BET protein Brd4 activates transcription in neurons and BET inhibitor Jq1 blocks memory in mice

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Precise regulation of transcription is crucial for the cellular mechanisms underlying memory formation. However, the link between neuronal stimulation and the proteins that directly interact with histone modifications to activate transcription in neurons remains unclear. Brd4 is a member of the bromodomain and extra-terminal domain (BET) protein family, which binds acetylated histones and is a critical regulator of transcription in many cell types, including transcription in response to external cues. Small molecule BET inhibitors are in clinical trials, yet almost nothing is known about Brd4 function in the brain. Here we show that Brd4 mediates the transcriptional regulation underlying learning and memory. The loss of Brd4 function affects critical synaptic proteins, which results in memory deficits in mice but also decreases seizure susceptibility. Thus Brd4 provides a critical link between neuronal activation and the transcriptional responses that occur during memory formation.

The nervous system requires tight control of transcription in response to external signals. Rapid activation of immediate early genes (IEGs) in response to stimulation is critical for synaptic plasticity and is observed in vivo during learning and memory. Misregulation of gene expression in the brain results in neuronal deficits and neurodevelopmental disorders1,2, and inhibition of transcription immediately after neuronal stimulation blocks the mechanisms underlying memory formation3–6. This inducible transcription requires that transcriptional activators bind to promoters of target genes and recruit other proteins such as RNA polymerase II (PolII)7,8. Recent work found that, in several non-neuronal cell types, the protein Brd4 is critical in regulating the recruitment of protein complexes such as positive transcription elongation factor b (P-TEFb) to allow PolII phosphorylation and the subsequent elongation of target genes in response to a signal9–12.

Brd4 is a member of the BET protein family and functions as a chromatin ‘reader’ that binds acetylated lysines in histones13,14. Knockout of Brd4 in mice is lethal15, and recent elegant work indicates that small molecule inhibitors of BET proteins represent a promising therapeutic strategy for several types of cancer16–18. Brd4 also regulates stimulus-dependent transcription in postmitotic cells by recruiting P-TEFb to target promoters in response to extracellular signals13,19. While P-TEFb recruitment is necessary for transcriptional elongation in neurons20, the link between neuronal stimulation and the proteins that directly interact with histone modifications to activate transcription remains unclear.

Brd4 is well positioned to regulate transcription in neurons in response to neuronal activation. Acetyl marks are critical to brain function and are linked to memory formation and neurological disorders21. Brd4 activity is regulated by casein kinase 2 (CK2)14, which is activated in response to neuronal stimulation22. In addition, a full understanding of whether and how Brd4 functions in the brain is of particular importance now because BET protein inhibitors are in clinical trials.

Here we show that Brd4 is critical to neuronal function and mediates the transcriptional regulation underlying learning and memory. We found that Brd4 regulates IEG transcription in neurons in response to activity and is regulated by CK2. Loss of Brd4 function affects critical synaptic proteins and the BET inhibitor Jq1 results in memory deficits and decreases seizure susceptibility in mice. These results are, to our knowledge, the first demonstration of Brd4 function in the brain and provide a critical link between neuronal activity and transcriptional activation that underlies memory formation. In addition, our data call attention to the potential for small molecule inhibitors of BET proteins such as Jq1 to cause neuronal deficits. While BET protein inhibitors are a promising therapeutic strategy for several types of cancer17,18,23–25, modifications preventing blood-brain barrier penetrability may be necessary to prevent neurological side effects.

RESULTS
Brd4 is expressed in neurons

We examined Brd4 expression in adult mice using an antibody that detects the full-length form of Brd4 and found that it was expressed throughout the brain (Fig. 1a and Supplementary Fig. 1a). Brd4 positive cells typically expressed NeuN but not GFAP in both cortex and hippocampus (Fig. 1b–i), indicating that Brd4 is present in neurons while generally not seen in glial cells. In addition, we separately cultured cortical neurons and glia and found that neurons contained more Brd4 mRNA and protein than glial cells (Fig. 1j,k). Both CamKII-positive excitatory neurons and GABA-positive inhibitory neurons expressed Brd4 (Supplementary Fig. 1b,c). Finally, we treated cultured neurons with brain-derived neurotrophic factor (BDNF) to mimic physiological activation in the brain5, which resulted in small increases in Brd4 mRNA and protein (Supplementary Fig. 1d–f).

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Figure 1  Brd4 is expressed in neurons throughout the brain. (a) Brd4 staining of a sagittal adult mouse brain section. (b,f) Brd4 and NeuN staining of cortex (b) or hippocampus (f). (c,g) High-magnification image of Brd4 and NeuN staining of cortex (c) or hippocampus (g). (d,h) Brd4 and GFAP staining of cortex (d) or hippocampus (h). (e,i) High magnification image of Brd4 and GFAP staining of cortex (e) or hippocampus (i). (j) Western blot analysis of Brd4 protein from whole-cell lysate of cultured cortical neurons or glia. (k) Brd4 mRNA from cultured cortical neurons or glia (n = 3 biological replicates, paired two-tailed t test, P = 0.0057, t = 4.195). Full-length blots are presented in Supplementary Figure 10. In box plots, whiskers show minimum and maximum, box limits are first and third quartile, and center line is median. DAPI, 4′,6-diamidino-2-phenylindole. ***P < 0.001. Scale bars, 1 mm (a) and 10 μm (b–i).
Brd4 regulates IEG transcription in neurons

Like other post-mitotic cells that require Brd4 (refs. 13,19), neurons activate a subset of genes (IEGs) in response to external signals. This rapid response is critical to the consolidation of synaptic modifications underlying synaptic plasticity and memory formation3-6. We examined whether Brd4 is involved in transcriptional activation in neurons using the small molecule inhibitor Jq1, which blocks BET proteins from binding to acetylated histones18. After pretreatment with Jq1 or the negative enantiomer (−)Jq1, we stimulated cultured cortical neurons with BDNF (Fig. 2a). As expected, BDNF caused a rapid increase in transcripts of the IEGs Arc and Fos. However, pretreatment with Jq1 blocked the BDNF-induced increase (Fig. 2b,c). Rapidly induced IEGs such as Arc and Fos have PoII poised on their promoters to allow immediate activation, while other IEGs such as Nr4a1 must both recruit and phosphorylate PoII to activate transcription20. We found that Jq1 also prevented the activity-induced increase of Nr4a1 (Fig. 2d), indicating that Jq1’s effects are not limited to IEGs with poised PoII.

