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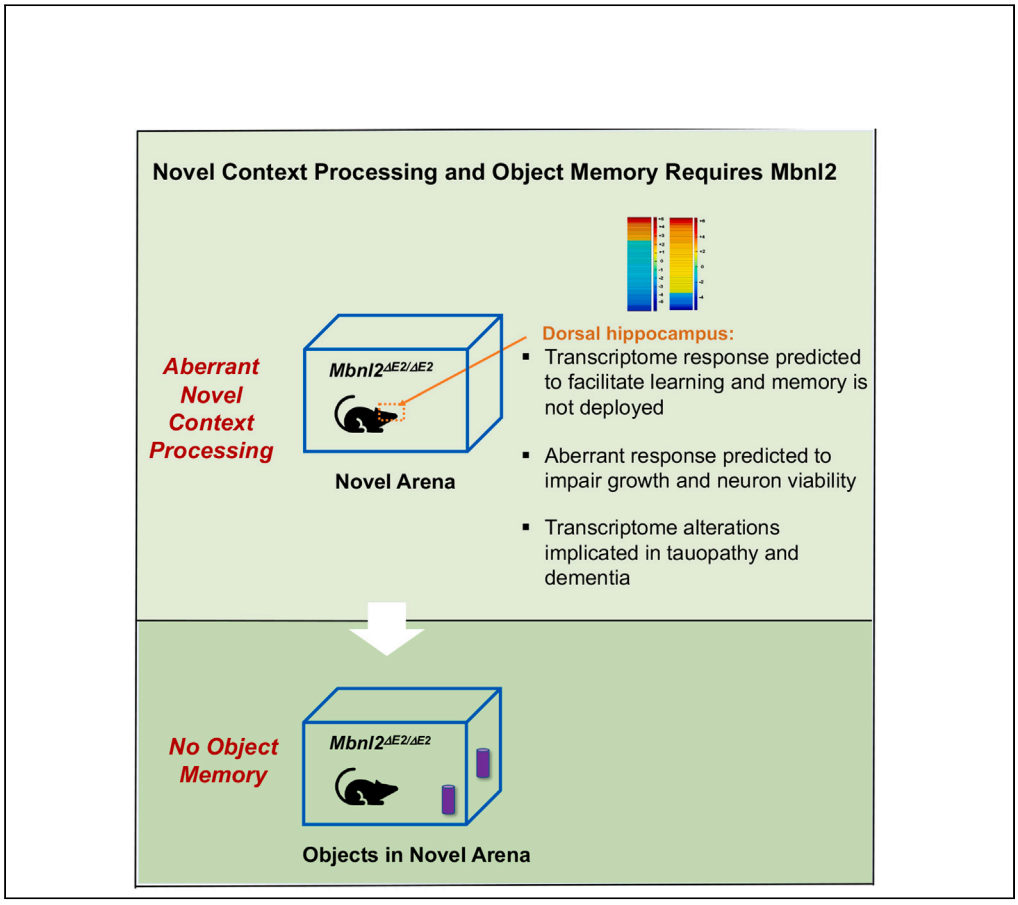

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Highlights

Mbnl2 is required for long-term object memory in the novel object recognition test

The hippocampal transcriptome response facilitating context memory requires Mbnl2

Context processing in *Mbnl2*$^{\Delta E2/\Delta E2}$ hippocampus alters genes implicated in dementia

Saturation effects may impair context processing in the *Mbnl2*$^{\Delta E2/\Delta E2}$ hippocampus

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Mbnl2 loss alters novel context processing and impairs object recognition memory

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SUMMARY

Patients with myotonic dystrophy type I (DM1) demonstrate visuospatial dysfunction and impaired performance in tasks requiring recognition or memory of figures and objects. In DM1, CUG expansion RNAs inactivate the muscleblind-like (MBNL) proteins. We show that constitutive Mbnl2 inactivation in *Mbnl2*$^{\Delta E2/\Delta E2}$ mice selectively impairs object recognition memory in the novel object recognition test. When exploring the context of a novel arena in which the objects are later encountered, the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus responds with a lack of enrichment for learning and memory-related pathways, mounting instead transcriptome alterations predicted to impair growth and neuron viability. In *Mbnl2*$^{\Delta E2/\Delta E2}$ mice, saturation effects may prevent deployment of a functionally relevant transcriptome response during novel context exploration. Post-novel context exploration alterations in genes implicated in tauopathy and dementia are observed in the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus. Thus, MBNL2 inactivation in patients with DM1 may alter novel context processing in the dorsal hippocampus and impair object recognition memory.

INTRODUCTION

Myotonic dystrophy (DM1) is a neuromuscular disorder where CNS dysfunction occurs with a comparable incidence to pathologies in skeletal and cardiac muscle.[1,2] DM1 results from a CTG repeat expansion in the 3′ untranslated region of the *DMPK* gene, with longer expansions generally correlating with increased severity and an earlier onset of the disease.[3–5] CNS manifestations differ with the age of onset. Intellectual disability and autism spectrum features predominate in the congenital form of the disease.[1,6,7] Behavioral and emotional manifestations in the childhood and juvenile-onset forms of the disease overlap with those observed in congenitally affected patients and include learning disabilities, memory deficits, autism spectrum features, dysexecutive syndrome, avoidant personality traits with difficulties in establishing relationships in social life, attention-deficit hyperactivity disorder, and sleep-related abnormalities.[1,6–9] Patients with adult-onset DM1 show similar but less severe symptoms.[1,10] Other behavioral alterations reported in adult-onset DM1 include apathy, anhedonia, and obsessive-compulsive traits.[11–13] Visuospatial dysfunction is prominent in DM1[14–18] and impairment in tasks requiring memory or recognition of objects and figures is reported in all forms of the disease.[14–18] Structural examination of the brain demonstrates brain atrophy, regional neuronal loss, and the formation of neurofibrillary tangles consisting of pathologic tau proteins in several brain regions including the hippocampus.[15,19–21]

In DM1, mutant CUG expansion RNAs sequester and functionally inactivate the muscleblind-like (MBNL) family of RNA-binding proteins.[22–25] We have previously demonstrated that Mbnl1 inactivation in mice results in depressive behaviors including anhedonism and complex behavioral alterations in the Morris water maze with apathy playing a role in the lack of task completion.[26] In subsequent experiments, Mbnl2 loss has been shown to alter sleep and impair learning and memory in the Morris water maze.[27] In this study, we concurrently examined behavior and the transcriptome response of the dorsal hippocampus in *Mbnl2*$^{\Delta E2/\Delta E2}$ mice to better elucidate the mechanistic role of MBNL2 in DM1 CNS dysfunction. The performance of *Mbnl2*$^{\Delta E2/\Delta E2}$ mice was studied in behavior paradigms related to the characteristic behaviors impaired in patients with DM1. This test battery demonstrated that Mbnl2 loss selectively impairs long-term object recognition memory in the novel object recognition test (NORT). As object recognition memory in wild-type mice requires habituation, a preliminary phase of the NORT, when mice cognize the

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context of the arena in which the objects are later encountered, we studied the impact of Mbnl2 loss on the transcriptome response of the dorsal hippocampus, a region of the brain that plays a key role in contextual and spatial learning, during habituation.[28] We show that when exploring the context of a novel arena, the dorsal hippocampus of *Mbnl2*$^{\Delta E2/\Delta E2}$ mice responds with a striking lack of enrichment of pathways predicted to facilitate learning and memory in conjunction with the deployment of changes predicted to impair growth and neuron viability. Our analysis of the transcriptome of house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice supports the hypothesis that the aberrant pre-activation of a subset of mRNA level changes that manifest with normal habituation in the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus may prevent their significant redeployment when exploring a novel context, potentially due to saturation effects. Post novel context exploration, the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus shows transcriptome alterations that are predicted to contribute to tauopathy and dementia. Thus, this study demonstrates a regulatory role for Mbnl2 in the development of object recognition memory and in deploying a transcriptome response predicted to facilitate learning and memory of a novel context in the dorsal hippocampus. Our data support the hypothesis that altered cognition of the context of a novel arena may impair long-term memory of objects encountered later within that arena in *Mbnl2*$^{\Delta E2/\Delta E2}$ mice.

RESULTS

Mbnl2$^{\Delta E2/\Delta E2}$ mice demonstrate impaired object recognition memory

To test the role of MBNL2 inactivation in DM1 CNS dysfunction, we developed F2 129sv/C57BL/6j *Mbnl2*$^{+/+}$ mice (indicated as *Mbnl2*$^{+/+}$) and F2 129sv/C57Bl6j *Mbnl2*$^{\Delta E2/\Delta E2}$ mice (indicated as *Mbnl2*$^{\Delta E2/\Delta E2}$) as described in the Methods. *Mbnl2*$^{\Delta E2/\Delta E2}$ mice have a constitutive deletion of exon 2 (619 bps and encoding the ATG start codon) and show a compensatory increase in Mbnl1 levels as previously described in a similar mutant mouse strain.[27] Male mice 4–5 months of age were used for this study. The numbers of mice/genotype used in the four behavioral tests were *Mbnl2*$^{+/+}$ = 9; *Mbnl2*$^{\Delta E2/\Delta E2}$ = 10. Behavioral experiments were performed in the following order, social approach, novel object recognition, fear conditioning, and marble burying.

Marble burying

As patients with DM1 show autism spectrum features and obsessive-compulsive traits, we tested *Mbnl2*$^{\Delta E2/\Delta E2}$ mice in marble burying, which is a test for repetitive behaviors that characterize these disorders.[29] *Mbnl2*$^{\Delta E2/\Delta E2}$ mice showed equivalent marble burying behavior indicating normal repetitive and anxiety-like behavior (% Marbles buried *Mbnl2*$^{+/+}$: 41.1 + 34.9; *Mbnl2*$^{\Delta E2/\Delta E2}$: 22.5 + 18.4; $F(1,19) = 2.170$, $p = 0.159$).

Social approach

To test for avoidant personality traits and the difficulties in establishing social relationships, the social approach test in mice was used. This test has validity as a surrogate for measures of simple social approach behaviors in humans, which are often impaired in autism and in patients with DM1.[30] *Mbnl2*$^{\Delta E2/\Delta E2}$ mice showed the expected preferential investigation of a novel mouse over an empty cup (Effect of chamber side *Mbnl2*$^{+/+}$: Mouse chamber investigation: 397 + 76.0 s vs. Empty chamber investigation: 135.0 + 63.3 s, $F(1,8) = 32.508$, $p < 0.001$; and *Mbnl2*$^{\Delta E2/\Delta E2}$: Mouse chamber investigation: 346.9 + 76.26 s vs. Empty chamber investigation: 172.9 + 52.6 s, $F(1,9) = 20.276$, $p = 0.001$) indicating normal social approach behavior.

