# Activation-induced cytidine deaminase regulates activity-dependent BDNF expression in post-mitotic cortical neurons

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**ABSTRACT**

Activity-dependent gene expression depends, in part, on transcriptional regulation that is coordinated by rapid changes in the chromatin landscape as well as the covalent modification of DNA. Here we demonstrate that the expression of brain-derived neurotrophic factor (BDNF), a gene that is critically involved in neural plasticity and subject to epigenetic regulation, is regulated by the RNA/DNA editing enzyme, activation-induced cytidine deaminase (AID). Similar to previous reports, we observed an activity-dependent induction of BDNF exon IV mRNA expression, which correlated with a reduction in DNA methylation within the BDNF P4 promoter. Lentiviral-mediated knockdown of AID disrupted these effects and inhibited BDNF exon IV mRNA expression, an effect that was associated with decreased cAMP response element-binding protein occupancy within the BDNF P4 promoter. Thus, together with other epigenetic mechanisms, AID plays a key role in regulating activity-dependent BDNF expression in post-mitotic cortical neurons.

## INTRODUCTION

Activity-dependent changes in gene expression depend on epigenetic mechanisms, including post-translational histone modifications and DNA methylation (Zovkic *et al.*, 2013). DNA methylation, and the accumulation of one particular mark, 5-methylcytosine (5mC), is one of the most well characterized epigenetic modifications, and has been shown to regulate X chromosome inactivation, genomic imprinting, retrotransposon silencing, and the reprogramming of gene expression in embryonic stem cells (Jones & Baylin, 2007; Reik, 2007). More recently, it was discovered that, in the adult brain, the accumulation of 5-mC also mediates synaptic plasticity and the formation of fear-related memories (Holliday, 1999; Levenson *et al.*, 2006; Feng *et al.*, 2010; Miller *et al.*, 2010; Sultan *et al.*, 2012). Brain-derived neurotrophic factor (BDNF) is an activity-dependent gene driven by the transcription factor cAMP response element-binding protein (CREB), which is necessary for neuronal survival, synaptic plasticity and memory formation, and has been shown to be sensitive to various epigenetic modifications, including DNA methylation (West *et al.*, 2001; Martinowich *et al.*, 2003). The BDNF locus consists of nine 5' non-coding exons, each with its own promoter, and a 3' protein-coding region (Aid *et al.*, 2007). Although activity-dependent changes in CpG dinucleotide methylation, including reduced 5mC within BDNF gene promoters, is thought to regulate the spatial and temporal expression of individual BDNF isoforms (Martinowich *et al.*, 2003; Lubin *et al.*, 2008; Wu & Sun, 2009; Guo *et al.*, 2011), the mechanism by which this occurs is not yet known.

Activation-induced cytidine deaminase (AID) was originally identified as an RNA/DNA editing enzyme involved in immune cell somatic hypermutation and class switch recombination underlying antibody diversification (Muramatsu *et al.*, 2000). This epigenetic mechanism is also necessary for the dynamic reprogramming of pluripotent embryonic stem (ES) cells (Bhutani *et al.*, 2010), and the regulation of DNA methylation during early development (Morgan *et al.*, 2004; Rai *et al.*, 2008). More recently, AID has been implicated in active DNA demethylation by promoting Tet1-mediated DNA hydroxylation through its interaction with thymine DNA glycosylase and the genome stability protein Gadd45a (Cortellino *et al.*, 2011; Guo *et al.*, 2011). Thus, AID regulates the covalent modification of DNA during early neuronal development and within a neurogenic niche in the adult brain. Together with its role in regulating the immune system and in ES cell differentiation, AID is also highly expressed in distinct neuronal populations including pyramidal neurons in both the developing and adult cortex (Rommel et al., 2013); however, its role in terminally differentiated cortical neurons has not yet been fully elucidated. Here we report that AID also contributes to activity-dependent BDNF exon IV expression in post-mitotic cortical neurons through its effects on DNA methylation and subsequent influence on the binding of the activity-dependent transcription factor CREB to the BDNF P4 promoter.

## MATERIALS AND METHODS

**Primary cortical neuron culture.** Cortical neurons derived from embryonic day 14 (E14) C57Bl6/J mice were dissociated in 0.025% trypsin (Sigma) at 37°C for 5min and plated ($1\times10^6$ per well) on 6 well plates. Neurons were maintained in 2ml Neurobasal medium containing B27 supplement (2%; Invitrogen), penicillin-streptomycin (50µg/ml penicillin, 50 U/ml streptomycin, Invitrogen) and L-glutamine (2mM, Invitrogen) at 37°C and 5% $CO_2$ for 7 days in vitro (DIV), with half the medium being replaced with fresh warm medium on the 3rd and 6th days. All experiments followed protocols approved by the Animal Ethics Committee of the University of Queensland in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

KCl-induced depolarization and lentivirus infection of neurons. In experiments designed to test the effect of neuronal activity on BDNF gene expression and epigenetic regulation, cortical neurons (6 DIV) were incubated overnight in 1µM tetrodotoxin (Sigma) in order to suppress spontaneous neuronal activity. 24 hours later, the neurons were stimulated with 50mM KCl for 0, 1, 3, 5, 7 and 10 hours relative to time-matched control samples that were incubated in phosphate buffered saline (PBS). We used standard PBS, which contains disodium hydrogen phosphate ($Na_2HPO_4$) at a concentration of 10mM. In each plate, we added 40µl of 2.5M KCl solution, containing 2ml of Neurobasal mediaum. Therefore, the final concentration of $Na_2HPO_4$ is reduced to 0.2mM, which is a normosomotic concentration of $Na^+$ that does not evoke changes in neural activity in the control conditions. At each time-point, total RNA and genomic DNA were harvested for mRNA expression and DNA methylation analysis. For experiments examining the effect of AID knockdown, a lentivirus harbouring an shRNA directed toward AID was added directly to the culture medium at a multiplicity of infection (MOI) of 5-10 at 4 DIV. The culture was immediately returned to the incubator for 24 hours, after which half of the culture medium was replaced. Following lentivirus infection, the concentration and timing of both tetrodotoxin and KCl treatment remained the same as described above.

