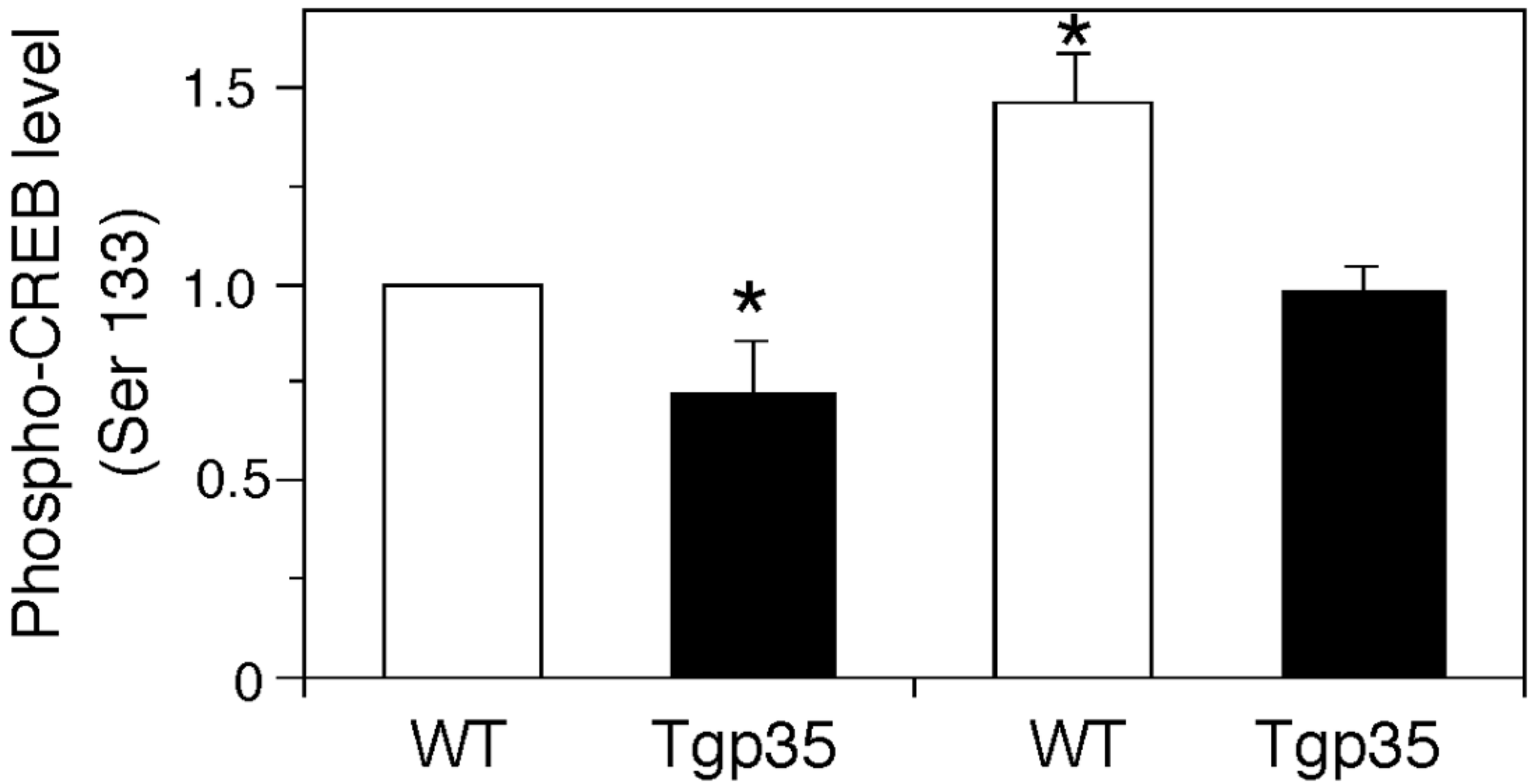
Tgp35