Fear conditioning

Mbnl2$^{\Delta E2/\Delta E2}$ mice have been reported to demonstrate impaired task completion in the Morris water maze, which is a test for visuospatial learning and memory.[27] As it is unclear if visuospatial dysfunction or other hippocampus-mediated learning and memory defects contribute to deficits in the Morris water maze that have been reported previously in *Mbnl2*$^{\Delta E/\Delta E2}$ mice,[27] we studied learning and memory in the fear conditioning test, where tone-shock pairings are examined. This test provides an independent measure of hippocampus-mediated associative learning.[31] For fear conditioning, on day 1, mice were placed in the experimental chamber and then administered 3 tone-shock pairings (Tone: 2800 Hz, 80 dB, Shock: 0.5 mA). *Mbnl2*$^{\Delta E2/\Delta E2}$ mice showed normal acquisition of fear such that freezing increased with training similar to *Mbnl2*$^{+/+}$ mice (Effect of acquisition time bin: $F(5,85) = 6.180$, $p < 0.001$, no genotype interaction or between subject genotype effect). The activity burst response to the shock, a measure of pain sensitivity, did not differ between the genotypes (*Mbnl2*$^{+/+}$: 649.9 + 188.2 arbitrary units (au); *Mbnl2*$^{\Delta E2/\Delta E2}$: 685.3 + 262.3 au, $F(1,17) = 0.112$, $p = 0.742$). On day 2, mice were returned to the conditioning chamber to test fear of the conditioning context. *Mbnl2*$^{\Delta E2/\Delta E2}$ mice showed a reduced mean value for freezing averaged

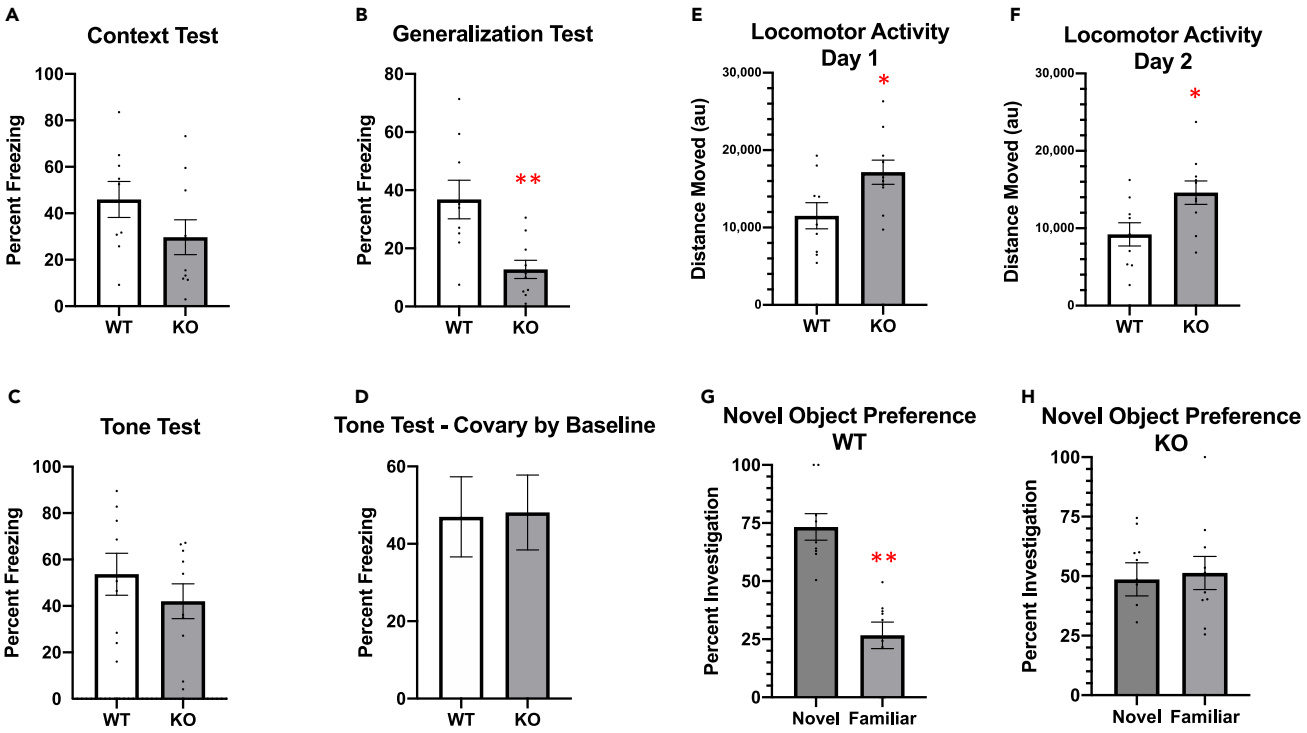

Figure 1. *Mbnl2*$^{\Delta E2/\Delta E2}$ mice demonstrate impaired object recognition memory

Fear conditioning A–D: A: Percent freezing during the context test.

(B) Percent freezing during the generalization test.

(C) Percent freezing in the tone test: raw freezing scores.

(D) Percent freezing in the tone test: covaried by baseline level of freezing. Covariance is computed at the group level so individual data points cannot be plotted. NORT E–H: (E and F). Locomotor activity in the open field on days 1 and 2.

(G and H) Preference for investigation of the novel object on the test day. For all behavioral tests, the numbers of male mice 4–5 months of age used were *Mbnl2*$^{+/+}$ = 9; *Mbnl2*$^{\Delta E2/\Delta E2}$ = 10. WT: *Mbnl2*$^{+/+}$; KO: *Mbnl2*$^{\Delta E2/\Delta E2}$. Means +SEM are shown. In all cases, * and ** indicate levels of significance where $p < 0.05$ and $p < 0.01$, respectively.

across the 8-min test. However, as this difference was not significant, the data suggest relatively normal retrieval of hippocampus-dependent contextual fear ($F(1,18) = 2.86$, $p = 0.149$) (Figure 1A).

On day 3, mice were placed in a modified conditioning chamber to assess generalization of contextual fear to a similar, but distinct, environment. *Mbnl2*$^{\Delta E2/\Delta E2}$ mice showed decreased freezing in this modified context ($F(1,17) = 11.336$, $p = 0.004$) (Figure 1B). On day 4, they were placed back in this modified context and presented with the same 2800 Hz tone that was paired with shock during training. *Mbnl2*$^{\Delta E2/\Delta E2}$ mice again showed reduced freezing in this modified context during the baseline period prior to tone presentation ($F(1,17) = 16.775$, $p = 0.001$). Freezing to the tone did not differ in the *Mbnl2*$^{\Delta E2/\Delta E2}$ mice with respect to *Mbnl2*$^{+/+}$ mice ($F(1,17) = 0.998$) (Figure 1C). As differences in baseline freezing can sum with tone freezing, we analyzed tone freezing with baseline freezing as a covariate, as previously validated in the study by Jacobs et al.,[32] to account for the lower baseline freezing in the *Mbnl2*$^{\Delta E2/\Delta E2}$ mice. As these scores were similar to the raw freezing scores, this confirmed that *Mbnl2*$^{\Delta E2/\Delta E2}$ mice have normal levels of hippocampus-independent tone fear ($F(1,16) = 0.005$, $p = 0.945$) (Figure 1D). Thus, overall, Mbnl2 loss does not appear to lead to major impairments of hippocampus-dependent contextual fear learning and memory but does reduce fear in a novel context. This suggests that there may be deficits in hippocampus-dependent context processing and/or compensation by extra-hippocampal structures.[33,34] In contrast, hippocampus-independent tone fear learning and memory appears to be intact.

Novel object recognition

As impairments in tasks requiring recognition and memory of objects and figures is observed in congenital, juvenile, and adult patients with DM1,[14–18] we tested object recognition memory using the NORT.[35] During

habituation on day 1 and 2 of the NORT, mice are familiarized to the arena in which the objects are subsequently encountered. The arena used was an open field testing environment consisting of a square open field chamber with a white flooring and an open ceiling (denoted as the empty chamber) for 10 min. Locomotor activity in these two sessions can be used to analyze changes in exploratory activity and anxiety-related behavior. *Mbnl2*$^{\Delta E2/\Delta E2}$ mice were hyperactive during both day 1 and 2 (Day 1: $F(1,17) = 6.019$, $p = 0.025$), Day 2: $F(1,17) = 6.385$, $p = 0.022$) (Figures 1E and 1F). Despite this overall hyperactivity, the *Mbnl2*$^{\Delta E2/\Delta E2}$ mice did show a normal reduction in exploration on day 2 relative to day 1 (Repeated Measures Effect of Day: $F(1,18) = 18.592$, $p > 0.001$, Genotype by Day interaction: ($F(1,18) = 0.295$, $p = 0.594$) and there were no differences in the time spent in the center of the open field, which is a measure of anxiety[36] (Day 1: $F(1,17) = 1.889$, $p = 0.187$), Day 2: $F(1,17) = 0.444$, $p = 0.514$).

In the 10-min object familiarization session on day 3, two identical objects were placed into the empty chamber in order to familiarize the mice with these objects. *Mbnl2*$^{\Delta E2/\Delta E2}$ mice showed normal levels of object investigation during this familiarization period (*Mbnl2*$^{+/+}$ mice = 55.0 + 18.7 s; *Mbnl2*$^{\Delta E2/\Delta E2}$ mice = 64.5 + 31.2 s, $F(1,17) = 0.631$, $p = 0.438$). On day 4, in a 10-min test phase, one of the objects was replaced with a novel object and investigation of the novel versus familiar object was measured. *Mbnl2*$^{+/+}$ mice showed the expected preferential investigation of the novel object (As a measure of raw investigation time: $F(1,8) = 9.660$, $p = 0.014$ and normalized by total investigation time: Novel Investigation Time/Total Investigation Time x 100, versus Familiar Investigation Time/Total Investigation Time x 100: $F(1,8) = 16.639$, $p = 0.004$), whereas *Mbnl2*$^{\Delta E2/\Delta E2}$ mice did not (raw investigation time: $F(1,9) = 0.490$, $p = 0.502$, normalized investigation time: $F(1,9) = 0.037$, $p = 0.851$); *Mbnl2*$^{+/+}$ mice: 73.2% investigation of the novel object versus 26.68% investigation of the familiar object, *Mbnl2*$^{\Delta E2/\Delta E2}$ mice: 48.9% investigation of the novel object versus. 51.1% investigation of the familiar object) (Figures 1G and 1H). These results demonstrate that wild-type mice develop long-term memory of the familiar objects on day 3 and prefer to investigate the novel object on day 4. In contrast, *Mbnl2*$^{\Delta E2/\Delta E2}$ mice do not interact preferentially with the novel object, indicating a deficit in long-term object recognition memory.