**Quantitative real time-PCR.** Control and stimulated neurons were lysed and total RNA was isolated. 1µg RNA was reverse transcribed using an optimized blend of oligo-dT and random primers according to the manufacturer's instructions (Qiagen). BDNF exon I and IV and PGK mRNA (as a reference) were amplified using 2x Rotor-Gene SYBR Green (Qiagen) in a Rotorgene Q real-time quantitative PCR (qPCR) detection system using the following primers: BDNF exon IV 5'-GCAGCTGCCTTGATGTTTAC-3' and 5'-CCGTGGACGTTTACTTCTTTC-3', BDNF exon I 5'-GCCGGCTGGTGCAGAAAAGC-3' and 5'-GCACGCCGATCCTTTGCCCA-3', AID 5'-CCACCTTCGCAACAAGTCTGGCT-3' and 5'-CCGGGCACAGTCATAGCACGG-3', and PGK 5'-TGCACGCTTCAAAAGCGCACG-3' and 5'-AAGTCCACCCTCATCACGACCC-3'. Each sample reaction was run in triplicate and the relative expression levels analysed by 2-ΔΔCt with

normalization relative to PGK. Data from at least 2 experiments were collected and were expressed as mean ± standard error (S.E.M.). A student's t-test (unpaired) was used for comparison between two groups at each time point.

**AID shRNA design and validation.** A lentiviral construct was designed to knock down AID by inserting an shRNA sequence 5'-GACTTGCGAGATGCATTTC-3' or scrambled control 5'-GGCTTCGCGCCGTAGTCTTATC AAGAGTAAGACTACGGCGCGAAGCTTTTTTG-3' into psiHIV-U6 (HIV based, Genecopoeia). To test the efficiency of this AID shRNA, we co-transfected 0.25 µg of an AID overexpression vector with 2µg of AID shRNA into HEK293T cells. Transfection was performed using lipofectamine (Invitrogen). Cells were harvested 3 days post-transfection and, after confirmation of GFP expression, total RNA was isolated using Trizol (Invitrogen). 1µg RNA was reverse transcribed and AID mRNA and GAPDH mRNA were measured by qPCR as described above. The primer sequences of GAPDH were 5'-AGGTCGG TGTGAACGGATTTG-3' and 5'-TGTAGACCATGTAGTTGAGGTCA-3'.

**Western blot.** Four days after lentivirus infection, neurons were lysed in 2X Laemmli sample buffer, then sonicated and run on a 12% SDS-PAGE gel. The protein was transferred to a PVDF membrane and incubated overnight with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Membranes were incubated with primary antibody diluted in TBS-T overnight at 4°C. Primary antibodies included mouse anti-AID 1µg/ml (Invitrogen) and rabbit anti-β tubulin 1:5000 (Cell Signaling). The blots were washed and incubated with horseradish peroxidase-labelled secondary antibody for 1 hour and then washed for 30 min. The secondary antibodies used for detection were ECL anti-mouse IgG (Amersham) and ECL anti-rabbit IgG (Amersham). Signals were visualized using chemiluminescence (Perkin Elmer).

**Selection of genomic regions of the BDNF gene for DNA methylation analysis.** DNA methylation within CpGs located proximal to regulatory elements for plasticity-related transcription factors is generally considered to be important for gene silencing. Thus, we chose proximal promoter regions of the BDNF gene upstream of exon I and exon IV that contain these elements as targets for DNA methylation analysis. The UCSC genome browser was used to identify the promoter sequence of exon I (MM10_NM_007540_chr2:109673700-109674699) and exon IV (MM10_NM_001048141 chr2:109691436-109692435). The following primer pairs were designed: BDNF exon I; 5'-aggaagagagTTGTGATTTTTTTGGTAAAAAGGAA-3' and 5'-cagtaatacgactca ctatagggagaaggctCTCTCATTTAAAAAACCATAAACCA-3', and BDNF exon IV; 5'-aggaag agagTTGTTGTTTAGATAATGATAGGTTTGG-3' and 5'-cagtaatacgactcactatagggagaaggct AACAAAAACTAAAAAATTTC ATACTAACTC-3'.

**DNA methylation analysis by nucleotide mass spectrometry.** DNA was extracted from KCl-treated neurons with the AllPrep DNA/RNA Micro Kit (Qiagen) according to the manufacturer's instructions. 1 µg of genomic DNA was treated with bisulphite conversion reagent using a two-step modification procedure outlined in the EZ DNA methylation kit (Zymo). Each assay involved PCR amplification of the region of interest, shrimp alkaline phosphatase (SAP) treatment to remove excess dNTPs, addition of T7-RNA polymerase to generate RNA copies, and RNase A for 'T'-specific cleavage of the RNA. This step was followed by clean up of the extension reaction and cation exchange resin, spotting of the extension product onto a 96 well SpectroCHIP, and analysis of the spotted product by MALDI-TOF nucleotide mass spectrometry (MassArray 4 system, Sequenom).

**Chromatin immunoprecipitation**. Primary cortical neurons were fixed in 1% formaldehyde and cross-linked cell lysates sheared by sonication in 1% SDS lysis buffer. Sonication generated chromatin fragments with an average length of 100-200 bp. Samples were incubated overnight at 4°C with an antibody specific to CREB (1:500, Santa Cruz) or an equivalent amount of control IgG (anti-rabbit; 1:500, Santa Cruz). Next, protein-DNA complexes were precipitated with protein G-magnetic beads at 4°C for 1 hour, followed by two washes in low salt buffer, two washes in high salt buffer, and three washes with TE buffer. After washing, the precipitated DNA-protein complexes were eluted using 1% SDS and 0.1 M $NaHCO_3$. In order to reverse the formaldehyde cross-link, eluted complexes were incubated overnight a 65°C in 200 mM NaCl. Before subjecting the samples to qPCR they were first treated with proteinase K digestion, followed by phenol-chloroform extraction and ethanol precipitation. Primer pairs specific for 200 bp segments corresponding to the BDNF P4 promoter region (5'-GCATGCAATGCCCTGGAACGG-3' and 5'-GAGGGCTCCACGCTGCCTTG-3') were used for qPCR and analysed relative to input, as described above. Three separate experiments were performed using independent biological replicates in each experiment.

## RESULTS

**BDNF mRNA expression is induced in post-mitotic neurons in an activity-dependent manner.** In accordance with previous studies (Martinowich *et al.*, 2003), there was a significant increase in BDNF exon I mRNA (n=6/group, unpaired two-tailed t-test, df=10; 3hr, t=6.34, p<.0001; 5hr, t=15.0, p<.0001; 7hr, t=5.95, p<.0001; 10hr, t=534, p<.001, Figure 1a), and BDNF exon IV expression (n=6/group, unpaired two-tailed t-test, df=10; 3hr, t=9.78, p<.0001; 5hr, t=10.68, p<.0001; 7hr, t=6.15, p<.0001; 10hr, t=4.05, p<.01, Figure 1b), with peak mRNA expression 7 hours after KCl-induced depolarization, *in vitro*.