We similarly examined the effects of Jq1 on tetrodotoxin (TTX) withdrawal which rapidly increases neuronal activity. Neurons were treated with TTX for 2 d, after which Jq1 or the negative enantiomer was added before TTX was removed from the medium. Jq1 pretreatment blocked the increase in nascent Arc in neurons stimulated for 30 min with BDNF following pretreatment with Jq1 or the negative enantiomer (−)Jq1 (unpaired two-tailed t test, for Arc n = 232, for BDNF n = 185, for Jq1 n = 245 and for Jq1 + BDNF n = 200 from 5 biological replicates per group; for control versus BDNF P = 8.52 × 10−25, t = 11.447; Jq1 versus Jq1 + BDNF P = 0.00084, t = 3.363; for BDNF versus Jq1 + BDNF P = 0.00013, t = 3.867). (m,n) Quantification (m) and staining (n) for Arc in neurons transfected with GFP and either a nontargeting siRNA pool or Brd4 siRNA (unpaired two-tailed t test, for control n = 105 neurons, for BDNF n = 80, for Brd2 siRNA n = 85, for Brd2 + BDNF n = 71, for Brd3 n = 88, for Brd3 + BDNF n = 63, for Brd4 n = 80 and for Brd4 + BDNF n = 71 from 11 biological replicates; for control versus BDNF P = 0.0012, t = 3.296; for BDNF siRNA versus Brd4 siRNA + BDNF P = 0.662, t = 0.438; for Brd2 siRNA versus Brd2 siRNA + BDNF P = 8.572 × 10−4, t = 4.035; for Brd3 siRNA versus Brd3 siRNA + BDNF P = 0.167, t = 2.42; for BDNF versus Brd4 siRNA + BDNF P = 0.0157, t = 2.445. *P < 0.05, **P < 0.01, ***P < 0.001. n.s., nonsignificant (P > 0.05). a.u., arbitrary units. Error bars represent s.e.m. In box plots, whiskers show minimum and maximum, box limits are first and third quartile, and center line is median. Scale bars, 10 μm.
again prevented rapid IEG induction (Fig. 2e–g). Notably, Jq1 could not prevent IEG activation after long periods of BDNF stimulation (Supplementary Fig. 2a–c), suggesting that Jq1 only affects the rapid increase in transcription, whereas at later times signaling may be robust enough to overcome BET inhibition. Because Jq1 also inhibits other members of the BET protein family, we tested whether loss of Brd4 is sufficient to block IEG induction. Partial knockdown of Brd4 with a lentivirus also blocked BDNF-induced IEG expression but did not block upstream pathways such as mitogen-activated protein kinase (MAPK) signaling (Fig. 2h–j and Supplementary Fig. 2d,e). These data fit with a model similar to that observed in other cell types, in which Brd4 recruits P-TEFb to promote PolII phosphorylation to allow rapid transcriptional elongation.

To confirm that the loss of transcriptional activation results in a corresponding change in protein levels, we examined Arc protein expression at 30 min, when newly transcribed mRNA has been translated into protein. As expected, Jq1-treated neurons showed less Arc protein induction than control neurons (Fig. 2k,l). Similarly, transfection of small interfering RNAs targeted against Brd4 blocked the BDNF-induced increase in Arc protein, whereas transfection with nontargeting siRNA or siRNAs targeted against the other BET family members did not (Fig. 2m,n and Supplementary Fig. 2f–i). BrdT is testis-specific, and so we did not test it. To ensure that the loss of Arc induction was not due to off-target effects, we tested two distinct Brd4 siRNAs. These also blocked BDNF-induced Arc expression (Supplementary Fig. 2j–l).

Finally, we sought to determine which of Brd4’s known functions is responsible for its effects in neurons. Full-length Brd4 can function by recruiting complexes such as P-TEFb and Mediator to trigger elongation, whereas both the long and short forms of Brd4 can promote PolII progression through acetylated nucleosomes after elongation begins26. We found that only the long form of Brd4 affects Arc expression (Supplementary Fig. 2m), indicating that Brd4 is likely functioning in neurons by recruiting coactivating complexes to target genes.

These data demonstrate that Brd4 regulates activity-induced IEG expression in neurons. However, inhibition of Brd4 may also disrupt transcriptional output in neurons even without a potent stimulus such as BDNF as a result of the cumulative loss of the response to endogenous signaling from other neurons over a long period of time. Indeed, we found that, by 24 h, Jq1 decreased Arc, Fos and Nt4a1 transcripts (Supplementary Fig. 2n). We also confirmed that long-term disruption of this BET function did not cause widespread disruption of chromatin acetylation (Supplementary Fig. 2o), as expected from its function as reader protein. Finally, we showed that Jq1 did not block upstream signaling pathways by demonstrating that MAPK phosphorylation is intact (Supplementary Fig. 2p). Together, these data demonstrate that Brd4 is responsible for transcription of IEGs in neurons.

**Genome-wide effects of Jq1**

While Brd4 clearly regulates specific IEGs, inhibition of Brd4 likely also affects a wider range of genes. We used RNA-sequencing to examine BDNF induction of IEGs after Jq1 treatment and long-term effects of Jq1. An extended pretreatment of Jq1 followed by BDNF stimulation (Supplementary Fig. 3a) recapitulated the effects of Jq1 on induction of the specific IEGs previously examined but did not affect the housekeeping gene Gapdh (Fig. 3a). We expanded this comparison to all genes significantly induced by BDNF and found a consistent decrease in induction in the presence of Jq1 (Fig. 3b and Supplementary Fig. 3b), supporting a model in which Brd4 mediates the rapid response to neuronal activity. In addition, we also examined the effects of 24 h of Jq1 treatment in the absence of exogenous stimulation. At this later time point, the effects of the loss of IEG transcription in response to basal levels of endogenous neuronal signaling will be apparent. We found a highly significant overlap between BDNF-induced genes and those regulated by Jq1 alone ($P < 0.000005$, paired two-tailed t-test). We also separately examined genes up- and down-regulated by Jq1, as downregulated genes are more likely to be direct targets of Brd4 as a result of its function as a transcriptional activator. Gene clusters most significantly downregulated by Jq1 included genes involved synapse function (Fig. 3c) and ion channel function (Fig. 3d). Jq1 treatment also increased genes involved in chromatin regulation and genes encoding nuclear proteins (Supplementary Fig. 3c). Such increases may be compensatory effects or indirect effects resulting from decreases in Brd4 target genes. To determine which histone modifications are responsible for recruiting Brd4 to target genes, we examined known Brd4 target acetyl marks and found that BDNF increased H3K14 and H4K16 acetylation at IEG promoters (Supplementary Fig. 3d), suggesting they may be involved in the stimulus-dependent recruitment of Brd4. As expected, Jq1 did not affect these marks (Supplementary Fig. 3e).
Brd4 is regulated by CK2 in neurons