Impaired performance in the object recognition task is unlikely to be due to a visual deficit in the *Mbnl2*$^{\Delta E2/\Delta E2}$ mice for the following reasons. To examine vision *per se*, the penlight vision test, where the pupil responds to light, was conducted. In this test, both genotypes showed pupil contraction when exposed to a penlight, thus demonstrating that the *Mbnl2*$^{\Delta E2/\Delta E2}$ mice are not blind. *Mbnl2*$^{\Delta E2/\Delta E2}$ mice behave normally in the visual placement test where the mouse is held by the tail and slowly lowered toward a clean cage placed on a tabletop. In this test, as the mouse gets closer, it should reach its front paws out to create a softer landing if it can see the cage. If the mouse cannot see, it will not reach out the forepaws until the vibrissae are touching the cage.[37] Both the *Mbnl2*$^{+/+}$ and the *Mbnl2*$^{\Delta E2/\Delta E2}$ mice behaved identically in the visual placement test and reached for the cage with their front paws before the vibrissae came in contact with the cage. A visual impairment would be expected to result in an impaired ability to see the objects, approach, and interact with them. We therefore performed an analysis on head orientation using an automated tracking system to determine if the *Mbnl2*$^{\Delta E2/\Delta E2}$ mice showed a similar degree of orienting their heads toward the objects as the *Mbnl2*$^{+/+}$ mice. Anymaze software was used to analyze the movement and orientation of the mice in reference to the four corners of the chamber, two of which have objects and two of which are empty. In theory, if the mice can see the objects, they should be orienting or moving toward them more than toward the empty corners. We calculated a ratio of time spent orienting toward versus away from the objects during the day 3 object familiarization session. This ratio was similar in the *Mbnl2*$^{\Delta E2/\Delta E2}$ and *Mbnl2*$^{+/+}$ mice ($F(1,18) = 0.069$, $p = 0.796$, *Mbnl2*$^{+/+}$: mean = 0.554 [0.507 - 0.601] 95% CE; *Mbnl2*$^{\Delta E2/\Delta E2}$: mean = 0.560 [0.515 - 0.604] 95% CE) indicating that they have a similar ability to orient toward the objects. Visually impaired mice would be expected to show a deficit in these ratios. As noted above, *Mbnl2*$^{\Delta E2/\Delta E2}$ mice show similar levels of object investigation as the *Mbnl2*$^{+/+}$ mice, in the day 3 object familiarization session, indicating that their propensity and capacity to interact with the objects is normal (Object investigation times: *Mbnl2*$^{+/+}$ = 64.5 + 8.244 s, *Mbnl2*$^{\Delta E2/\Delta E2}$ = 55.007 + 8.69 s, $F(1,19) = 0.631$, $p = 0.438$). Lastly, the absolute investigation times for the test phase on day 4 [*Mbnl2*$^{+/+}$: novel = 7.924 + 1.564 s, familiar = 2.650 + 0.796 s ($p = 0.014$); *Mbnl2*$^{\Delta E2/\Delta E2}$: novel = 8.534 + 1.536 s, familiar = 7.478 + 0.756 s ($p = 0.502$)] demonstrate that the interaction times with the familiar and novel objects for the *Mbnl2*$^{\Delta E2/\Delta E2}$ mice are comparable to the interaction time with the novel object for the *Mbnl2*$^{+/+}$ mice. The comparable interaction times of *Mbnl2*$^{\Delta E2/\Delta E2}$ mice with the novel and familiar objects demonstrate that the *Mbnl2*$^{\Delta E2/\Delta E2}$ mice were not novelty aversive or afraid to interact with the objects. Therefore, as the *Mbnl2*$^{\Delta E2/\Delta E2}$ mice do not prefer to spend time with the novel object over the

familiar object, these results are consistent with normal visual perception and an impairment in long-term object memory in the NORT in *Mbnl2*$^{\Delta E2/\Delta E2}$ mice.

Mbnl2$^{\Delta E2/\Delta E2}$ dorsal hippocampus initiates an aberrant transcriptome response when exploring the context of a novel arena

Tanimizu et al. have shown that in wild-type mice, object memory is impaired if habituation to the arena in which the objects are later encountered, does not occur.[28] This observation suggests that cognition of the context of the arena may be important in the formation of memories of objects encountered subsequently in that arena. Other experiments have shown that exposure to a novel context for 10 min produces a long-term memory for context.[38,39] As object recognition memory is impaired in *Mbnl2*$^{\Delta E2/\Delta E2}$ mice, we tested if long-term memory of the context of the arena occurs normally in *Mbnl2*$^{\Delta E2/\Delta E2}$ mice. Long-term memory requires gene transcription with a pattern of upregulated genes that enhance memory and downregulated genes, which impose inhibitory constraints on memory formation.[40–42] Therefore, to test if *Mbnl2*$^{\Delta E2/\Delta E2}$ mice cognize and develop a long-term memory of the context of the arena during the 10 min habituation phase of the NORT, we studied the transcriptome response of the dorsal hippocampus. The dorsal hippocampus was selected as it plays an important role in context and spatial learning and memory.[43–45] Specifically, RNA-seq was carried out in new cohort of mice and the dorsal hippocampi were dissected on day 1 of the NORT, prior to exploration of the novel arena (the empty chamber) (Not exposed to the empty chamber: denoted as house controls; n = 3/genotype) or immediately after exploration of the novel arena (the empty chamber) for 10 min (Exposed to the empty chamber: denoted as trained; n = 3/genotype). The number of significant transcriptome alterations ($p < 0.05$) observed in the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus was ~70% lower than the number of significant transcriptome changes observed in the dorsal hippocampus of *Mbnl2*$^{+/+}$ mice with exploration of the empty chamber. Trained versus house control *Mbnl2*$^{+/+}$ mice: Of the 1571 significant mRNA alterations, 798 (51%) mRNAs showed decreased levels and 773 (49%) mRNAs showed increased levels. Trained versus house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice: Of the 421 significant mRNA alterations, 296 (70%) mRNAs showed decreased levels and 125 (30%) mRNAs showed increased levels (Figures 2Ai, 2Aii, S1A, and S1B, Tables S1, S2, and S3). Thus, a diminished transcriptome response, with a decrease in the number of significant mRNA level alterations, was observed with exploration of the empty chamber, in the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus.

When all genes with significant p values ($p < 0.05$) and log2 fold change >1.5 (<-1.5) are considered, a pattern of mRNA level reductions predominates in *Mbnl2*$^{+/+}$ mice with exploration of the empty chamber. Specifically, of 111 significant mRNA alterations, 2.7% show a log2 fold change >1.5 and 97.3% show a log2 fold change <-1.5. This pattern of predominant mRNA level downregulation is altered in *Mbnl2*$^{\Delta E2/\Delta E2}$ mice. Specifically, of the 59 significant mRNA alterations, a ~5-fold increase in mRNAs showing a log2 fold change >1.5 (15.3%) is observed in *Mbnl2*$^{\Delta E2/\Delta E2}$ mice with exploration of the empty chamber. Pattern alterations with exploration of the empty chamber in *Mbnl2*$^{+/+}$ and *Mbnl2*$^{\Delta E2/\Delta E2}$ mice are shown as heatmaps demonstrating the range and distribution of significant ($p < 0.05$) mRNA alterations with log2 fold change >1.5 (<-1.5) (Figures 2Bi and 2Bii and Table S4). Taken together, these results show that both the number of significant mRNA changes and the pattern of up- and downregulated genes altered in the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus with exploration of the novel context of the empty chamber.

To determine the validity of our RNA-seq data analysis, we measured the levels of mRNAs chosen semi-randomly, which were both up- and downregulated significantly ($p < 0.05$), during novel context exploration in the *Mbnl2*$^{+/+}$ dorsal hippocampus by qPCR (Figure 2C). In this dataset, three genes implicated in the NORT were measured. The somatostatin receptor subtype *Sstr3* is critical for object recognition and *Sstr3* knockout mice are severely impaired in discriminating novel objects.[46] In contrast, Apelin and its receptor (*Aplnr*) are implicated in blocking long-term memory consolidation in the NORT.[47] Other studies have shown *FosB* induction in the dorsal hippocampus is required for novel object recognition.[48] Consistent with these observations, we observe *Sstr3* mRNA levels elevated ($p = 0.04$) and *Aplnr* mRNA levels reduced ($p = 0.02$) significantly in the *Mbnl2*$^{+/+}$ dorsal hippocampus but not in the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus with novel context exploration. Interestingly, *FosB* mRNA levels show significant increases in both *Mbnl2*$^{+/+}$ ($p = 0.02$) and in *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampi ($p = 0.03$), demonstrating that only a subset of functionally relevant transcriptome alterations may be impaired in *Mbnl2*$^{\Delta E2/\Delta E2}$ mice with exploration of a novel context.