**KCl-induced depolarization leads to a reduction in DNA methylation within the BDNF P4 promoter.** When DNA methylation was measured 7 hours after KCl-induced depolarization, there was no effect of neuronal activation on this epigenetic modification within the selected region of the

BDNF P1 promoter (Figure 2c), indicating that variations in DNA methylation at this particular locus may not support the activity-dependent induction of BDNF exon I mRNA expression. At the same time-point, there was a general trend toward decreased methylation in the BDNF P4 promoter. At two CpG sites, there was a significant decrease in DNA methylation (n=6/group; CpG-109, -10.63%, unpaired two-tailed t-test df=10, t=2.61, p<.05 and CpG-66, -30.80%, t=2.56, p<.05) following membrane depolarization (Figure 2d). There was also a trend towards decreased DNA methylation at CpG-35.

**Lentiviral-mediated AID knockdown disrupts activity-dependent changes in DNA methylation at the BDNF P4 promoter and inhibits BDNF exon IV mRNA expression.** The AID shRNA showed efficient transfection in primary cortical neurons (Figure 3a) and exhibited 80% knockdown of AID mRNA expression relative to scrambled controls in HEK293T cells (n=9/group, $F_{5,47}$ = 12.04, Figure 3b), with near complete knockdown of AID protein expression (Figure 3c). In primary cortical neurons transfected with AID shRNA 3 days prior to treatment, activity-dependent changes in DNA methylation within the BDNF P4 promoter were disrupted when examined 7 hours post-KCl-induced depolarization (n=6-9/group, CpG-109, $F_{3,22}$ = 3.51, p<.05; CpG-66, $F_{3,22}$ = 14.36, p<.001, Figure 4a). Importantly, BDNF exon IV mRNA expression was also significantly blunted at the same time-point ($F_{3,55}$ = 20.63, p<.0001, Figure 4b). Together, these data suggest a significant role for AID in the modulation of DNA methylation within the BDNF P4 promoter, which correlates with its effect on BDNF exon IV gene expression.

**AID-mediated changes in DNA methylation are necessary for CREB binding to the BDNF P4 promoter.** There was a significant effect of KCl-induced depolarization on CREB occupancy at the BDNF P4 promoter when assessed 7 hours after stimulation (n=3/group, $F_{5,17}$=15.47, p<.0001, Figure 5). These effects were completely blocked in cortical neurons transfected with AID shRNA 3 days prior to activation, suggesting a critical role for AID-mediated changes in DNA methylation and CREB binding at this locus.

## DISCUSSION

This study generated three main findings: 1) the expression of BDNF exon I and IV mRNA in post-mitotic cortical neurons is activity-dependent, 2) CpG sites proximal to regulatory elements within the BDNF P4 promoter are demethylated following KCl-induced depolarization, and 3) AID regulates DNA methylation within this region of the BDNF P4 promoter; an effect which influences CREB binding and subsequent BDNF exon IV mRNA expression. These data suggest that, together with its essential role as an RNA/DNA editing enzyme, AID also contributes to dynamic changes in DNA methylation in post-mitotic neurons, with subsequent effects on activity-dependent BDNF expression.

**BDNF gene expression is activity-dependent and the BDNF P4 promoter is subject to epigenetic regulation.** BDNF is encoded within a complex genomic locus, consisting of nine 5' non-coding exons and a protein-coding 3' region (Aid *et al.*, 2007), with differential usage of BDNF non-coding exons under varying conditions having been reported (Pattabiraman *et al.*, 2005; Chiaruttini *et al.*, 2008). BDNF exons I and IV are of interest because they are activated in response to a wide variety of stimuli, including early life stress, fear conditioning and fear extinction, and are sensitive to epigenetic modification (Martinowich *et al.*, 2003; Bredy *et al.*, 2007; Cokus *et al.*, 2008; Lister *et al.*, 2008; Fuchikami *et al.*, 2010). Our data corroborate the activity-dependent nature of BDNF exons I and IV in primary cortical neurons (Figure 1a and 1b). Accumulating evidence also indicates that activity-dependent induction of BDNF depends on variations in DNA methylation (Lubin *et al.*, 2008). The BDNF P1 and P4 promoters have CpG islands located proximal to their TSS. However, as most CpG islands are protected from methylation, we used nucleotide mass spectrometry to interrogate a 354bp CpG *shore* within the BDNF P1 promoter due to recent evidence indicating that tissue-specific changes in DNA methylation are more likely to occur within CpG *shores* (Doi *et al.*, 2009; Irizarry *et al.*, 2009). The CpG sites in this region are either part of, or in close proximity to, several transcription factor-binding sites, including activator protein 1 (AP-1), specificity protein 1 (Sp1), and neuronal PAS domain protein 4 (Npas4). AP-1 is a transcriptional regulator associated with DNA methylation changes (Ng *et al.*, 2013) and is highly regulated by neuronal activity in prefrontal cortical neurons (Covington *et al.*, 2010). Sp1 belongs to the C2H2-type zinc-finger protein family and is known to mediate enhancement of NMDA receptor subunit type 1 (NR1) promoter activity by binding to GC-rich regions in the promoter (Okamoto *et al.*, 2002). Npas4 is an immediate-early gene that shows very little expression prior to membrane depolarization, but very rapid and robust protein synthesis following stimuli that induce calcium influx into neurons (Lin *et al.*, 2008). We did not observe any changes in DNA methylation within this region of the BDNF P1 promoter following KCl-induced depolarization (Figure 2a). Given that we have only examined a single locus, we cannot rule out the possibility that dynamic variations in DNA methylation in response to neuronal activity may have occurred at distal promoter regions. Indeed, a region 4.8kb upstream of BDNF P1 harbours a consensus sequence for MEF2D, which also regulates transcriptional activity at the BDNF P1 promoter (Flavell *et al.*, 2008).