To better understand how Brd4 is targeted to chromatin in neurons, we next examined the mechanism underlying activation of Brd4 itself. We found that BDNF stimulation increased Brd4 association with promoter regions of IEGs, suggesting that neuronal activity targets Brd4 to acetylated histones immediately after stimulation (Supplementary Fig. 4a–c).

This effect was not observed for Brd2 or Brd3 (Supplementary Fig. 4d–i). To investigate how Brd4 is targeted to promoters, we explored the role of CK2. In the human embryonic kidney (HEK) 293 cell line, CK2 phosphorylates Brd4, which triggers Brd4 binding to acetylated histones at target gene promoters to activate transcription14. In neurons, CK2 is important in regulating synaptic strength27,28 and is activated by BDNF stimulation22. We found that BDNF-induced targeting of Brd4 was blocked by pretreatment with the CK2 inhibitor 4,5,6,7-tetra bromo benzotriazole (TBB), as well as Jq1 (Fig. 4a–c), suggesting that Brd4 is activated by CK2 in response to neuronal activity. This short TBB pretreatment did not prevent MAPK phosphorylation, demonstrating upstream signaling is intact (Supplementary Fig. 4j). Neither TBB nor Jq1 prevented BDNF-induced CREB binding protein (CBP) association with chromatin, indicating that other transcriptional cofactors are still recruited to target genes (Supplementary Fig. 4k–m). If Brd4 activation by CK2 is necessary for the activity-dependent transcription, then CK2 inhibition should also block the activity-induced increase in IEGs. Fitting with this model, pretreatment with TBB blocked the increase in Arc, Fos and Nr4a1 mRNA (Fig. 4d–i), as well as Arc protein levels (Fig. 4j–k). To control for off-target effects of TBB, we confirmed that transfection of Cskn2a1 (CK2) siRNA also blocked Arc protein induction (Supplementary Fig. 4n–p).
Figure 5 Phosphorylation of Brd4 is critical for its function. (a) Model of Brd4 and the critical amino acids in the CK2 phosphorylation site. (b) Western blot for phosphorylated Brd4 (pBrd4) shows an increase with BDNF but not after TBB pretreatment or phosphatase treatment of lysates. Representative of 3 biological replicates. (c,d) Staining (c) and quantification (d) for Arc and Brd4 in neurons transfected with GFP and Brd4 bearing deletions or mutations in the CK2 site (unpaired two-tailed t test, for GFP n = 68, for Brd4 n = 61, for CK2 deletion n = 46, for deletion 492–494 n = 44, for S492A n = 54, for Brd4-pm n = 51 from 5 biological replicates; for GFP versus Brd4 P = 1.223 × 10^{-8}, t = 6.09; for GFP versus Brd4-pm P = 2.00 × 10^{-13}, t = 3.36; for Brd4 versus CK2 deletion P = 0.0011, t = 3.36; for Brd4 versus deletion 492–494 P = 0.00037, t = 2.53; for Brd4 versus S492A P = 0.0075, t = 2.72; for Brd4 versus Brd4-pm P = 0.0204, t = 2.35). (e) Quantification of the mobile fraction from FRAP performed on Brd4 with mutations in the CK2 domain (unpaired two-tailed t test, for Brd4 n = 57 neurons, for S492A n = 52 and for Brd4-pm n = 38 from 3 biological replicates; for Brd4 versus S492A P = 0.0001, t = 4.04; for Brd4 versus Brd4-pm P = 0.0085, t = 2.488). (f) Pearson correlation coefficient for H4K16ac colocalization with Brd4 with CK2 site mutations (two-sided, two-tailed t test for Brd4 n = 35 neurons, for Brd4 + BDNF n = 21, for S492A n = 29 and for Brd4-pm n = 18 from 3 biological replicates; for Brd4 versus S492A + BDNF P = 0.0164, t = 2.477, for Brd4 versus S492A P = 0.00167, t = 3.226; for Brd4 versus S492A P = 0.035, t = 2.166). *P < 0.05. ***P < 0.001. a.u., arbitrary units; p’ase, phosphatase. Full-length blots are presented in Supplementary Figure 4. Error bars represent s.e.m. Scale bar, 10 μm.

To further support our proposed mechanism of Brd4 phosphorylation-induced chromatin targeting, we examined the movement of Brd4 after neuronal stimulation using fluorescence recovery after photobleaching (FRAP). Using live neurons, we photobleached a region of the nucleus of expressing EGFP-Brd4 and observed the recovery of the signal in the bleached region over time to measure the mobile fraction of Brd4. As expected, Iq1 increased the mobile fraction (Supplementary Fig. 4g). The mobile fraction of Brd4 also increased after BDNF treatment, presumably as it is activated and relocates to acetylated chromatin. However, the enhanced mobility of Brd4 was blocked by TBB (Fig. 4l–n), indicating that CK2 is responsible for this effect.

To better understand the mechanism of Brd4 activation in neurons, we examined the specific serine residues in the CK2 site in Brd4, Ser492 and Ser494, that are critical to Brd4 activation (Fig. 5a). We developed a site-selective antibody against a peptide containing the Brd4 CK2 site with phosphorylated Ser492. Using a dot blot assay, we found that the affinity-purified antibody specifically bound a peptide phosphorylated at Ser492, as well as a peptide phosphorylated at both Ser492 and Ser494, but did not recognize the unphosphorylated peptide (Supplementary Fig. 5a). Next we used neuronal lysates to determine the specificity of the antibody in cells by western blot and observed a band matching the size of full-length Brd4 that was not present in lysates treated with phosphatase, although other low-molecular-weight bands were also observed at high exposures (Supplementary Fig. 5b). We observed a robust BDNF-induced increase in the phospho-Brd4 signal that was lost with TBB pretreatment (Fig. 5b).