To test if transcription repressor mRNA level alterations correlate with transcriptome pattern distortions observed in the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus, we examined the PR/SET domain protein, *Prdm8*,

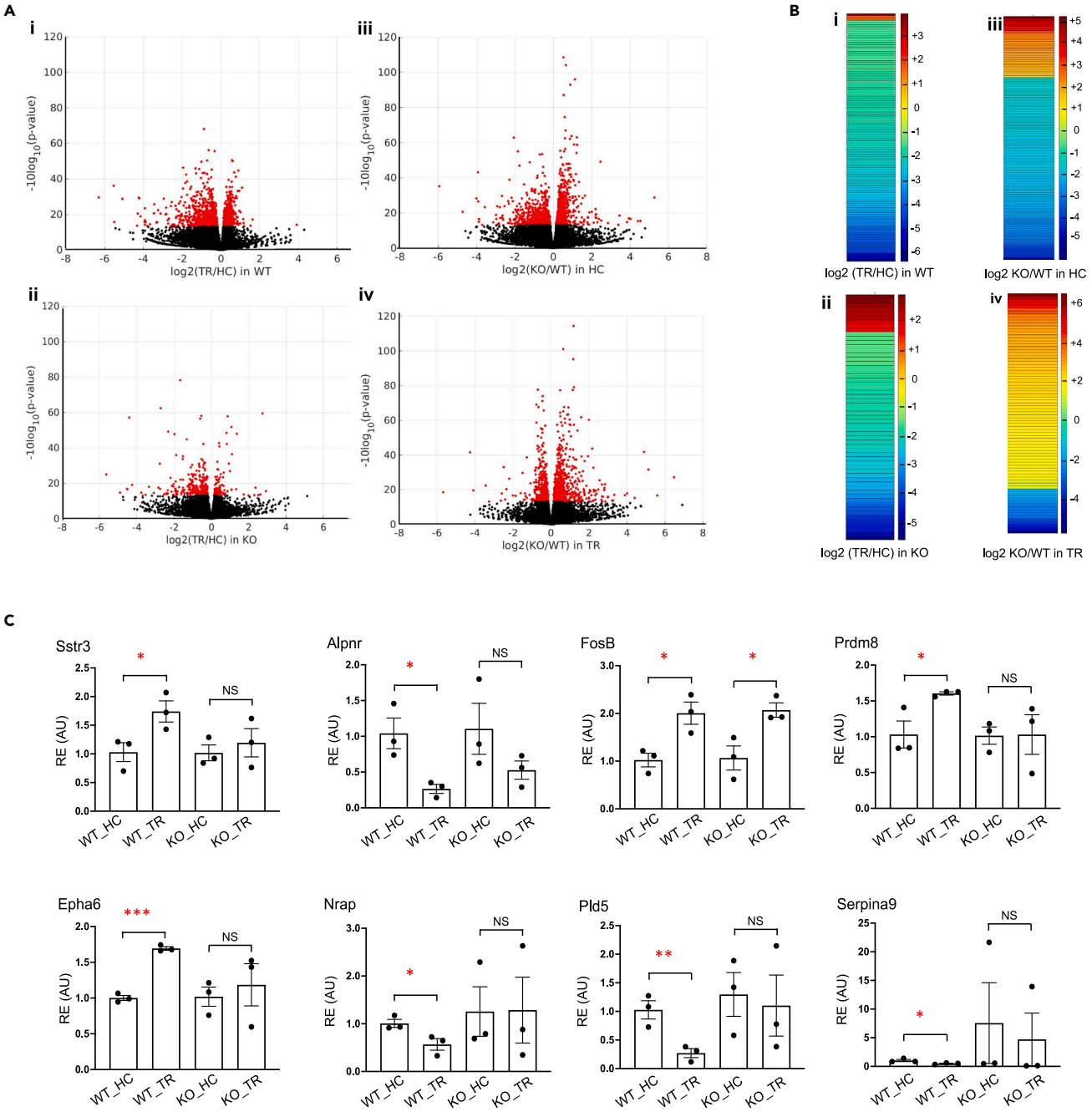

Figure 2. *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus initiates an aberrant transcriptome response when exploring the context of a novel arena

(A) Volcano plots of transcriptome differences in the dorsal hippocampi of trained versus house control *Mbnl2*$^{+/+}$ mice (Ai), trained versus house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice (Aii), house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice versus house control *Mbnl2*$^{+/+}$ mice (Aiii), and trained *Mbnl2*$^{\Delta E2/\Delta E2}$ mice versus trained *Mbnl2*$^{+/+}$ mice (Aiv) are shown. In all cases, the log2 fold change and the corresponding p value are plotted on the x axis and y axis, respectively. Significant gene alterations ($p < 0.05$) are shown in red and non-significant alterations are shown in black. All corresponding histograms are shown in Figure S1. Data are tabulated in Tables S1, S2, S3, S9, and S11.

(B) Heatmaps show the range and distribution of genes with significant p values ($p < 0.05$) and log2 fold change >1.5 (<-1.5). Dorsal hippocampi from trained versus house control *Mbnl2*$^{+/+}$ mice (Bi), trained versus house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice (Bii), house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice versus house control *Mbnl2*$^{+/+}$ mice (Biii), and trained *Mbnl2*$^{\Delta E2/\Delta E2}$ mice versus trained *Mbnl2*$^{+/+}$ mice (Biv) are shown. Data are tabulated in Table S4.

(C) qPCR analysis of steady-state mRNA level alterations in the dorsal hippocampi of house control and trained *Mbnl2*$^{+/+}$ and *Mbnl2*$^{\Delta E2/\Delta E2}$ mice are shown. Dorsal hippocampi were derived from n = 3 mice/genotype/condition. Means +SEM are shown. WT: *Mbnl2*$^{+/+}$, KO: *Mbnl2*$^{\Delta E2/\Delta E2}$, HC: house control, TR: trained. In all cases, *, **, and *** indicate levels of significance where $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively; NS: not significant.

which mediates repression of target genes to regulate neural circuitry.[49] We observe that *Prdm8* mRNA levels increase with exploration of the novel context in the $Mbnl2^{+/+}$ ($p = 0.04$) but not in the $Mbnl2^{\Delta E2/\Delta E2}$ dorsal hippocampus. Similarly, *Epha6*, implicated in neural circuits underlying multiple aspects of learning and memory,[50] shows a significant increase in mRNA levels only in the $Mbnl2^{+/+}$ ($p = 0.00009$) but not in the $Mbnl2^{\Delta E2/\Delta E2}$ dorsal hippocampus with exploration of a novel context. Three other mRNAs downregulated in the $Mbnl2^{+/+}$ dorsal hippocampus with novel context exploration, which were measured were *Nrap*, which regulates NMDA receptors to modify synaptic strength,[51] *Pld5,* which is associated with autism,[52] and *Serpina9,* an mRNA whose upregulation is implicated in Alzheimer disease.[53] The levels of all 3 mRNAs decrease significantly only in the $Mbnl2^{+/+}$ but not in the $Mbnl2^{\Delta E2/\Delta E2}$ dorsal hippocampus with novel context exploration [$Mbnl2^{+/+}$ mice: *Nrap* ($p = 0.04$), *Pld5* ($p = 0.01$), and *Serpina9* ($p = 0.05$)]. These experiments validate our RNA-seq data analysis by demonstrating that the qPCR measurements of the semi-randomly selected mRNAs were comparable to the mRNA level alterations ($p < 0.05$) identified by RNA-seq data analysis (Tables S2 and S3).

$Mbnl2^{\Delta E2/\Delta E2}$ dorsal hippocampus responds to the context of a novel arena with a lack of enrichment for cellular and functional pathways implicated in learning and memory

Scatterplots comparing all significant transcriptome alterations ($p < 0.05$) occurring with the exploration of the context of a novel arena (empty chamber) in the $Mbnl2^{+/+}$ and the $Mbnl2^{\Delta E2/\Delta E2}$ dorsal hippocampi demonstrates that a large fraction of the significant changes occurring in the $Mbnl2^{+/+}$ dorsal hippocampus are not significantly altered in the $Mbnl2^{\Delta E2/\Delta E2}$ dorsal hippocampus and vice versa (Figures 3A and 3B). We therefore hypothesized, first, that transcriptome changes occurring selectively in the $Mbnl2^{+/+}$ dorsal hippocampus may contain alterations required for the cognition of the novel context of the empty chamber. Second, transcriptome alterations in the dorsal hippocampus, shared by $Mbnl2^{+/+}$ and $Mbnl2^{\Delta E2/\Delta E2}$ mice, may reflect events either not associated with cognition or are insufficient to facilitate cognition of the novel context of the empty chamber. Third, unique transcriptome changes deployed in the $Mbnl2^{\Delta E2/\Delta E2}$ dorsal hippocampus may contain events that do not facilitate cognition, are insufficient to facilitate cognition, or can potentially impair cognition of the novel context of the empty chamber.

To test this hypothesis, the Ingenuity Pathway Analysis (IPA) was used to study these three sets of transcriptome alterations.[54] Examination of transcriptome changes ($p < 0.05$) selectively manifesting only in the $Mbnl2^{+/+}$ dorsal hippocampus demonstrates significant, non-random enrichment (activation *Z* score >2 or < −2) for cellular events associated with learning and memory[40,55,56] including increased neurotransmission, synaptic transmission, potentiation of the synapse, long-term potentiation, microtubule dynamics, quantity, transport and endocytosis of synaptic vesicles, branching of neurons, extension of cellular protrusions, sprouting, shape change and branching of neurites, and dendritic growth and branching (Figure 3C; Individual gene alterations for all functions are shown in Data S1A–S1Q and in Tables S5 and S6). Therefore, based on the transcriptome response occurring selectively in the dorsal hippocampus, $Mbnl2^{+/+}$ mice appear to cognize and develop a long-term memory of the novel context of the empty chamber during the 10-min exploratory phase.