With respect to the BDNF P4 promoter, we selected a 337bp region encompassing a CpG shore for investigation (Figure 2b). This locus is important because it contains three $Ca^{2+}$ response elements (CaREs), which have been shown to be critical for the activity-dependent induction of BDNF exon IV (Tao *et al.*, 1998; Zheng *et al.*, 2011). These include CREB, upstream stimulatory factors 1/2 (USF1/2) and calcium-responsive transcription factor (CaRF) (Shieh *et al.*, 1998; Tao *et al.*, 1998; Timmusk *et al.*, 1999; Tabuchi *et al.*, 2002; Tao *et al.*, 2002; Chen *et al.*, 2003). Furthermore, this region of the BDNF P4 promoter has also been shown to be sensitive to

epigenetic modification (Martinowich *et al.*, 2003). DNA methylation at this locus was generally reduced following KCl-induced depolarization (Figure 2b), and the two CpG sites, which exhibited the greatest decrease in DNA methylation, are closely positioned near consensus sequences for Sp1 and CaRE1, which is known to be critically involved in the activity-dependent regulation of BDNF exon IV (Lyons & West, 2011; Pruunsild *et al.*, 2011). Although the range of the observed reduction in DNA methylation (10-30%) is similar to previous reports (Martinowich *et al.*, 2003), we cannot rule out the possibility that a greater reduction in DNA methylation within distal regions (including enhancers) may have led to a cumulative effect on the transcriptional output of the BDNF P4 promoter. Nonetheless, together these data lend further support to the notion that dynamic changes in DNA methylation can regulate transcription (Cokus *et al.*, 2008; Lister *et al.*, 2008; Rai *et al.*, 2008; Bhutani *et al.*, 2010), and highlight the selectivity and complexity of activity-dependent epigenetic regulatory processes, as not all CpG sites were affected by this form of neuronal stimulation.

**AID regulates DNA methylation and influences BDNF exon IV gene expression through indirect effects on CREB binding at the BDNF P4 promoter.** The involvement of AID in the regulation of DNA methylation has been demonstrated using interspecies heterokaryons (Bhutani *et al.*, 2010), zebrafish (Rai *et al.*, 2008) and ES cells (Cokus *et al.*, 2008; Lister *et al.*, 2008). Recently, Song and colleagues found that AID promotes active DNA demethylation within the adult hippocampus by preferentially targeting hydroxylated methylcytosine (5-hmC) (Guo *et al.*, 2011). As a caveat, the hippocampal formation contains a neurogenic niche, which contains a heterogeneous population of cells at various stages of development, thereby limiting the general interpretation of these findings. Whether the activity of AID is functionally relevant in fully differentiated cortical neurons has remained an open question. In order to address this, we explored the role of AID-mediated regulation of DNA methylation within the BDNF P4 promoter, and its effect on BDNF exon IV mRNA expression. Firstly, we observed a significant increase in AID mRNA expression in primary cortical neurons following KCl-induced depolarization, which suggests that AID is induced in differentiated cortical neurons in an activity-dependent manner (data not shown). In primary cortical neurons transfected with AID shRNA, the dynamic changes in DNA methylation within the BDNF P4 promoter that occur as a function of KCl-induced depolarization were clearly disrupted (Figure 4a) and this effect was accompanied by a significant reduction in BDNF exon IV mRNA expression (Figure 4b), as well as decreased CREB occupancy at the BDNF P4 promoter (Figure 5). The effect of stimulation on DNA methylation at CpG-109 and CpG-66 was restored in an activity-dependent manner. Interestingly, DNA methylation at CpG-35 increased in the presence of AID shRNA. These observations suggest that there is a complex interaction between KCl-induced changes in DNA methylation and AID-mediated effects on this epigenetic process. A limitation of our approach is that the bisulphite conversion of modified DNA cannot distinguish between 5-mC and the newly identified DNA modification, 5-hmC (Huang *et al.*,

2010; Booth *et al.*, 2012). Thus, it is possible that following AID knockdown, we may be observing an increase in the accumulation of 5-hmC or some other oxidative mark. Nonetheless, the data suggest that, together with its essential role as an RNA/DNA editing enzyme, AID contributes to dynamic changes in DNA methylation in post-mitotic neurons, with subsequent effects on activity-dependent BDNF exon IV expression.

Although the mechanistic details of this process remain to be elucidated, there are a number of potential ways in which AID may regulate gene expression. For example, activity-induced DNA demethylation has been shown to depend on a combination of AID, TET, and Gadd45a activity (Ma *et al.*, 2009; Guo *et al.*, 2011; Franchini *et al.*, 2012), and emerging evidence indicates that AID and TET1 predominantly co-occupy promoter regions (Guo *et al.*, 2011). It is possible that AID belongs to a complex involving other epigenetic regulatory proteins that are directed to specific genomic loci in response to KCl-induced depolarization, as may be the case with the BDNF P4 promoter. In addition, it has recently been shown that members of the RNA processing/degradation complex act as co-factors in targeting AID to double-stranded DNA substrates (Basu *et al.*, 2011; Fruhbeis *et al.*, 2012), which may also promote the selective recruitment of AID to methylated CpGs along the BDNF P4 promoter. Finally, the long noncoding RNA p68/Ddx5 colocalizes with the RNA binding protein Gadd45a (Schwarz *et al.*, 2000). Long non-coding RNAs function as decoys for transcription-related factors, as modular scaffolds, or as guides to direct chromatin-modifying complexes to their genomic sites of action (Rinn & Chang, 2012; Spadaro & Bredy, 2012; Mercer & Mattick, 2013). Thus, we are currently testing the hypothesis that the tethering and targeting of an AID/Gadd45a complex by p68/Ddx5 promotes CpG-specific changes in DNA methylation within the BDNF P4 promoter and regulates the activity-dependent induction of BDNF exon IV.

In summary, we have confirmed that the induction of BDNF exon IV mRNA expression in cortical neurons is activity-dependent and correlates with a reduction in DNA methylation at specific CpG sites within the proximal BDNF P4 promoter. Lentiviral-mediated knockdown of AID disrupted the effect of KCl-induced depolarization on DNA methylation and blocked BDNF exon IV mRNA expression, an effect that was associated with decreased CREB occupancy within the BDNF P4 promoter. Thus, these findings highlight the important role of AID in promoting the epigenetic regulation of activity-dependent BDNF expression in post-mitotic neurons.

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**Figure 1. BDNF mRNA expression is induced in post-mitotic neurons in an activity-dependent manner.** Transcript levels of (a) BDNF exon I and (b) BDNF exon IV in primary cortical neuronal cells exhibit a time-dependent increase in expression following KCl-induced depolarization. Values represent mean ± S.E.M. (n=6 for each condition; Student's t-test KCl- vs KCl+ at each time point, **p<.01, ***p<.001; ****p<.0001).