Next, to ensure that the effects of CK2 inhibition and knockdown were due to its effects on Brd4 and not an indirect effect of other CK2 targets, we tested the critical target residues in the CK2 phosphorylation site in Brd4. We created a full deletion (delCK2) and partial deletion (del492–494) and a point mutation (S492A) in the CK2 site in Brd4. Transfection of wild-type Brd4 increased Arc expression even in the absence of exogenous stimulation. However, this effect was greatly reduced when the CK2 site was mutated or deleted. Conversely, phosphomimetic (Brd4-pm) mutations at the key serines in Brd4 (S492E and S494E) resulted in an even greater increase in Arc expression (Fig. 5c,d). This demonstrates that phosphorylation of the CK2 site in Brd4 is necessary for its ability to activate transcription of Arc. We also repeated our FRAP assay and found the S492A mutant decreased Brd4 mobility, presumably by preventing activation of Brd4 from endogenous signaling, while the phosphomimetic Brd4 behaved similarly to BDNF-stimulated Brd4, showing an increased mobile fraction (Fig. 5e). Finally, we sought to confirm that this increase in the mobile fraction corresponded to a translocation to active chromatin. We focused on acetylated H4K16ac, which recruits Brd4 and increases Brd4 affects neuronal receptor proteins

In neurons, IEGs regulate the response to activity by changing the receptor content of synapses both by directly modifying synaptic...
proteins and altering gene expression of these proteins\textsuperscript{29,30}. Thus, the prolonged loss of IEG activation resulting from Jq1 treatment may affect neuronal function by changing critical synaptic proteins. In addition, RNA-sequencing data suggest that Jq1 treatment affects transcription of synaptic proteins and receptors in neurons (Fig. 3c,d). We therefore examined the GluA1 subunit of the AMPA receptor (AMPA), the main excitatory receptor in neurons. Jq1 treatment decreased transcript levels of Gria1 (Fig. 6a), the gene encoding GluA1, and decreased total GluA1 protein (Fig. 6b). Jq1 did not affect Gria2, which, unlike Gria1, lacks activity-responsive promoter regions\textsuperscript{31,32}. These changes, as well as those observed on IEGs, occurred slowly, over the course of several hours of Jq1 treatment (Supplementary Fig. 6a). To confirm that decreasing the total pool of available GluA1 results in decreased surface expression, we used a surface-staining assay that specifically stains receptors expressed on the exterior of dendrites. We found that both Jq1 and Brd4 siRNA, but not Brd2 or Brd3 siRNA, decreased GluA1 surface expression in both cortical and hippocampal neurons without affecting spine number (Fig. 6c–g and Supplementary Fig. 6b–e). Increasing Brd4 expression resulted in a small, but significant, increase in GluA1 surface expression (Fig. 6g–i). We again compared the long and short forms of Brd4 and found that full-length Brd4 increased GluA1 surface expression while the short isoform did not (Supplementary Fig. 6f), indicating that Brd4 functions by recruiting coactivating complexes to the promoter of target genes. Gria1 may be a direct target of Brd4, as Brd4 chromatin immunoprecipitation (ChIP) assays showed a high basal level of Brd4 at Gria1 regulatory elements in the promoter region and the small BDNF-induced increase in Brd4 binding is not seen with Jq1 (Supplementary Fig. 6g). In addition, we observed a nonsignificant increase in histone acetyl marks at the Gria1 promoter following BDNF treatment (Supplementary Fig. 6h). These data provide support for RNA-sequencing data showing that Jq1 affects synaptic proteins and demonstrate that Brd4 affects the expression of a critical subunit of a major excitatory receptor in neurons.

Jq1 treatment affects memory formation

Because of the importance of Brd4 in controlling critical neuronal proteins, we examined whether Jq1 affects brain function in mice. We injected wild-type adult male mice with Jq1 (50 mg/kg) daily for 1 week or 3 weeks before performing behavioral tests. Jq1 has excellent blood-brain barrier permeability\textsuperscript{33}, and, as in previous reports, we found that Jq1 was well tolerated in mice at this dose and schedule\textsuperscript{17,18,22,23} (Supplementary Fig. 7a). In an open field test, Jq1 did not affect distance traveled or zone preference, indicating that Jq1 does not cause anxiety or problems with mobility (Fig. 7a and Supplementary Fig. 7b–d).

We next used a novel object–recognition task (Fig. 7b) in which mice were briefly exposed to two identical objects and later presented with one familiar and one novel object. If mice remember the previous objects, they will subsequently spend more time with a novel object\textsuperscript{44}. All groups behaved similarly during habituation and the initial exposure, although mice receiving Jq1 for 3 weeks explored less during testing (Supplementary Fig. 7c–g). Strikingly, while control mice preferred the novel object as expected, Jq1-treated mice showed no preference (Fig. 7c). However, when mice were tested immediately after the initial exposure, control and Jq1 treated mice performed equally well (Supplementary Fig. 7h–j), suggesting that Jq1 does not disrupt learning or short-term memory but instead affects long-term memory. To control for possible health issues resulting...
Figure 7 Jq1 affects mouse behavior. (a) Time spent in the inner or outer zone of an open field in mice treated with vehicle or with Jq1 for 1 or 3 weeks (for DMSO n = 10 mice, for 1 week Jq1 n = 9 mice and for 3 weeks Jq1 n = 10 mice; for DMSO versus 1 week Jq1 P = 0.0107, t = 2.88; for DMSO versus 3 weeks Jq1 P = 0.0094, t = 2.92; one-sample t test for DMSO P = 0.00127, t = 4.85). (d) Discrimination index of time spent with a novel versus familiar object 1 d after initial exposure to the objects (unpaired two-tailed t test, for DMSO n = 10 mice, for 1 week Jq1 n = 9 mice and for 3 weeks Jq1 n = 10 mice; for DMSO versus 1 week Jq1 P = 0.0135, F = 4.67; unpaired two-tailed t test for DMSO versus 3 weeks Jq1 P = 0.0036, t = 3.34, df = 54). Discrimination index on a scale of −1 to 1 (% time with novel object − % time with familiar object)/(% time with novel object + % time with familiar object). ***P < 0.001 with univariate analysis. *P < 0.05. **P < 0.01. n.s., nonsignificant. Error bars represent s.e.m.

from long-term treatments, we injected mice with a single dose of Jq1 or DMSO either 6 h before or within 30 min after their initial exposure to objects and tested them the following day. We found no preference for the novel object in mice that received a single dose after training (Fig. 7d). This suggests that Jq1 given during the process of memory consolidation can block long-term memory formation.