Transcriptome alterations shared by the $Mbnl2^{+/+}$ and the $Mbnl2^{\Delta E2/\Delta E2}$ mice during novel context exploration showed non-random enrichment (activation *Z* score >2 or < −2) for an increase in the quantity of neurites and a decrease in the proliferation of neuroglia, quantity of CNS cells, and self-renewal of cells (Figure 3C; Individual gene alterations for all functions are shown in Data S2A–S2D and in Tables S5 and S7). Unique transcriptome alterations selectively manifesting only in the dorsal hippocampus of the $Mbnl2^{\Delta E2/\Delta E2}$ mice with novel context exploration show significant, non-random enrichment (activation *Z* score >2 or < −2) for decreased development of the CNS, decreased formation of the brain, the telencephalon and the forebrain, decreased growth of nervous tissue, decreased cell viability, and decreased cell viability of neurons (Figure 3C; Individual gene alterations for all functions are shown in Data S3A–S3G and in Tables S5 and S8). This analysis demonstrates a conspicuous lack of enrichment for cellular functions implicated in learning and memory in the dorsal hippocampus of $Mbnl2^{\Delta E2/\Delta E2}$ mice during novel context exploration. The absence of transcriptome changes predicted to facilitate learning and memory during novel context exploration in the dorsal hippocampus may therefore contribute to impaired learning and memory of the novel context of the empty chamber in $Mbnl2^{\Delta E2/\Delta E2}$ mice. Enrichment for cellular pathways that decrease cell viability and the viability of neurons with novel context exploration in the dorsal hippocampus of $Mbnl2^{\Delta E2/\Delta E2}$ mice may have additional deleterious effects on context learning and memory that

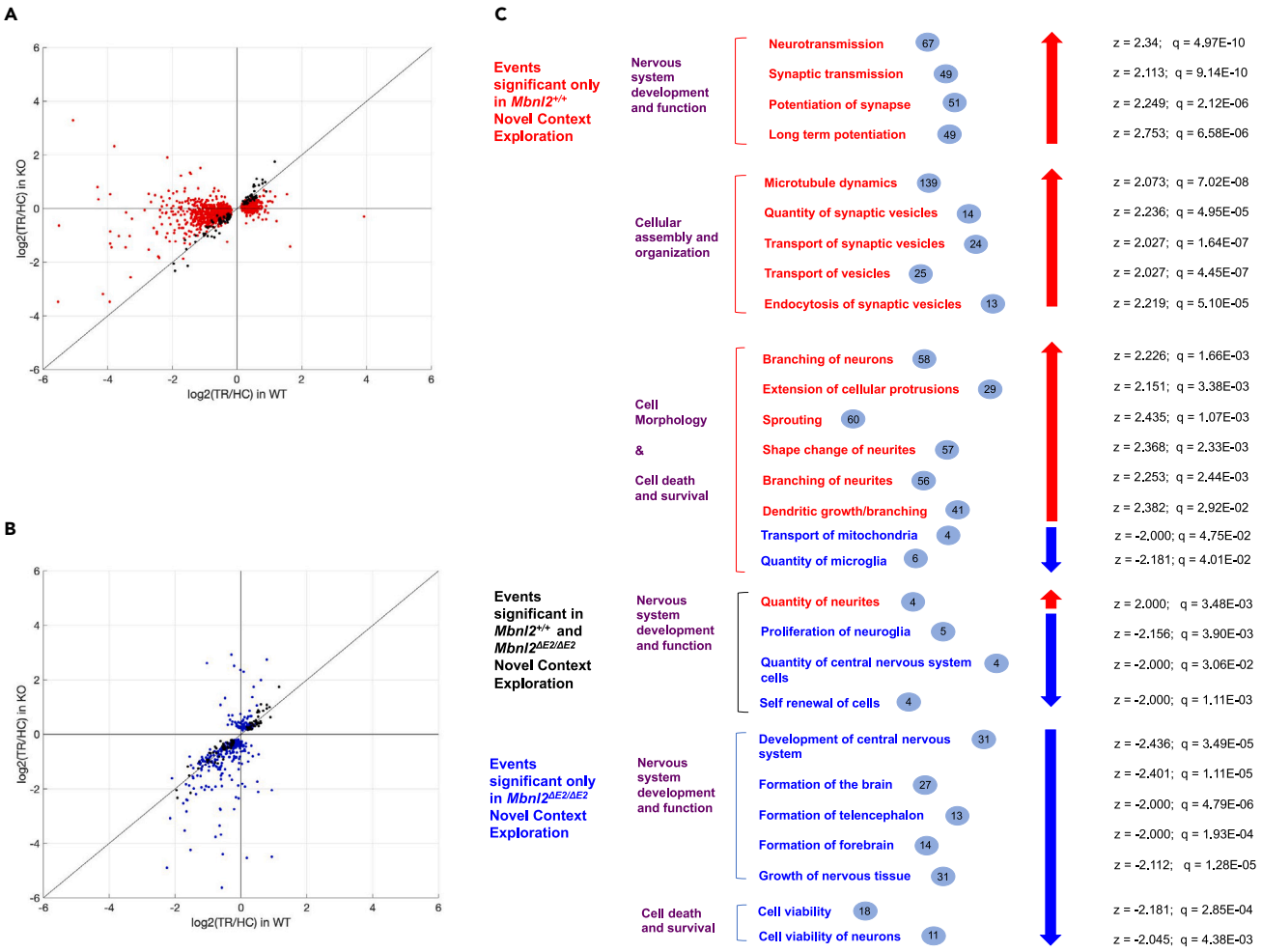

Figure 3. *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus responds to the context of a novel arena with a lack of enrichment for cellular and functional pathways implicated in learning and memory

(A and B) Scatterplots of log2(TR/HC) in WT mice (*Mbnl*$^{+/+}$ novel context exploration) versus log2(TR/HC) in KO mice (*Mbnl2*$^{\Delta E2/\Delta E2}$ novel context exploration) are shown. Genes significantly altered in both cases are indicated in black. Genes that are significantly altered only with *Mbnl*$^{+/+}$ but not with *Mbnl2*$^{\Delta E2/\Delta E2}$ novel context exploration are shown in red and assigned their true values in Panel a, and genes that are significantly altered only with *Mbnl2*$^{\Delta E2/\Delta E2}$ but not with *Mbnl*$^{+/+}$ novel context exploration are indicated in blue and assigned their true values in Panel B. WT: *Mbnl2*$^{+/+}$, KO: *Mbnl2*$^{\Delta E2/\Delta E2}$, HC: house control, TR: trained.

(C) All cellular functions showing significant non-random enrichment only with *Mbnl*$^{+/+}$ but not with *Mbnl2*$^{\Delta E2/\Delta E2}$ novel context exploration, with both *Mbnl*$^{+/+}$ and *Mbnl2*$^{\Delta E2/\Delta E2}$ novel context exploration and only with *Mbnl2*$^{\Delta E2/\Delta E2}$ but not with *Mbnl*$^{+/+}$ novel context exploration are shown. IPA activation *Z* score < −2 or >2 and q values (B–H multiple testing correction p values) for the cellular functions are indicated. The number of genes for each cellular function is shown in blue circles. Individual gene alterations for the cellular functions in Panel c are shown in Data S1–S3 and in Tables S5, S6, S7, and S8. The IPA prediction legend is shown in Figure S2.

are yet to be understood and/or may contribute to the gray matter and neuron loss reported in the hippocampus of *Mbnl2*$^{\Delta E2/\Delta E2}$ mice and patients with DM1.[15,57]

The transcriptome of the house control *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus overlaps with the transcriptome alterations occurring in the *Mbnl2*$^{+/+}$ dorsal hippocampus with novel context exploration

The dorsal hippocampus of house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice shows alterations ($p < 0.05$) in 1368 genes when compared with the dorsal hippocampus of house control *Mbnl2*$^{+/+}$ mice. Of the significant gene alterations, 620 (45%) genes showed decreased levels and 748 (55%) genes showed increased levels (Figures 2Aiii and S1C and Table S9). Pattern alterations shown as heatmaps demonstrating the range and distribution of significant ($p < 0.05$) mRNA alterations with log2 fold change >1.5 (<-1.5) show that

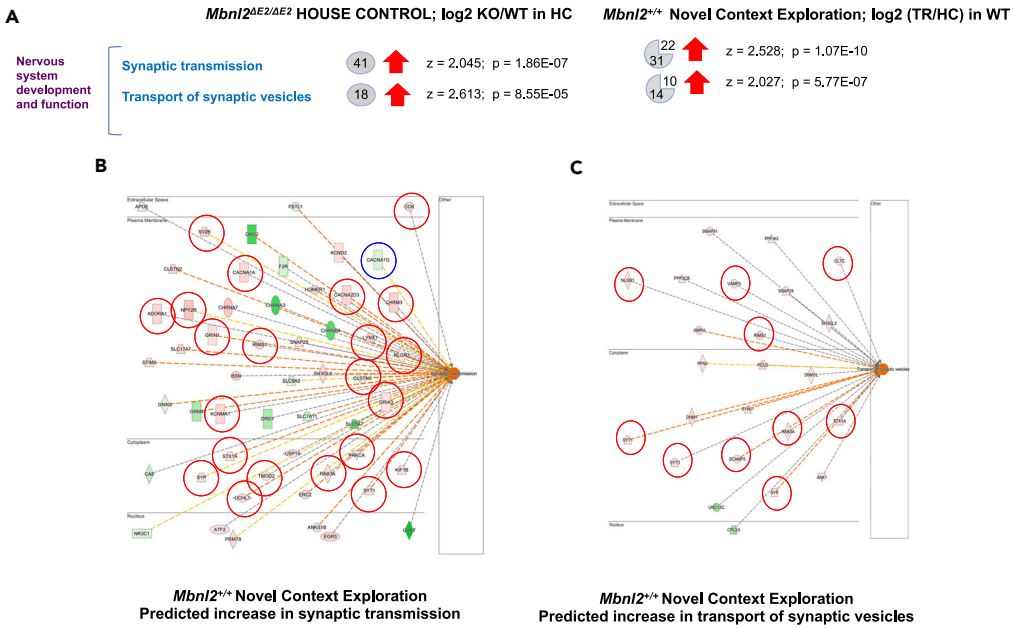

Figure 4. The transcriptome of the house control *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus overlaps with the transcriptome alterations occurring in the *Mbnl2*$^{+/+}$ dorsal hippocampus with novel context exploration

(A) Shared cellular functions showing significant non-random enrichment in house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice (house control *Mbnl2*$^{\Delta E2/\Delta E2}$ versus house control *Mbnl2*$^{+/+}$ mice) and in *Mbnl*$^{+/+}$ mice with novel context exploration (trained versus house control *Mbnl*$^{+/+}$ mice) are shown. IPA activation *Z* score < −2 or >2 and q values (B–H multiple testing correction p values) for the cellular functions are indicated. The number of genes for each cellular function of the house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice is shown in gray circles. The numbers of shared gene alterations for each cellular function in *Mbnl*$^{+/+}$ novel context exploration and in house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice are indicated above the gray pies. WT: *Mbnl2*$^{+/+}$, KO: *Mbnl2*$^{\Delta E2/\Delta E2}$, HC: house control, TR: trained.

(B and C) Individual gene alterations for increased synaptic transmission (B) and increased transport of synaptic vesicles (C) occurring with novel context exploration in the dorsal hippocampus of *Mbnl*$^{+/+}$ mice are shown. Genes comparably altered in the house control *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus are ringed in red and genes that are inversely altered are ringed in blue. Individual gene alterations for the cellular functions in Panel a are shown in Data S4 and S5 and in Tables S2, S9, and S10. The IPA prediction legend is shown in Figure S2.

the fraction of significant mRNA level increases (log2 fold change >1.5) is elevated in the dorsal hippocampus of house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice (23.88%) when compared with house control *Mbnl2*$^{+/+}$ mice (Figures 2Biii and Table S4). These data suggest that the aberrant transcriptome of the house control *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus may contribute to the diminished transcriptome response of the dorsal hippocampus during novel context exploration in *Mbnl2*$^{\Delta E2/\Delta E2}$ mice, potentially due to saturation effects.