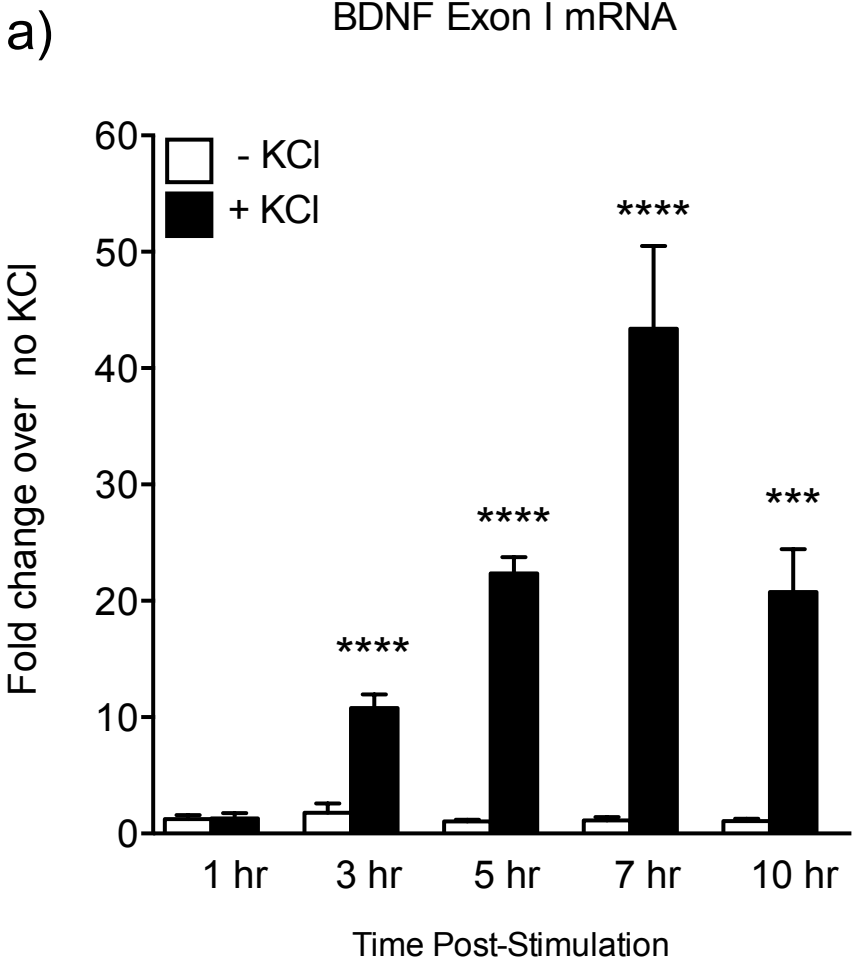
**Figure 2. KCl-induced depolarization leads to a reduction in DNA methylation within the BDNF P4 promoter.** DNA methylation was measured at CpG sites proximal to regulatory elements within the (a) BDNF P1 and (b) BDNF P4 gene promoters. Nucleotide mass spectrometry analysis revealed (c) no effect on DNA methylation within the P1 promoter, and (d) a significant decrease in DNA methylation at two specific CpG sites, CpG-109 and CpG-66, within the BDNF P4 promoter. Percent methylation represented as mean ± S.E.M. (n=6 for each condition; Student's t-test KCl- vs KCl+ at each CpG site, *p<.05).

**Figure 3. Validation of AID knockdown construct.** (a) Representative image of AID shRNA lentiviral transfection in primary cortical neurons, (b) The efficiency of various AID shRNAs were determined by ectopic expression of AID and simultaneous transfection of AID shRNA, which revealed an 80% reduction in AID mRNA by AID shRNA2 when measured 3 days after viral treatment. Values represent mean ± S.E.M. (n=9 for each condition; Dunnett's post-hoc test, AID Ox vs. AID Ox + AID sh2 ****p<.0001), c) The effect of AID shRNA on mRNA levels was accompanied by a reduction in AID protein expression.

**Figure 4. Lentiviral-mediated AID knockdown disrupts activity-dependent changes in DNA methylation at the BDNF P4 promoter and inhibits BDNF exon IV mRNA expression.** (a) Nucleotide mass spectrometry analysis revealed a disruption in the effect of neuronal stimulation on DNA methylation within the BDNF P4 promoter in primary cortical neurons pre-treated for 3 days with AID shRNA and examined 7 hours after KCl-induced depolarization. Percent methylation represented as mean ± S.E.M. (n=6-9/group, CpG-109; $F_{3,22} = 14.36$, p<.001, -KCl vs +KCl p<.05; -KCl AID shRNA vs +KCl AID shRNA, **p<.01; CpG-66; $F_{3,22} = 6.18$, p<.01, -KCl vs +KCl p<.01; -KCl AID shRNA vs +KCl AID shRNA, *p<.05). b) Diminished expression of BDNF exon IV in the neurons transfected with AID shRNA, when measured at the same time-point. Values represent mean ± SEM (n=6-9 for each condition; +KCl control vs +KCl AID shRNA, **p<.01).

**Figure 5. AID-mediated changes in DNA methylation are necessary for CREB binding to the BDNF P4 promoter.** Activity-dependent CREB binding to the BDNF P4 promoter in both mock- and scrambled control-treated samples is blocked in primary cortical neurons transfected with AID shRNA for 3 days, when examined 7 hours after KCl-induced depolarization. Values represent

mean ± S.E.M. (Dunnett's post-hoc analysis relative to empty vector KCl- revealed a significant increase in CREB binding following KCl treatment in both empty (***p<.001) and control-treated wells (*p<.05), but not in neurons treated with AID shRNA.


a)
BDNF Exon I mRNA
- KCl
+ KCl
Fold change over no KCl
60
50
40
30
20
10
0
****
****
***
1 hr
3 hr
5 hr
7 hr
10 hr
Time Post-Stimulation