Figure 8 Jq1 decreases seizure susceptibility. (a) Seizure induction procedure. (b) Seizure susceptibility score of mice treated for 1 week with either DMSO or Jq1 and given pentylenetetrazol (PTZ) to induce seizures (unpaired two-tailed t test, n = 7 mice for DMSO and 6 mice for Jq1, P = 0.002, t = 3.155). (c) Latency to the return of normal movement after PTZ injection (unpaired two-tailed t test, n = 7 mice for DMSO and 6 mice for Jq1, P = 0.036, t = 2.388). (d) Seizure induction procedure. (e) Seizure susceptibility score of mice on day 30 of kindling testing (one-sample t test, day 13 P = 0.00555, t = 2.494; day 15 P = 0.0486, t = 0.928; unpaired two-tailed t test for day 30 P = 0.0265, t = 2.715). *P < 0.05 with univariate analysis. **P < 0.01. ***P < 0.001. Error bars represent s.e.m. in box plots, whiskers show minimum and maximum, box limits are first and third quartile, and center line is median.
The smaller effect observed after a dose given before training may be due to a smaller amount of Jq1 remaining in the brain during the consolidation process several hours later.

We also tested a fear-conditioning protocol of three tones paired with shocks to determine the extent of the memory deficits (Fig. 7e). All mice learned both the cued conditioning and context-dependent conditioning (Supplementary Fig. 7k,l), demonstrating that Jq1 did not affect this simple behavior dependent on the amygdala. However, mice given Jq1 for 3 weeks froze more in a new context, suggesting they were less able to distinguish between the training context and a new context. This indicates that the more difficult hippocampus-dependent test of context discrimination training context and a new context. This indicates that the more difficult hippocampus-dependent test of context discrimination may also require BET protein function (Fig. 7f). Together these data provide in vivo support of our cell-based data demonstrating that Jq1 disrupts the transcriptional responses that are critical to neuronal function.

Jq1 treatment decreases seizure susceptibility

To confirm that Jq1 has similar effects on neurons in vivo as it does in vitro, we examined tissue from mice after behavioral testing. Despite the heterogeneity of cortical tissue, we found either trends or significant decreases in IEGs, Gria1 and GluA1 protein (Supplementary Fig. 8a,b). Long-term decreases in Gria1 and other synaptic proteins (Fig. 3c) may dampen synaptic strength, which also has implications for other aspects of mouse behavior. We hypothesized that if Jq1 effectively decreases neuronal firing through regulation of synaptic proteins, then Jq1 treatment might decrease seizure susceptibility because seizures result from excess synaptic excitability. We injected adult male mice with Jq1 or DMSO for 1 week and then induced seizures with pentylenetetrazol (PTZ) (50 mg/kg) (Fig. 8a), which inhibits GABA_A receptors, resulting in increased excitatory activity. Jq1-treated mice showed decreased seizure susceptibility (Fig. 8b), as measured by a modified Racine scale, which measures both severity of seizure and latency to onset of each seizure stage35,36. In addition, while approximately 30% of control mice died after seizure induction, all Jq1 treated mice survived and recovered faster as measured by a return to normal movement (Fig. 8c and Supplementary Fig. 8c,d). Similar but more variable effects were observed in female mice (Supplementary Fig. 8e,f).

We also tested the kindling method of seizure induction by giving DMSO or Jq1 1 h before mice receive a subthreshold dose of PTZ37,38. This was repeated every 2 d for 2 weeks, and experimental mice as well as an additional unkindled group were tested again 2 weeks later (Fig. 8d). Mice typically show increased seizure induction over time as PTZ-induced increases in neuronal firing enhance the strength of neuronal connections, intensifying the response to future doses37. Mice are considered kindled if they show enhanced susceptibility that is maintained for several weeks. Jq1 had little effect during initial treatments, possibly because mice had only received a few doses of Jq1. However, on day 30, only the DMSO-treated mice showed kindling as compared to the unkindled group (Fig. 8c–g and Supplementary Fig. 8g,h). These data indicate that Jq1 treatment has effects on neuronal function in vivo similar to those we observed in vitro and raise the possibility of using BET inhibitors for treatment of epilepsy.

DISCUSSION

Our results demonstrate that Brd4 is expressed throughout the brain and is critical to activity-dependent transcription. Neuronal activity acts through CK2 to increase Brd4 association with chromatin. Brd4 then promotes transcription of critical IEGs and synaptic proteins (Supplementary Fig. 9). We found that Jq1 inhibition of Brd4 and its family members blocked novel object preference, indicating impairments in memory consolidation. In addition, consistent with its effects on synaptic proteins, Jq1 treatment also decreased seizure susceptibility in mice. This is, to our knowledge, the first demonstration that Brd4 has a critical function in neurons and that BET protein inhibition affects memory consolidation.

Implications for the clinical use of BET inhibitors

Our data demonstrate that Brd4 is necessary for rapid activation of genes. As has been demonstrated by many researchers, the first few minutes following a burst of neuronal activity are of critical importance for the activation of an appropriate transcription response in a cell3–6. Loss of this rapid response may represent the mechanism through which Brd4 inhibition prevents long-term memory formation.

BET protein inhibitors have been proposed as a treatment for several types of cancer and are now in clinical trials. Initial mouse studies reported that Jq1 was well tolerated17,18,23,33, and we did not find obvious deficits in the health or mobility of mice. However, our study provides new evidence that use of such inhibitors causes memory deficits in mice and thus may also cause neurological problems in patients receiving these drugs. Our results suggest that compounds that do not cross the blood-brain barrier may pose less risk of neurological side effects.