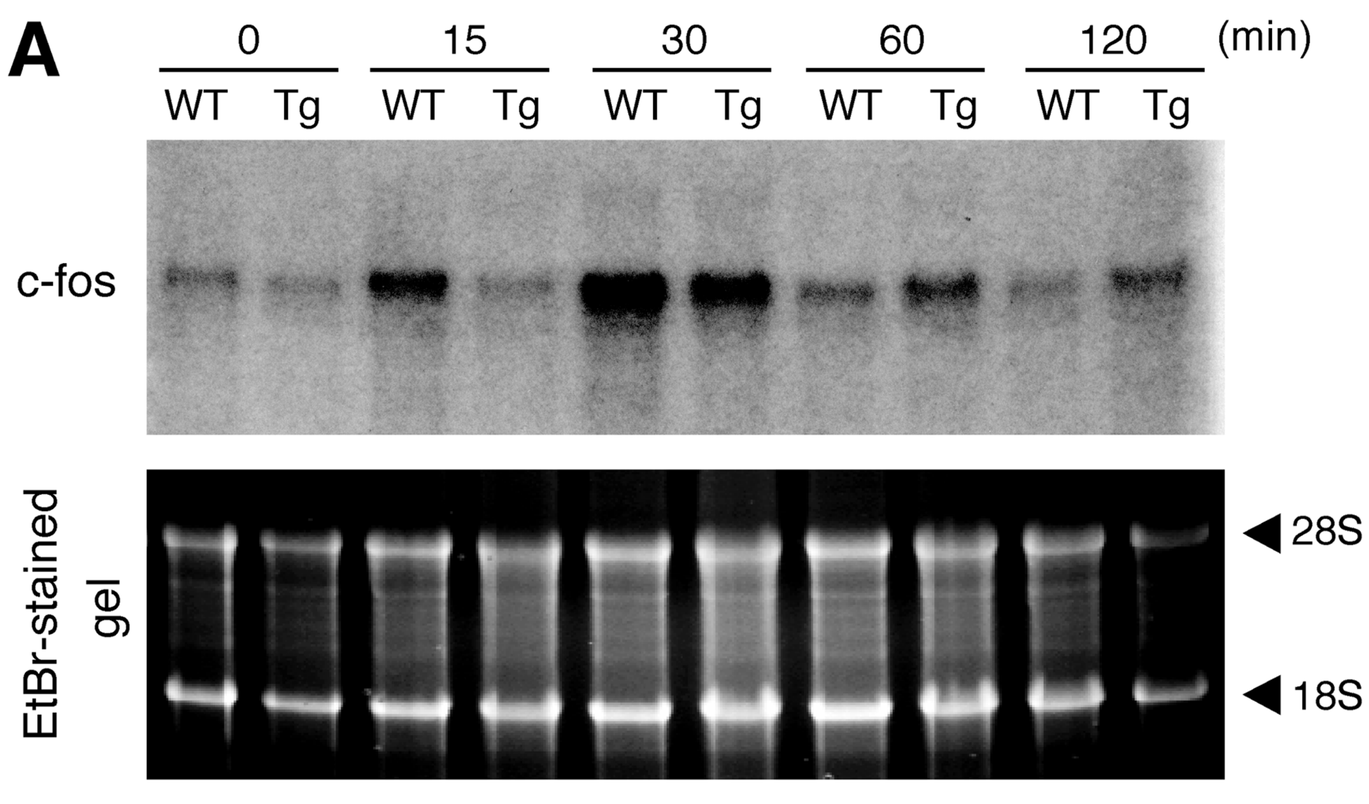
WT

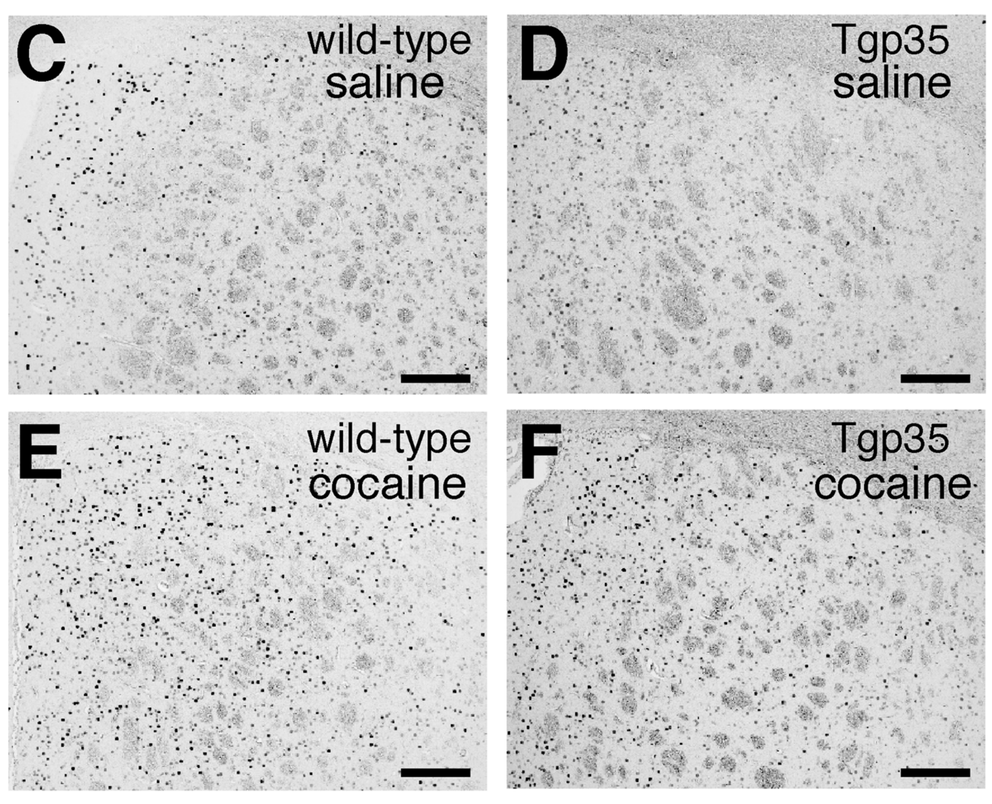