To test this hypothesis, we examined if one or more cellular functions, predicted by IPA to be significantly enriched during novel context exploration in the *Mbnl2*$^{+/+}$ dorsal hippocampus, are also enriched in the house control *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus. Consistent with this hypothesis, IPA analysis demonstrates that the house control *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus shows significant, non-random enrichment (activation *Z* score >2 or < −2) for the shared functions of increased synaptic transmission and transport of synaptic vesicles (Figure 4A; Individual gene alterations for each function in the house control *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus are shown in Data S4 and in Table S10).

If saturation effects are responsible for the diminished transcriptome response in the *Mbnl2*$^{\Delta E2/\Delta E2}$ hippocampus during novel context exploration (Figure 2Aii), two events are predicted: First, a subset of transcriptome alterations that occur with novel context exploration in the *Mbnl2*$^{+/+}$ dorsal hippocampus must also occur in a comparable fashion in the house control *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus. Second, the presence of shared transcriptome changes in the house control *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus must preclude, at least for one or more functionally relevant genes, a further significant change during novel context exploration in the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus. When the individual mRNA level alterations

occurring with novel context exploration in the dorsal hippocampus of *Mbnl2*$^{+/+}$ mice were compared with mRNA level changes observed in the dorsal hippocampus of house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice, a subset of mRNA level alterations were found to be comparably changed in the shared cellular functions of synaptic transmission and transport of synaptic vesicles. Specifically, 22 of 53 mRNA changes predicted to increase synaptic transmission and 10 of 24 mRNA changes predicted to increase transport of synaptic vesicles, which occur in the dorsal hippocampus of *Mbnl2*$^{+/+}$ mice with novel context exploration comparably altered in the dorsal hippocampus of house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice (Figure 4A, Data S4 and S5, Tables S2, S9, and S10).

Significantly, only 1 (Gria3:log2FC = 0.18, p = 0.03) of the 22 mRNA changes predicted to increase synaptic transmission, which are comparably altered in the *Mbnl2*$^{+/+}$ dorsal hippocampus during novel context exploration and in the dorsal hippocampus of house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice (Figure 4B), shows a significant change in the dorsal hippocampus of *Mbnl2*$^{\Delta E2/\Delta E2}$ mice with novel context exploration (Table S3). Similarly, no comparably altered mRNA changes predicted to increase the transport of synaptic vesicles (Figure 4C) show significant changes in the dorsal hippocampus of *Mbnl2*$^{\Delta E2/\Delta E2}$ mice with novel context exploration (Table S3). This analysis therefore supports the hypothesis that the aberrant pre-activation of a subset of mRNA level changes in the house control *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus, which normally manifest with novel context exploration in the *Mbnl2*$^{+/+}$ dorsal hippocampus, prevents their significant re-deployment, possibly due to saturation effects, when *Mbnl2*$^{\Delta E2/\Delta E2}$ mice explore the novel context of the empty chamber. Thus, alterations in the untrained *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus may contribute to the impaired transcriptome response mounted in response to the novel context of the empty chamber in *Mbnl2*$^{\Delta E2/\Delta E2}$ mice.

Post novel context exploration, the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus shows changes in genes implicated in tauopathy and dementia

Post novel context exploration, the dorsal hippocampus of the trained *Mbnl2*$^{\Delta E2/\Delta E2}$ mice shows significant alterations ($p < 0.05$) in 1117 genes, with 438 (~39%) genes showed decreased levels and 679 (~61%) genes showed increased levels when compared with the dorsal hippocampus of trained *Mbnl2*$^{+/+}$ mice (Figures 2Aiv and S1D and Table S11). The fraction of significant mRNA level increases of log2 fold change >1.5 was further elevated in the dorsal hippocampus of trained (81.48%) *Mbnl2*$^{\Delta E2/\Delta E2}$ mice when compared with trained *Mbnl2*$^{+/+}$ mice (Figures 2Biv and Table S4). To test if gene alterations that occur in *Mbnl2*$^{\Delta E2/\Delta E2}$ mice during novel context exploration can impact other behaviors, we first examined if significant enrichment was observed for genes implicated in other behaviors by IPA in the dorsal hippocampus of house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice. Subsequently, we tested if such gene cohorts were impacted by novel context exploration. This analysis demonstrated significant enrichment for genes implicated in tauopathy in the dorsal hippocampus of house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice (house control *Mbnl2*$^{\Delta E2/\Delta E2}$ versus house control *Mbnl2*$^{+/+}$) (Figure 5A, Tables S9 and S12). Examination of mRNA alterations in the dorsal hippocampus of trained *Mbnl2*$^{\Delta E2/\Delta E2}$ mice (trained *Mbnl2*$^{\Delta E2/\Delta E2}$ versus trained *Mbnl2*$^{+/+}$) showed enrichment for genes impacting tauopathy and dementia (Figures 5B and 5C, Tables S11 and 12). Comparison of these gene cohorts showed that post novel context exploration, in addition to mRNA alterations observed in the dorsal hippocampus of house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice, the dorsal hippocampus of trained *Mbnl2*$^{\Delta E2/\Delta E2}$ mice showed significant alterations in new genes impacting these behaviors. These data suggest that on encountering the novel context of the empty chamber, the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus initiates mRNA changes that can impact other CNS dysfunctions reported in DM1 including tauopathy and cognitive decline with time.[19–21,58,59]

DISCUSSION

CNS disorders are a prominent feature of DM1 with visuospatial dysfunction and impairments in tasks requiring recognition or memory of objects and figures reported in all forms of the disease.[14–18] The mechanism whereby these defects manifest is currently unknown. Here, we demonstrate that Mbnl2 loss in mice plays a causal role in impaired long-term object recognition memory and in altering the hippocampal response to the context of a novel arena in which the object is later encountered. In particular, when a mouse explores the novel context of the empty chamber, Mbnl2 inactivation precludes the deployment of transcriptome changes predicted to facilitate learning and memory in the dorsal hippocampus, and mounts instead an aberrant response predicted to impair growth and neuron viability. Our analysis supports the hypothesis that saturation effects in the house control *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus may prevent the deployment of a functionally relevant transcriptome response when the novel context of the

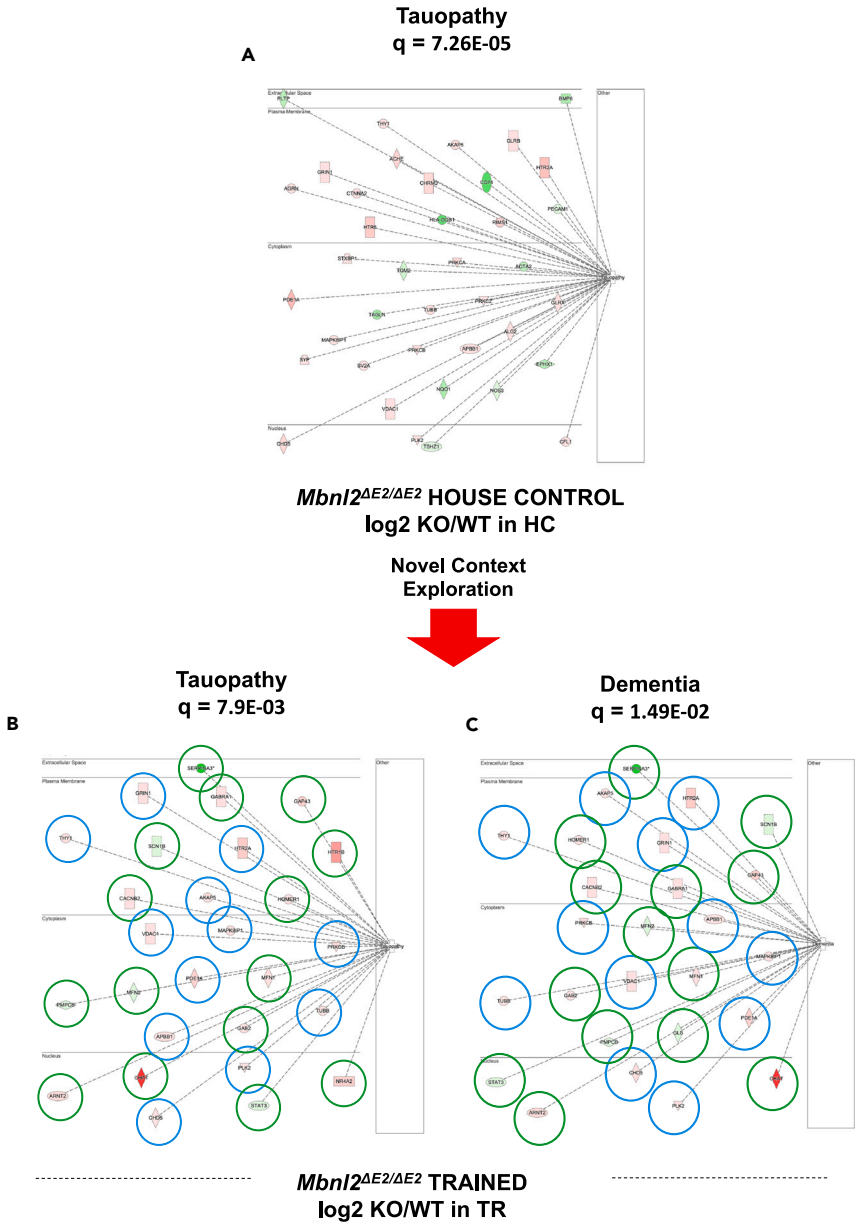

Figure 5. Post novel context exploration, the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus shows changes in genes implicated in tauopathy and dementia

(A–C) q values (B-H multiple testing correction p values) of genes implicated in tauopathy (A) in the dorsal hippocampus of house control *Mbnl2*$^{\Delta E2/\Delta E2}$mice (house control *Mbnl2*$^{\Delta E2/\Delta E2}$ versus house control *Mbnl2*$^{+/+}$ mice; indicated as KO/WT in HC) and q values of genes implicated in tauopathy (B) and dementia (C) in trained *Mbnl2*$^{\Delta E2/\Delta E2}$mice (trained *Mbnl2*$^{\Delta E2/\Delta E2}$ versus trained *Mbnl2*$^{+/+}$ mice; indicated as KO/WT in TR) are shown. In panels B and C, all genes that are comparably altered in the dorsal hippocampus of the house control *Mbnl2*$^{\Delta E2/\Delta E2}$ mice and the trained *Mbnl2*$^{\Delta E2/\Delta E2}$ mice are ringed in blue and genes that are altered significantly only in the dorsal hippocampus of trained *Mbnl2*$^{\Delta E2/\Delta E2}$mice are ringed in green. Individual gene alterations are shown in Tables S9, S11, and S12. The IPA prediction legend is shown in Figure S2.

empty chamber is explored. Lastly, these data suggest that the aberrant transcriptome changes occurring with exploration of the novel context in the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus may impact other CNS dysfunctions reported in DM1 including tauopathy and cognitive decline. These results support the hypothesis that the functional inactivation of MBNL2 by CUG expansion RNAs in patients with DM1 may impair the formation of object memories and alter context processing at the transcriptome level in the dorsal hippocampus (Figure 6).