CK2 in neurons

CK2 has several established functions in neurons in addition to regulating Brd4. CK2 phosphorylates the GluA1 and GluA2 subunits of the AMPA receptor to promote its expression27 and regulates the composition of the NMDA receptor28. These synaptic actions of CK2 promote synaptic strength, as does the function we propose for CK2 in regulating Brd4. This dual function would allow CK2 to act immediately on the synapse by directly phosphorylating synaptic proteins while also acting through Brd4 to promote expression of these same genes in order to consolidate synaptic changes. The effects of the CK2 inhibitor TBB have also been tested in vivo in an epilepsy model39. TBB treatment blocks recurrent epileptiform discharges in hippocampal slice preparations after magnesium removal. We found the Jq1 resulted in decreased seizure susceptibility (Fig. 7), suggesting that some of CK2’s effects on epileptiform discharges may be the result of its action on Brd4 as well as its effects on the synapse.

BET inhibitors as epilepsy drugs

We found that Jq1 decreased the seizure susceptibility, potentially by decreasing levels of the GluA1 subunit of AMPARs, which have been linked to epilepsy46–47. Decreased levels of other gene targets that regulate synaptic function may also contribute to the seizure effect though mechanisms such as phosphorylation of GluA1 (ref. 48). Although the dose we tested resulted in memory deficits, it is possible that in an overactive, epileptic brain Jq1 would restore normal levels of synaptic proteins. Most epilepsy treatments directly target synaptic proteins and receptors. Jq1 treatment represents a novel approach by targeting a protein responsible for the transcriptional regulation of these synaptic receptors instead of modifying proteins already present at the synapse. While many cases of epilepsy respond to available treatments, a portion are refractory to existing drugs38. It is possible that this approach of targeting transcriptional regulators of synaptic proteins rather than targeting synaptic proteins directly may provide a more robust method of dampening the heightened synaptic activity that leads to seizures and could provide new avenues of treatments for these patients.
METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. GEO: GSE63809.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

E.K. wrote the manuscript and designed and carried out experiments. M.H. helped carry out behavioral testing and molecular studies. I.Z.-S. helped with seizure testing. R.B.D. helped initiate and design the project and provided feedback, and C.D.A. provided support, feedback and guidance.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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DNA complexes were left on cells overnight. Cells were harvested for analysis in DMEM using Lipofectamine 2000 (Life Technologies). Lipofectamine and tested for Biosystems) on an Applied Biosystems quantitative PCR system run using the Qiagen RNAeasy kit and reverse-transcribed using an Applied Biosystems. Primers used for qPCR were as follows:

**Gapdh forward**: AACTCCCTCTCAAGATGGTGTAAGCAA.

**Gapdh reverse**: GGCATCGACTGTGGTCTAGTA.

**Arc forward**: TAACCTGCGTCTCCCTGCTAGATC.

**Arc reverse**: GGGATAAGTGTAAGCCCA and GTACA.

**Brd4 forward**: TAACCTGCGTCTCCCTGCTAGATC.

**Brd4 reverse**: GGGATAAGTGTAAGCCCA and GTACA.

**CK2 forward**: CCGAAGAAGATTTCTCAGACCT.

**CK2 reverse**: CCGAAGAAGATTTCTCAGACCT.

**Brd2 forward**: CCGAAGAAGATTTCTCAGACCT.

**Brd2 reverse**: CCGAAGAAGATTTCTCAGACCT.

**Brd4 forward**: AAATACGTCCACACAGGCTTG.

**Brd4 reverse**: TCTTGGGGCTGTAGGGTTG.

**Brd2 forward**: AAACGGATGATCTAGGCTTGAAGGCT.

**Brd2 reverse**: CTTGTCGATTGATGAACTTGG.

**Brd4 forward**: GACATCTGCTCGGAACAGT.

**Brd4 reverse**: CATCTGCTCGGAACAGT.

**Bdnf forward**: TGTCATCGTCTCCCTCAGCAGT.

**Bdnf reverse**: TGAGCTGGTCCTGCTCTTCTCATGGGC.

Primers used for ChIP were as follows:

**Arc forward**: ATAAATAGCCGCTGGTGCGC.

**Arc reverse**: CGGCCCGCCAGAAGGT.

**Fos forward**: TAACCTGGTGTCCCTCCTAGATC.

**Fos reverse**: GGAAAGACTTCTCAGCAGCTT.

**Bdnf forward**: TGTCATCGTCTCCCTCAGCAGT.

**Bdnf reverse**: HGAGCTGGTCCTGCTCTTCTCATGGGC.

Primers used for qPCR were as follows:

**Gapdh forward**: AACTCCCTCTCAAGATGGTGTAAGCAA.

**Gapdh reverse**: GGCATCGACTGTGGTCTAGTA.

**Arc forward**: TAACCTGCGTCTCCCTGCTAGATC.

**Arc reverse**: GGGATAAGTGTAAGCCCA and GTACA.

**Brd4 forward**: AAATACGTCCACACAGGCTTG.

**Brd4 reverse**: TCTTGGGGCTGTAGGGTTG.

**Brd2 forward**: AAACGGATGATCTAGGCTTGAAGGCT.

**Brd2 reverse**: CTTGTCGATTGATGAACTTGG.

**Brd4 forward**: GACATCTGCTCGGAACAGT.

**Brd4 reverse**: CATCTGCTCGGAACAGT.

**Bdnf forward**: TGTCATCGTCTCCCTCAGCAGT.

**Bdnf reverse**: TGAGCTGGTCCTGCTCTTCTCATGGGC.

RNA-sequence sampling preparation and analysis. RNA was collected and prepared using a Qiagen RNAeasy kit and the TruSeq RNA Sample Preparation Kit v2 (Illumina). Sequencing was performed with an Illumina HiSeq2500 system. Reads were aligned to the mouse mm9 reference genome using TopHat 2.0.11 (ref. 50). Reads with two or fewer mismatches with a maximum of 20 hits for each read were used. Transcript levels were analyzed with cufflinks 2.2.1 (ref. 51). Expressed genes were defined as those with an FPKM of 1 or above. BDNF-induced genes were defined as those with a significant increase in expression with 10 min BDNF treatment using a P value with a Bonferroni correction.