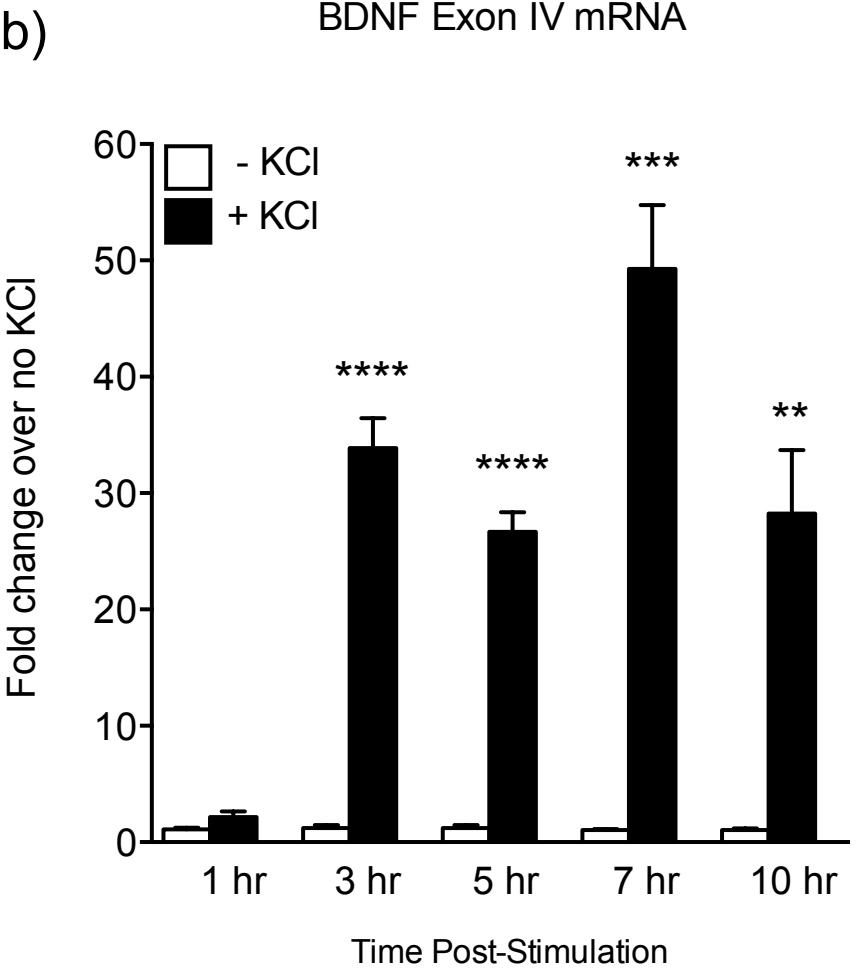
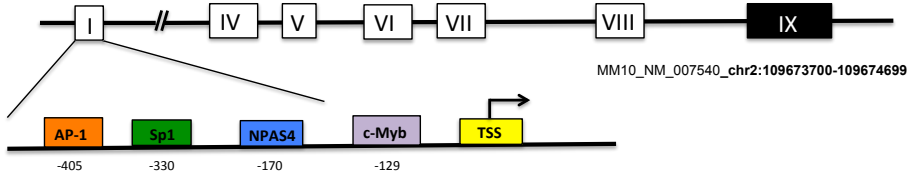

b)
BDNF Exon IV mRNA
- KCl
+ KCl
Fold change over no KCl
60
50
40
30
20
10
0
****
****
***
**
1 hr
3 hr
5 hr
7 hr
10 hr
Time Post-Stimulation

Figure 1

a) BDNF P1 promoter (-440 to -87)




CTCTCATTTGAGAAGCCATAAGCCATTAGAGCAAACGCAGTCATAACTTCATTCAACTCAAGCCGCTTGAGAGCTTGGCTTGCACAGGTTCCTGTGGG
CAACTAGTGGCTCGCCCGGGTGCCTCTCGCCTAGTCATCAGTCCCTAAGAGGAAAAGGGAAAGTTGTGGGGCTGATGCGCTCTTCGATTCACGCAGT
TGTTCCCTAGAACAAGTCACTCCCGCTCCATCACCCAGCCCCCCCCCCCCTCCCATTTGATCATCACTCACGACCACGTCCGCTGGAGACCCTTAGTCA
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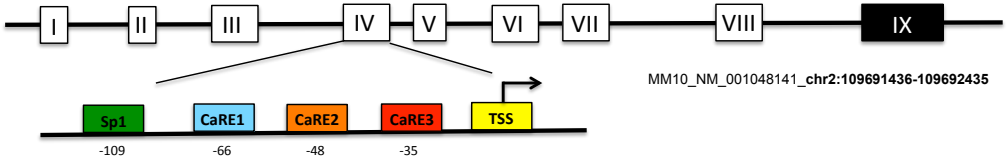
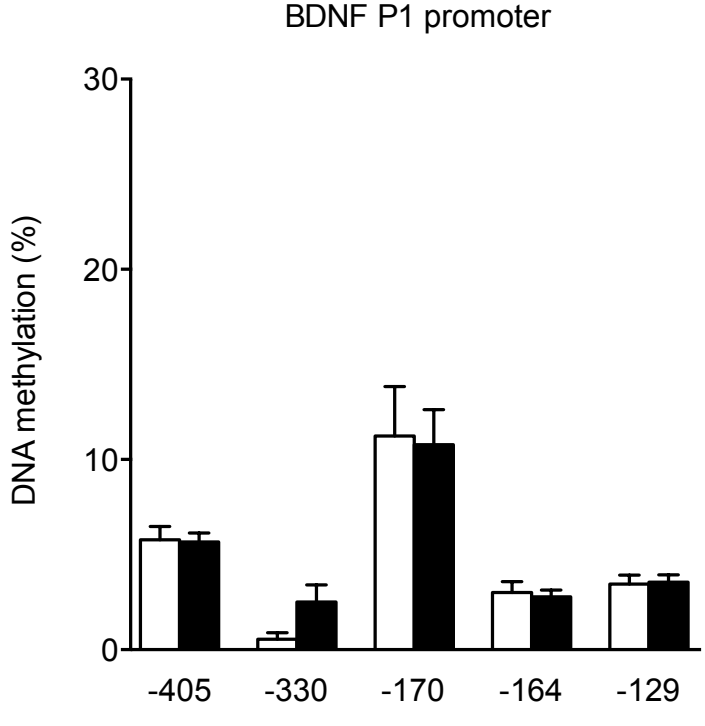
b) BDNF P4 promoter (-242 to 95)




TTGCTGCCTAGATAATGACAGGCTTGGCTTCTGTGTGCGTGAATTTGCTAGGACTGGAAGTGAAAACATCTACAAAGCATGCAATGCCCTGGAACGGAATT
CTTCTAATAAAAGATGTATCATTTTAAATGCGCGGAATTCTGATTCTGGTAATTCGTGCACTAGAGTGTCTATTTCGAGGCAGAGGAGGTATCATATGACA
GCTCACGTCAAGGCAGCGTGGAGCCCTCTCGTGGACTCCCACCCACTTTCCCATTCACCGAGGAGAGGACTGCTCTCGCTGCCGCTCCCCCCACCCACCCCC
GGCGAGCTAGCATGAAATCTCCCAGCCTCTGCC

c)




d)