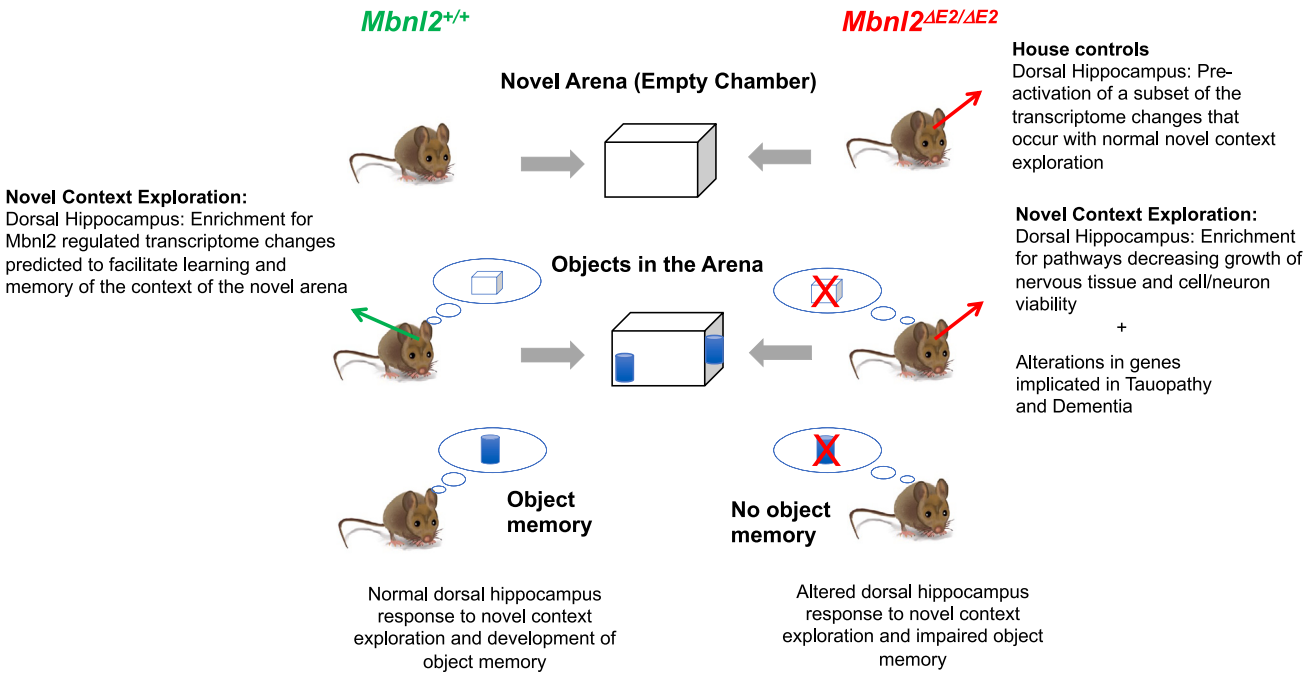

Figure 6. Mbnl2 inactivation impairs novel context processing and object recognition memory

The validity of the study design in identifying transcriptome alterations that initiate learning and long-term memory of the novel context of the empty chamber during the 10-min habituation phase of the NORT is demonstrated at multiple steps: First, consistent with long-term memory requiring both activation and repression of gene transcription and the key role that the dorsal hippocampus plays in context and spatial learning and memory,[40–45] we observe wide-spread transcriptome alterations that both increase and decrease mRNA steady-state levels subsequent to the 10 min novel context exploration phase of the NORT in the *Mbnl2*$^{+/+}$ dorsal hippocampus (Figure 2Ai). Second, pathway analysis of the transcriptome changes occurring with exploration of the novel context demonstrates enrichment for cellular events predicted to facilitate learning and memory in the *Mbnl2*$^{+/+}$ dorsal hippocampus (Figures 3A and 3C).[40,55,56] Lastly, qPCR validation studies of individual mRNA alterations in the *Mbnl2*$^{+/+}$ dorsal hippocampus with novel context exploration demonstrates the predicted changes in mRNA levels of genes previously implicated in the NORT, including *Sstr3*, *Aplnr*, and *FosB* (46–48) (Figure 2C).

We have utilized a filtering strategy to identify the transcriptome-wide mRNA level alterations predicted to increase neurotransmission, synaptic transmission, potentiation of synapse, long-term potentiation, microtubule dynamics, quantity, transport and endocytosis of synaptic vesicles, sprouting, extension of cellular protrusions, branching of neurons and neurites, shape changes of neurites, and dendritic growth and branching that are initiated during novel context exploration in the dorsal hippocampus of wild-type mice (Figure 3C). A parallel analysis uncovers the unusual transcriptome response of the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus, where cellular pathways associated with brain development, growth, and cell and neuron viability are predicted to be inhibited, during exploration of a novel context (Figure 3C). This comparison supports the hypothesis that Mbnl2 loss impairs context processing by altering the transcriptome response deployed by the dorsal hippocampus.

A previous observation from Tanimizu and colleagues suggests linkage between context familiarization during habituation with memories of objects encountered subsequently within that context.[28] Consistent with the observation of Tanimizu and colleagues, *Mbnl2*$^{\Delta E2/\Delta E2}$ mice demonstrate both impaired object recognition memory and an aberrant transcriptome response in the dorsal hippocampus, which plays an important role in context and spatial memory, during the habituation or context familiarization phase of the NORT. Thus, our results when taken in conjunction with those of Tanimizu et al. suggest that the altered dorsal hippocampus transcriptome response during context exploration of the empty chamber may contribute to the impaired object recognition memory of *Mbnl2*$^{\Delta E2/\Delta E2}$ mice in the NORT. How this linkage

occurs mechanistically is of interest. As suggested by a previous study, neuronal ensembles activated during novel context exploration and subsequently with object exploration may overlap or interact in ways that have yet to be fully understood to allow long-term object recognition memories to develop.[39] In this regard, it will be of interest to test if small-molecule-mediated displacement of MBNL2 from CUG expansion RNAs in DM1 ameliorates visuospatial dysfunction and/or performance in tasks requiring recognition or memory of objects in patients. The transcriptome-wide changes that occur with novel context exploration in *Mbnl2*$^{+/+}$ and *Mbnl2*$^{\Delta E2/\Delta E2}$ mice reported here could provide valuable molecular markers to test the efficacy of CNS therapies in DM1.

It is unclear how RNA-binding proteins such as Mbnl2 regulate the transcriptome response predicted to facilitate learning and memory in the dorsal hippocampus when exploring a novel context. Both our previous studies and those of others have shown that the Mbnl proteins regulate target RNA splicing, transport, and translation.[27,60–63] IPA analysis of the 568 Mbnl2 target RNAs that are altered during *Mbnl2*$^{+/+}$ novel context exploration demonstrates significant enrichment for cellular functions implicated in learning and memory[27] (Figure S3 and Table S13). It is however unclear how selective impairment of long-term object memory occurs in *Mbnl2*$^{\Delta E2/\Delta E2}$ mice. Curation of mRNA level alterations occurring with context exploration in the *Mbnl2*$^{+/+}$ and the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus provides intriguing clues, demonstrating changes in mRNA levels of genes regulating transcription and chromatin structure, such as *Prdm8*, which facilitate gene repression to regulate neural circuitry.[49] Specific alterations in mRNA levels of genes implicated in the NORT, such as *Sstr3* and *Aplnr* may further steer this selectivity.[46,47] Thus, it is possible that Mbnl2 may alter mRNA levels by changing transcription rates or mRNA half-lives of genes required for context processing in ways that are yet to be fully understood. Implication of RNA-binding proteins in transcriptional control and RNA stability[64,65] suggest that comparable Mbnl2 functions may also be relevant in shaping the transcriptome to facilitate novel context learning in the dorsal hippocampus.

Limitations of the study

These data support the hypothesis that the functional inactivation of MBNL2 by CUG expansion RNAs in patients with DM1 can contribute to long-term object memory impairments and altered novel context processing. The following aspects are limitations of this study. It is unclear how closely Mbnl2 function in mice reflects MBNL2 function in humans. The transcriptome analysis supports but does not prove the hypothesis that saturation effects can impair the transcriptome response of the *Mbnl2*$^{\Delta E2/\Delta E2}$ dorsal hippocampus to a novel context. An observation not directly proved by this analysis is the potential impact that the aberrant response to novel context exploration mediated by Mbnl2 loss may have on other CNS dysfunctions including tauopathy and dementia.

These analyses were not performed on female mice as estrous cycles could potentially interfere with the behavioral analyses.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106732.