**Immunohistochemistry.** Adult mice were perfused with 4% paraformaldehyde and brains were removed and kept in paraformaldehyde overnight. Tissue was then washed in PBS and processed for paraffin embedding at the Molecular Cytology Core Facility of Memorial Sloan Kettering Cancer Center using a Leica ASP6025 tissue processor. Brains were embedded in paraffin, and paraffin sagittal sections of 5 µm were cut on a Leica RM2155 microtome and collected on Superfrost Plus slides (Fisher). Slides were baked for 1 h at 60 °C before deparaffinization and staining.

Staining was performed at the Molecular Cytology Core Facility of Memorial Sloan Kettering Cancer Center using a Discovery XT processor (Ventana Medical Systems). For GFAP and Brd4 co-staining, slices were first stained for Brd4 (Bethyl A301-985A, 2 µg/mL). The tissue sections were blocked for 30 min in 10% normal goat serum and 2% BSA in PBS. The incubation with the primary antibody was done for 3 h, followed by 60 min incubation with biotinylated goat anti-rabbit IgG (Vector Labs PK6101, 1:200). Detection was performed with Strepavidin-HRP D (Ventana Medical Systems) followed by incubation with tyramide–Alexa Fluor (Invitrogen T20948, 1:200). For GFAP, sections were blocked for 30 min in

**Western blotting.** Cells were lysed in RIPA buffer and lysates were separated by SDS-PAGE and transferred to PVDF membrane. Antibodies used were to Brd4 (Bethyl A301-985A, 2 µg/mL), NeuN (Millipore MAB377, 1:500), GFAP (Abcam ab10062, 1:1,000), Gapdh (Abcam ab8245, 1:500), MAPK (Cell Signal 4695P, 1:3,000), PhosMAPK (Cell Signal 4370, 1:3,000), H3 (Abcam ab1791, 1:4,000), H4 (Abcam ab10158, 1:4,000), H3K14ac (Active Motif 39697, 1:500), H4K16ac (Active Motif 39167, 1:500). The phospho–Brd4 antibody was developed with Millipore. The antiserum was raised in rabbit against peptide DSSD(p)-SDDSDST. For immunization, the peptides were conjugated to KLH. For affinity purification, peptides were bound to Sulfolink (Thermo Fisher). The antibody was affinity-purified on a phosphorylated peptide–coupled Sulfolink column and then the collected material was depleted on an unmodified control peptide–coupled Sulfolink column. The formulation of the purified material was 0.1 M Tris-glycine (pH 7.4), 150 mM NaCl, 0.05% sodium azide. The best bleeds were affinity-purified against the phosphorylated target peptide and immunodepleted with unmodified Brd4. Blots were imaged on an LAS3000 system (FujiFilm).

Reverse transcription, quantitative PCR and ChIP. RNA was purified using the Qiagen RNAeasy kit and reverse-transcribed using an Applied Biosystems kit. qPCR was performed with Power SYBR Green PCR master mix (Applied Biosystems) on an Applied Biosystems quantitative PCR system run using StepOne software. ChIP was done as previously described. Chromatin shearing was performed with a Bioruptor300 (Diagenode) at 4 °C for 55 cycles of 30 s on and off. Immunoprecipitation was performed using 5 µg of antibody bound to 50 µL of magnetic Dynabeads M280 (Life Sciences). DNA was purified using the QIAGEN QIAquick PCR purification kit. Antibodies used were to Brd4 (Bethyl A301-985A, 2 µg/mL), Brd2 (Bethyl A302-583A, 5 µg/mL), Brd3 (Bethyl A302-368A, 5 µg/mL), H3K9ac (Millipore 07-352, 2 µg/mL), H3K14ac (Active Motif 39697, 5 µg/mL) and CBP (Santa Cruz 7300, 5 µg/mL). Primers used for qPCR were as follows:

**Gapdh forward**: AACTCCCTCTCAAGATGGTGTAAGCAA.

**Gapdh reverse**: GGCATCGACTGTGGTCTAGTA.

**Arc forward**: TAACCTGCGTCTCCCTGCTAGATC.

**Arc reverse**: GGGATAAGTGTAAGCCCA and GTACA.

**Fos forward**: TAACCTGCGTCTCCCTGCTAGATC.

**Fos reverse**: GGGATAAGTGTAAGCCCA and GTACA.

**Brd4 forward**: AAATACGTCCACACAGGCTTG.

**Brd4 reverse**: TCTTGGGGCTGTAGGGTTG.

**Brd2 forward**: AAACGGATGATCTAGGCTTGAAGGCT.

**Brd2 reverse**: CTTGTCGATTGATGAACTTGG.

**Brd4 forward**: GACATCTGCTCGGAACAGT.

**Brd4 reverse**: CATCTGCTCGGAACAGT.**
Immunocytochemistry. Cells were fixed in 4% paraformaldehyde for 10 min, washed with PBS and permeabilized in 0.1% Triton in PBS for 10 min. Cells were then blocked for 1 h in 2% serum, 3% BSA and 0.1% Triton in PBS and then primary antibody was added in the same solution overnight at 4°C. Cells were washed in PBS for three 10 min washes and put in secondary antibody for 1 h at room temperature. After three more washes, coverslips were mounted for 3 h at room temperature and followed by 60 min incubation of biotinylated mouse secondary (Vector Labs, MOM Kit BMK-2202, 1:200 dilution, 5.75 µg/mL). The detection was performed with Secondary Antibody Blocker, Blocker D, Streptavidin-HRP D (Ventana Medical Systems) and DAB Detection Kit (Ventana Medical Systems) according to manufacturer instructions.