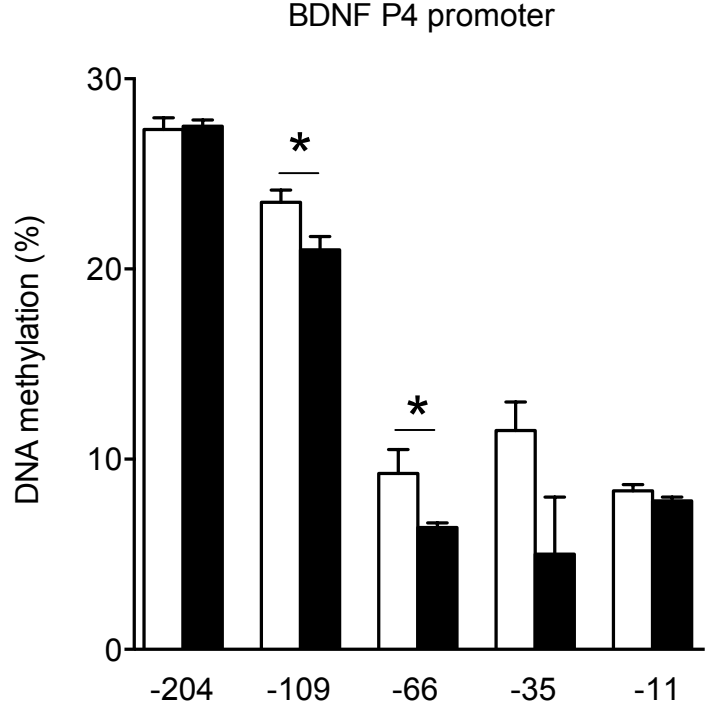



Figure 2

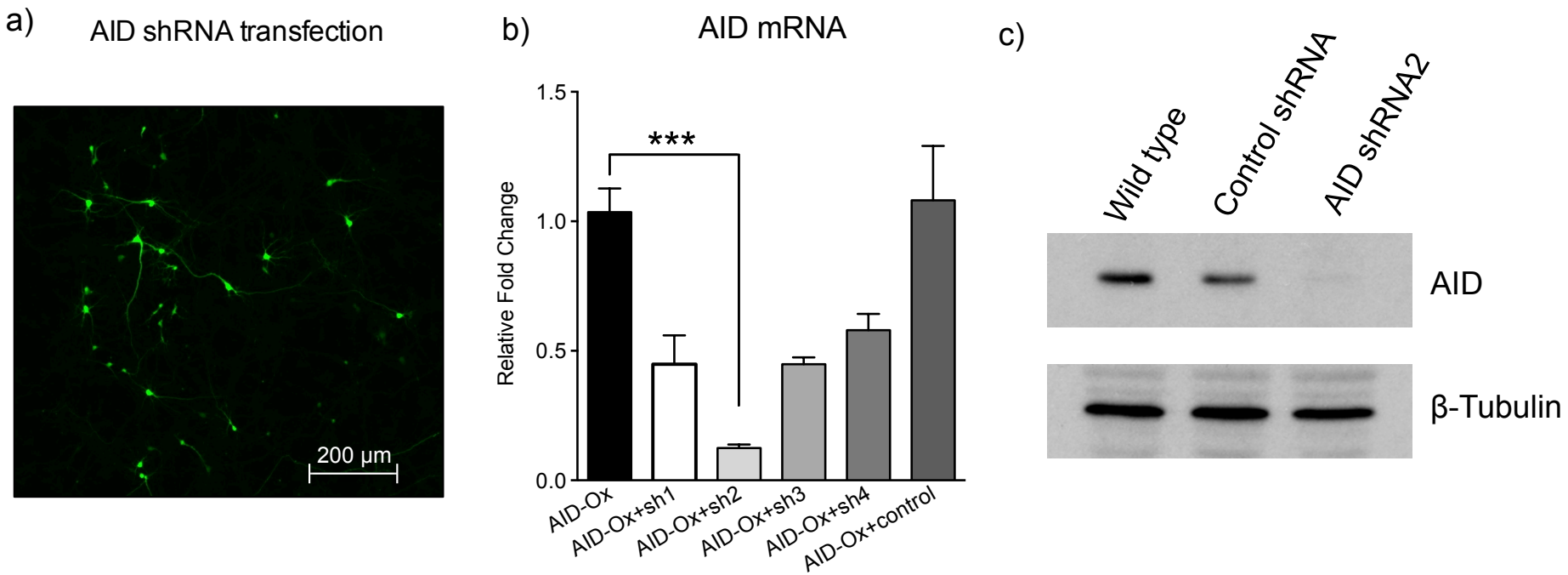

a)
AID shRNA transfection
200 μm
b)
AID mRNA
Relative Fold Change
1.5
1.0
0.5
0.0
***
AID-Ox
AID-Ox+sh1
AID-Ox+sh2
AID-Ox+sh3
AID-Ox+sh4
AID-Ox+control
c)
Wild type
Control shRNA
AID shRNA2
AID
β-Tubulin