Tgp35

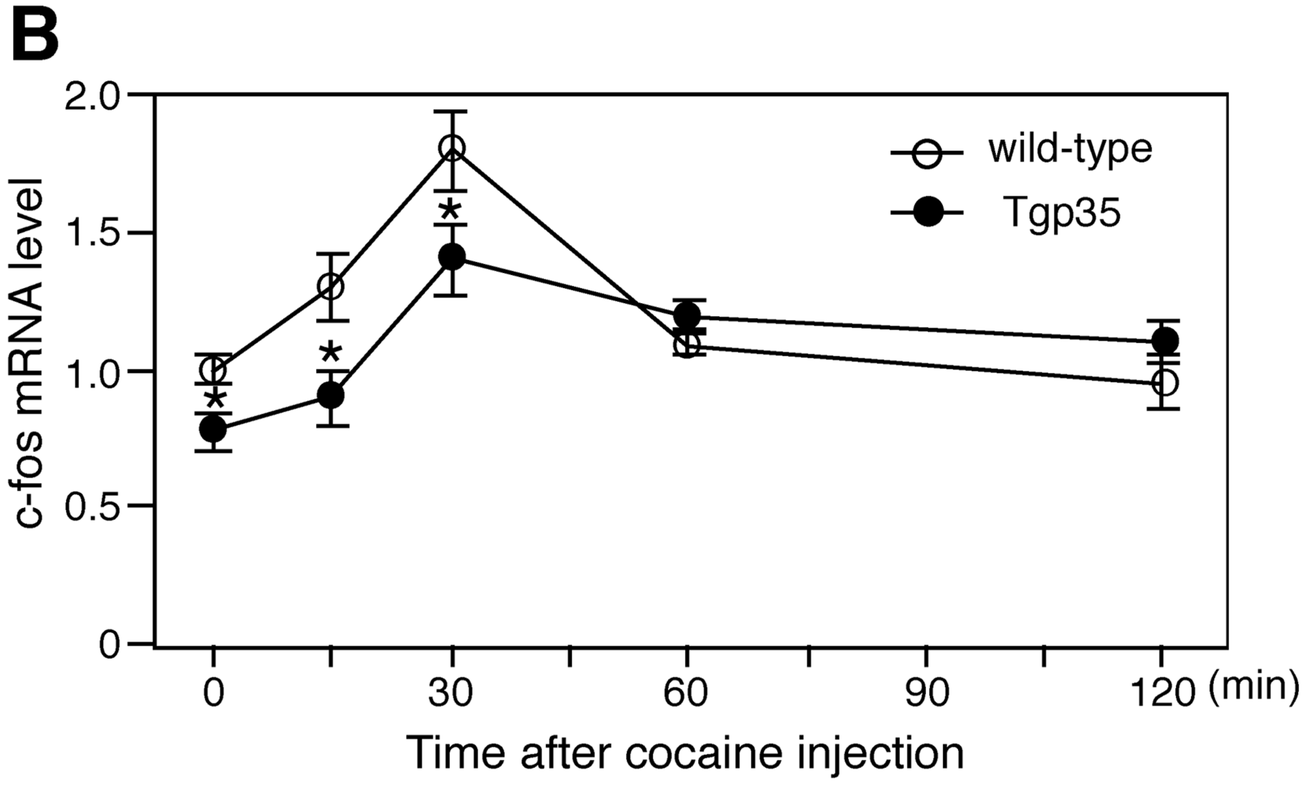
Phospho-CREB (Ser133)