Microscopy equipment and settings. Slides were imaged at room temperature on an inverted Leica DMI 6000, TCS SP laser scanning confocal microscope with a 405-nm laser and a fully tunable white light laser (470–670 nm) with an acousto-optical beam splitter. The microscope uses three gated HyD detectors and one PMT detector and both a conventional scanner and a resonant scanner. Objectives used were a 63× HC PL APO CS2 oil objective with an NA of 1.40 and, for whole-brain images, a 10× HCX PL APO DS dry objective with an NA of 0.4. Type F immersion liquid (Leica) was used for oil objectives. For brain images, the Leica Super-z stage and rapid tiling system was used to compute images. For GluA1 surface staining, z-stacks spaced at 0.5 µm were used to image the entire dendrite. For 63× images, images were 184.52 by 184.52 µm, 1,052 by 1,052 pixels, (5.701 pixels per µm) and 8 bits per pixel. For 10× images, images were 1,162.5 by 1,162.5 µm, 1,052 by 1,052 pixels (0.881 pixels per µm) and 8 bits per pixel.

ImageJ was used to crop images and merge channels into composite RGB images. Photoshop was used to adjust individual channels. In all cases, identical adjustments were applied across all images used in an experiment for each channel. No deconvolution software was used. All image analysis was performed in ImageJ. For Arc staining quantification, a region of interest was selected in the cell body outside the nucleus and the average intensity was measured. Regions were selected using DAPI and GFP channels and then applied to the Arc channel such that the analysis was performed blind to the Arc staining. For Brd4 staining quantification, the same process was used but inside the nucleus. For surface GluA1 quantification, the z-stacks were summed using ImageJ to create one image per channel. GFP images were converted to binary and used to create a mask surrounding the transfected dendrite. The mask was then applied to the GluA1 image and the average intensity within the dendrite was measured. This was automated using ImageJ macros to prevent user bias. For all image analysis, an average background intensity value was subtracted from each intensity value. To allow comparisons across experiments, the average control cell value was set to 100 and all conditions were normalized to this value.

Behavior. All experiments were approved by the Institutional Animal Care and Use Committee of the Rockefeller University. C57BL/6 male mice (Jackson) were housed up to 5 mice per cage in a 12:12-light-dark cycle. Jq1 (APExBIO) was administered to mice 2–3 months old via intraperitoneal injections. Each mouse was injected daily for 1 week or 3 weeks before testing began with either Jq1 at 50 mg/kg dissolved in DMSO or DMSO alone, diluted into cyclohextrin (Sigma). Mice were randomly assigned to groups and groups were then checked to ensure that the average weight per mouse of each group was equivalent at the beginning of the experiment. Injections continued during the week of behavioral testing and testing was performed during the light cycle. Open field testing was performed first and activity was measured for 1 h. Fusion 3.2 was used to track mice and analyze movement. One day after open field testing, mice were habituated to the novel object recognition box for 10 min. One day later mice were habituated for an additional 2 min and then two identical objects (either a faucet or a Lego pyramid) were placed in the box and mice were given 10 min to explore. On the following day, mice were returned to the box with one object they had previously seen and one new object in place of the original object and allowed to explore for 10 min. All sessions were recorded using EthoVision software. Time spent interacting with each object was manually analyzed. Discrimination index was calculated as (% time with novel object – % time with familiar object)/(% time with novel object + % time with familiar object). Fear conditioning tests began 1 d after novel object recognition. Mice were placed in a small box and allowed to explore for 2 min. A tone was played for 20 s followed by a 0.7-mA shock. This was repeated once per minute for three shocks total. After an additional 2 min, mice were removed from the box. One day later mice were returned to the same box for 7 min to measure context-dependent freezing. Then the flooring, wall covering and smell of the box was changed and mice were returned to the box. The tone was then played in the same pattern as the original training session without a subsequent shock to measure cued learning. Fear conditioning sessions were run and recorded using FreezeFrame 3 software and scored manually in random order. All experiments were carried out and analyzed with the experimenter blind to the treatment group. One mouse was excluded from analysis because the lights went off in the facility during the discrimination test so the data could not be analyzed.

For testing of novel object learning, mice were habituated to the novel object recognition box for 10 min. One day later mice were given 10 min to interact with two identical objects. Mice were then removed and one object was replaced with a novel object and mice were returned to the box and again allowed to explore for 10 min. For single-dose tests, one cohort received a dose of DMSO in the morning approximately 6 h before testing and a second dose of DMSO within 30 min of exposure to objects. One cohort received Jq1 in the morning and DMSO after testing and the final cohort received DMSO in the morning and Jq1 following testing. Mice were tested for novel object preference 1 d after the first exposure to objects. All sessions were recorded using EthoVision software. Time spent interacting with each object was manually analyzed. All experiments were carried out and analyzed with the experimenter blind to the treatment group and which object was considered novel.

Seizure testing. Jq1 (APExBIO) was administered to 3- to 4-month-old C57BL/6 male or female mice (Jackson) via intraperitoneal injections. For acute seizure testing, each mouse was injected daily for 1 week before testing began with either Jq1 at 50 mg/kg dissolved in DMSO or DMSO alone, diluted into cyclohextrin (Sigma). Pentylenetetrazol (PTZ) (Sigma) dissolved in PBS was injected at 50 mg/kg via intraperitoneal injections. For kindling seizure testing, Jq1 was administered 1 h before PTZ injection. Mice were observed up to 1 h after injection or until recovery from seizure (defined by a return to normal movement). The modified Racine scale45 used to measure seizure induction was as follows:

Stage 1: hypoactivity culminating in behavioral arrest with contact between abdomen and the cage.
Stage 2: partial clonus (PC) involving the face head or forelimbs.
Stage 3: generalized clonus (GC) including all four limbs and tail, rearing or falling.
Stage 4: generalized tonic-clonic seizure (TC).

Seizure susceptibility score was calculated as: (0.2)(1/PC latency) + (0.3)(1/GC latency) + (0.5)/(1/TC latency).

Statistics. An α level of 0.05 was used for all statistical analyses. Two-sided t-tests were performed in Excel. A Bonferroni correction was applied
when comparing multiple groups. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications\textsuperscript{20,30,37}. Data distribution was assumed to be normal, but this was not formally tested. For fear conditioning, two-way ANOVA was performed in R with post hoc Bonferroni corrections for individual comparisons.

For novel object testing, context discrimination was calculated by time spent with objects: (novel − familiar)/(novel + familiar). Univariate analysis was used for each individual group to compare to a context discrimination of zero. Degrees of freedom were calculated as the biological replicates minus 1.

For all other behavioral testing, t-tests with a Bonferroni correction were used to compare between multiple groups.

A Supplementary Methods Checklist is available.

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