Figure 3

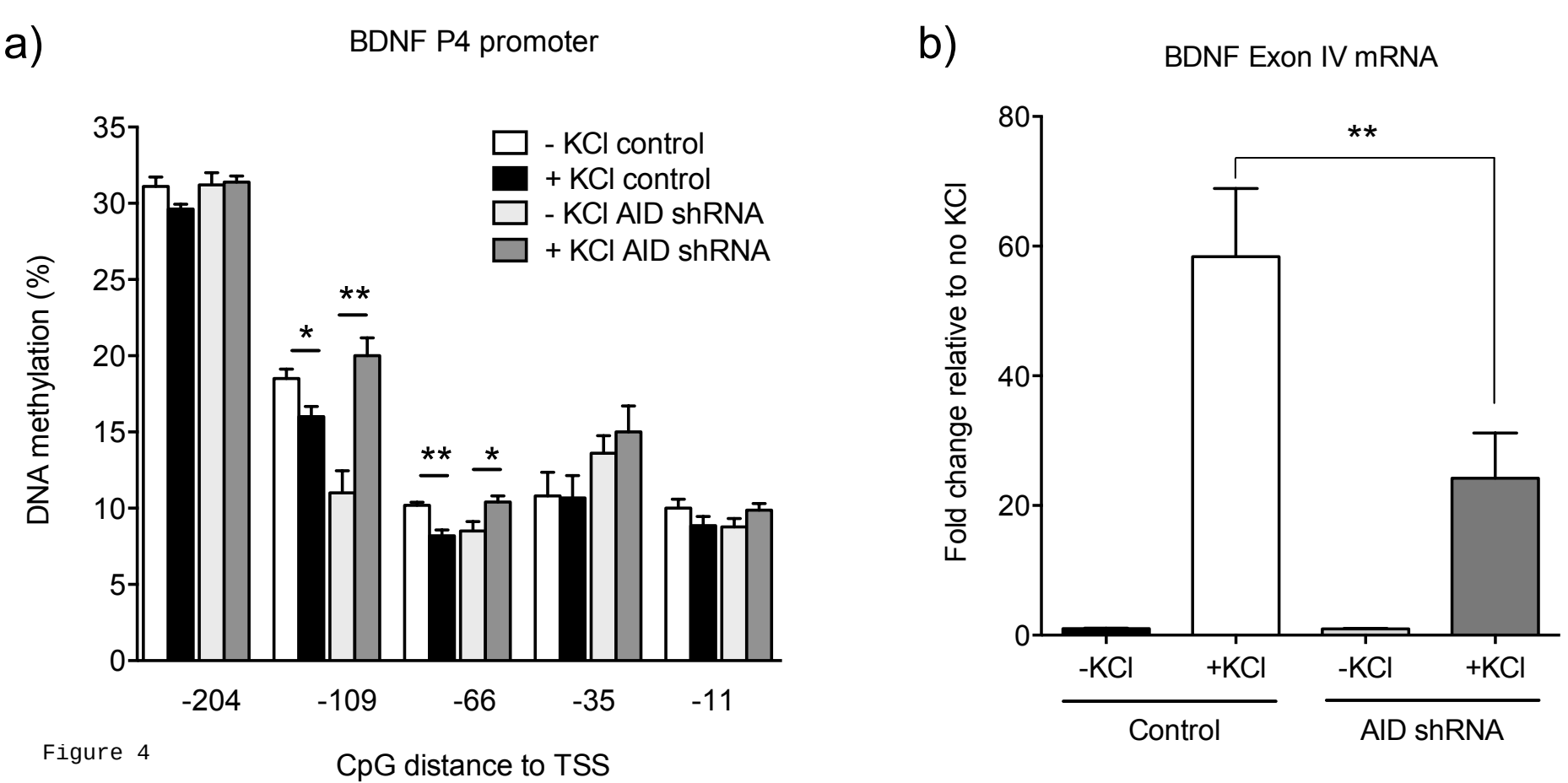

a)
BDNF P4 promoter
- KCl control
+ KCl control
- KCl AID shRNA
+ KCl AID shRNA
DNA methylation (%)
35
30
25
20
15
10
5
0
*
**
**
*
-204
-109
-66
-35
-11
CpG distance to TSS
b)
BDNF Exon IV mRNA
Fold change relative to no KCl
80
60
40
20
0
**
-KCl
+KCl
-KCl
+KCl
Control
AID shRNA

Figure 4

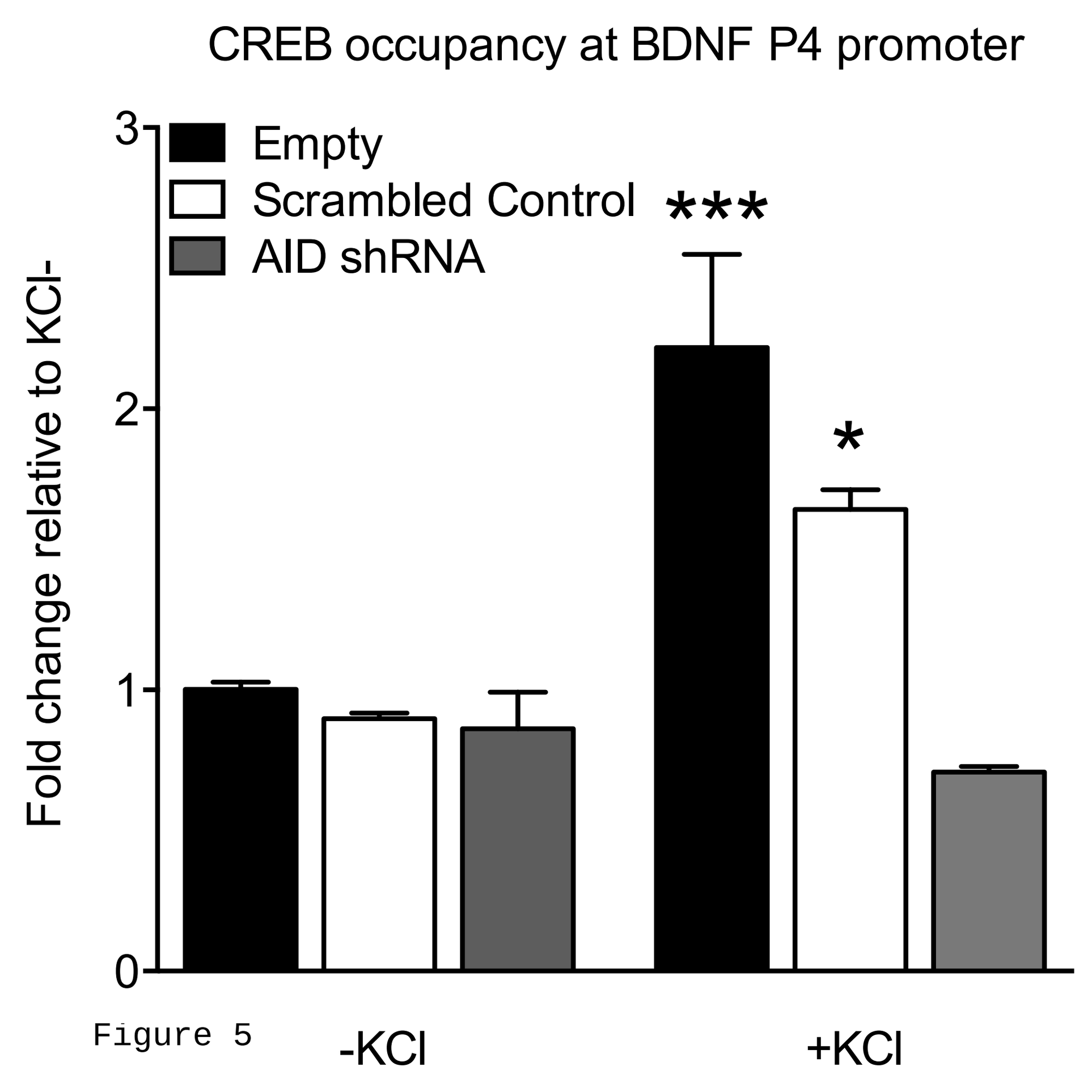

CREB occupancy at BDNF P4 promoter
Empty
Scrambled Control
AID shRNA
***
*
Fold change relative to KCl-
3
2
1
0
-KCl
+KCl

Figure 5