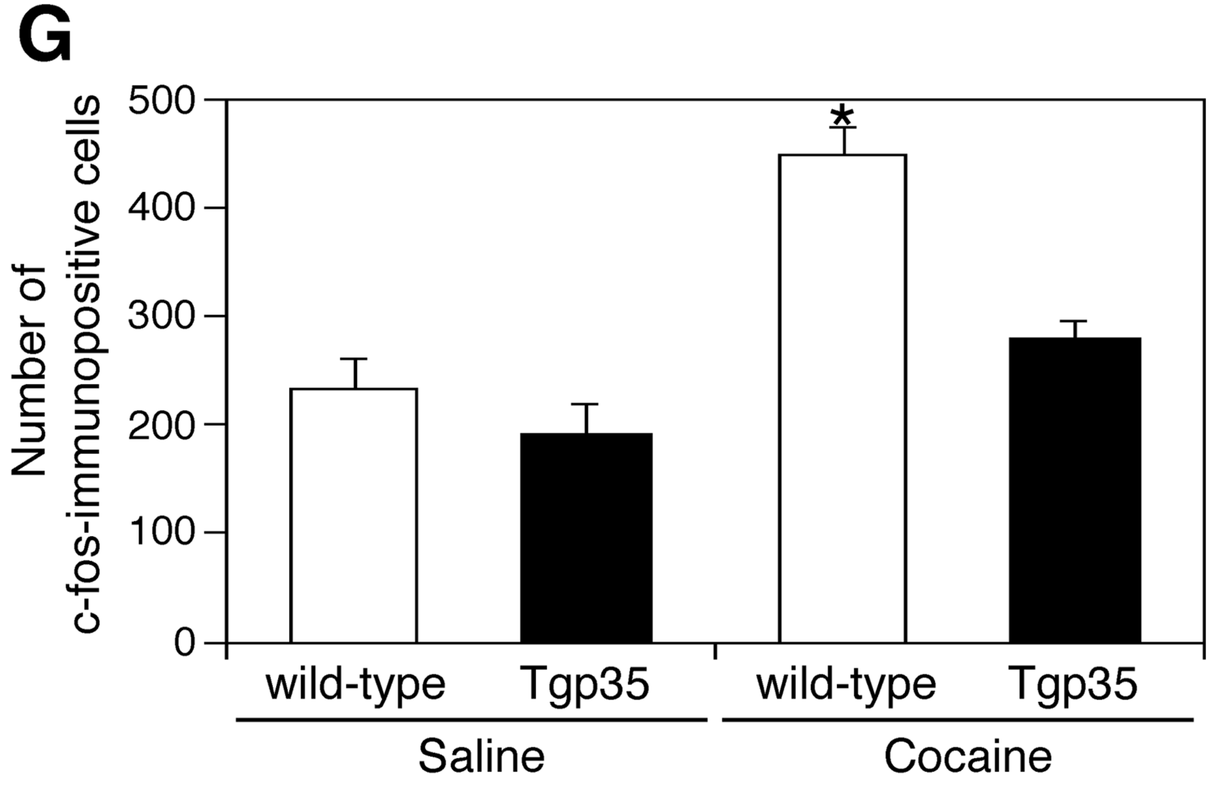
Total-CREB




A
0
15
30
60
120
(min)
WT
Tg
c-fos
EtBr-stained gel
28S
18S


C
wild-type
saline
D
Tgp35
saline
E
wild-type
cocaine
F
Tgp35
cocaine


B
2.0
1.5
1.0
0.5
0
c-fos mRNA level
wild-type
Tgp35
*
0
30
60
90
120 (min)
Time after cocaine injection


G
500
400
300
200
100
0
Number of c-fos-immunopositive cells
*
wild-type
Tgp35
wild-type
Tgp35
Saline
Cocaine