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AUTHOR CONTRIBUTIONS

S.R. designed the study, analyzed the data, and wrote the manuscript. S.R. and L.C. jointly supervised this work. J. Cushman and I.Z. carried out the behavioral studies and analyzed the data. J. Cushman, I.Z., and S.R. wrote the sections relevant to the behavioral analysis. X.L., C.Z., J. Choi, and P.V. developed the mouse cohorts and carried out behavioral studies. J. Choi and A.R. developed the RNA-seq libraries. A.K., J. Choi, and S.R. analyzed the RNA-seq data. A.K., J. Cushman, and J. Choi contributed equally to this work. S.R., L.C., J. Cushman, A.K., and J. Choi have verified the underlying data.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Mouse dorsal hippocampus	This study	N/A
Chemicals, peptides, and recombinant proteins		
Trizol	Invitrogen	Cat# 15596026
Critical commercial assays		
RNeasy Mini Kit	Qiagen	Cat# 74004
Agilent 2100 Bioanalyzer	Agilent	Cat# G2939BA, RRID: SCR_019389
NEBNext Poly(A) mRNA Magnetic isolation Module	NEB	Cat# E7490S
NEBNext Ultra II RNA library prep kit for Illumina	NEB	Cat# E7770S
Deposited data		
mm10: Mouse reference genome NCBI build 38, GRCm38	Genome Reference Consortium	http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/, RRID: SCR_006553
polyA database	Gordon et al.[66]	http://hannonlab.cshl.edu/fastx_toolkit/, RRID: SCR_005534
RNA-seq data	This study	GEO (GSE226191)
Experimental models: Organisms/strains		
129sv/C57BL/6j *Mbnl2*$^{+/+}$	This study	N/A
129sv/C57Bl/6j *Mbnl2*$^{\Delta E2/\Delta E2}$	This study	N/A
129sv *Mbnl2*$^{\Delta E2/\Delta E2}$	Sta Maria et al.[57]	N/A
Oligonucleotides		
NEBNext Multiplex Oligos for Illumina (Index primers set 1)	NEB	Cat# E7335S
Primers for qPCR: see Table S15	This paper	N/A
Software and algorithms		
Cutadapt (v1.18)	Martin, M[67]	http://code.google.com/p/cutadapt/, RRID:SCR_011841
Galaxy	Useglalaxy.org	https://galaxyproject.org, RRID: SCR_006281
FASTX-Toolkit	Hannon Lab, CSHL	http://hannonlab.cshl.edu/fastx_toolkit/, RRID: SCR_005534
Bowtie for illumina (Galaxy version 1.1.2)	Langmead et al.[68]	http://bowtie-bio.sourceforge.net/index.shtml, RRID: SCR_005476
Tophat2 (v2.1.1)	Kim et al.[69]	http://ccb.jhu.edu/software/tophat/index.shtml, RRID: SCR_013035
HTseq (htseq-count Galaxy version 0.9.1)	Anders et al.[70]	http://htseq.readthedocs.io/en/release_0.9.1/, RRID: SCR_005514
DESeq2(v1.18.1)	Love et al.[71–73]	https://bioconductor.org/packages/release/bioc/html/DESeq2.html, RRID: SCR_015687
Ingenuity Pathway Analysis (IPA)	Kramer et al.[54]	http://www.ingenuity.com/products/pathways_analysis.html, RRID: SCR_008653
TopScan Behavioral Tracking software	CleverSys Inc	http://cleversysinc.com/CleverSysInc/?csi_products=topscan-suite, RRID: SCR_017141
Other		
Maxima H minus First strand cDNA kit	ThermoFisher Scientific	Cat# K1652
PerfeCTa SYBR Green SuperMix	Quanta	Cat# 95054-500

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sita Reddy (sitaredd@usc.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

RNA-seq files are deposited at GEO (GSE226191). No custom codes or mathematical algorithms were used in these analyses. Additional information required to reanalyze the data reported in this paper is available from the lead contact upon request. Source data for behavioral studies (Figure 1) are shown in Table S14. Source data for RNA-seq analysis (Figures 2, 3, 4, 5, and S1–S3; Data S1–S5) are shown in Tables S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, and S13. Source data for qPCR (Figure 2) are shown in Tables S15 and S16.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mbnl2$^{\Delta E2/\Delta E2}$ mice

129sv *Mbnl2*$^{\Delta E2/\Delta E2}$ mice with a constitutive deletion of exon 2 encoding the ATG start codon were developed as described in Sta Maria et al.[57] Inbred mouse strains differ in many behaviors and mutations studied on different backgrounds can lead to alterations in behavioral phenotypes.[74] As behavioral examinations on a mixed background can serve to minimize such biases, analysis of *Mbnl2*$^{\Delta E2/\Delta E2}$ mice was performed on a F2 C57BL/6jx129sv background.[75] Mice used in this study were developed by crossing 129sv *Mbnl2*$^{\Delta E2/\Delta E2}$ mice with wild-type C57BL/6j mice and the resulting F1 hybrid 129sv/C57BL/6j *Mbnl2*$^{+/\Delta E2}$ mice were crossed to develop F2 129sv/C57BL/6j *Mbnl2*$^{+/+}$ mice (indicated as *Mbnl2*$^{+/+}$) and 129sv/C57Bl/6j *Mbnl2*$^{\Delta E2/\Delta E2}$ mice (indicated as *Mbnl2*$^{\Delta E2/\Delta E2}$) mice. Male mice 4–5 months of age were used in this study. Animals were pathogen free and maintained in 12 light/12 dark cycles at room temperature and were fed with standard laboratory chow. Littermates of the same sex were randomly assigned to the relevant experimental groups. All experiments were performed in accordance with the institutional guidelines of the University of Southern California and the University of California Los Angeles. Protocol 11970 was approved by the Institutional Animal Care and Use Committee at the University of Southern California, Los Angeles.

METHOD DETAILS

Behavioral analysis

Numbers of male mice (4–5 months of age) used for the behavioral studies were *Mbnl2*$^{+/+}$ mice: n = 9; *Mbnl2*$^{\Delta E2/\Delta E2}$ mice: n = 10. All experimental mice were handled for 5 days prior to experiments. Behavioral experiments were performed in the following order.

Social interaction

Mice were placed in the center of a three-chamber apparatus, with the openings to the two side chambers closed off, for a total of 10 minutes, to allow the mice to acclimate to the environment and encourage exploration. After completion of the acclimation period, an empty pencil cup was placed in one of the side chambers upside-down and another was placed in the other side chamber with an interaction mouse of the same gender. The side chamber with the mouse was counterbalanced between mice of the same group. The dividers between the three chambers were removed and the mouse was allowed to explore the chambers for 10 minutes. Videos were recorded from cameras mounted above the arena and analyzed using TopScan Behavioral Tracking software (CleverSys Inc). The software calculates the percentage time spent in each chamber during the second 10-minute exploration period of the experiment.

Novel object recognition

This test was performed across 4 consecutive days. On day 1 and 2, mice were placed in a square open field chamber, 48 × 48 cm in size with a white flooring and an open ceiling, for 10 minutes. On day 3, two identical objects were placed in opposite corners of the open field chamber and the subjects were allowed to explore the chamber for 10 minutes. On day 4, 24 hours later, subjects were returned to the chambers with

one of the objects replaced with a novel object and allowed to explore the chamber for 10 minutes. To avoid any issues due to preference of one of the objects over another for reasons other than the prior experience, we counter-balanced which object is familiar, and which is novel between the subjects. Specifically, mice were randomly assigned in the training session to two identical objects which are either a clear plastic scintillation vial with white plastic cap and filled with distilled water for weight or a small glass Erlenmeyer flask filled with green tissue paper and sealed with a black glass marble, and on the test day, one of the objects was swapped between the two mice. In all cases, the objects and the open field were wiped down with 70% ethanol to deodorize and sanitize. The objects were then wiped down with tap water to remove the alcohol and wiped dry with a paper towel. Videos were recorded from cameras mounted above the arena and analyzed using TopScan Behavioral Tracking software (CleverSys Inc). Overall locomotion was measured across the first two days and the percentage of time spent in the center of the open field was measured as time spent in the inner 50% of the arena divided by the total time. The software calculates the percentage time spent near both objects on day 4 and a preference score is given to indicate whether the mouse prefers the novel object or had no preference.

The transcriptome analyses were carried out on an independent cohort of mice. These animals were utilized for the house controls and the trained animals, which were dissected on day 1 of the NORT, immediately after the animals had explored the empty chamber for 10 minutes.

Fear conditioning

On day 1, animals were placed in one of four conditioning chambers (30 cm × 25 cm × 25 cm; Med-Associates Inc.) with a shock grid floor, each in their own sound-attenuating cubicle. The chambers were thoroughly cleaned with 50% isopropyl ethanol between animals and a solution of 50% Simple Green cleaner was placed in the pan below the shock grid. After 2 minutes, a 30 second 2800 Hz 80 dB tone was presented that co-terminated with a .60 mA foot-shock. The tone and shock were presented two more times with a one-minute interval. Animals remained in the conditioning chamber for one minute following the last shock. On day 2, the animals were returned to the original training context for an 8-minute context test. On day 3, the animals were placed in a novel, modified context with a white plastic floor, curved white plastic back that was cleaned and scented with a 1% ascetic acid solution. They remained in this context for an 8-minute contextual generalization test. On day 4, the animals were returned to this modified context for the tone test. The three tones were presented following the identical parameters as the training day, except that the shock was omitted. Video was recorded at 30 frames per second and the software calculated the noise (standard deviation) for each pixel in a frame by comparing its gray scale value to previous and subsequent frames. This produced an "activity unit" score for each frame. Based on previous validation by a human observer, freezing was defined as sub-threshold activity [set at 19 activity units (AU)] for longer than 1 second]. Percent freezing was calculated as the number of seconds the animal was scored as freezing divided by the total time.

Marble burying

Sawdust bedding was placed in cage (25 cm × 46 cm, with 20 cm high walls) and firmly padded down. 20 marbles in an equally spaced 5 × 4 grid were placed on top of the bedding. The animals were placed in the cage for 10 minutes after which the number of marbles that were at least 75% covered were counted. This number was converted into a percentage score by dividing the number of covered marbles by 20.

Penlight vision test and the visual placement test

The penlight vision test and the visual placement test were performed as described in the results section on an independent cohort of 4–5-month-old male *Mbnl2*$^{+/+}$ and *Mbnl2*$^{\Delta E2/\Delta E2}$ mice (n = 10/genotype). Source Data for the behavioral analysis are shown in Table S14.

Dorsal hippocampus dissection, RNA preparation, RNA-seq and data processing

A brain matrix (Stoelting Co.) was used to isolate the dorsal hippocampus. Briefly, the sectioning blades were placed 1 mm apart after laying the brain inside the matrix. The dorsal hippocampus was manually dissected from 1 mm thick coronal sections using micro punch needles that were connected to a 1 mL syringe filled with 1XPBS. The boundaries for the punches were confirmed using Allen Brain atlas.

Total RNA from the dorsal hippocampus of mice was isolated by using the RNeasy Mini kit (Qiagen, USA) in accordance with the manufacturer's protocol. The quality of RNA was analyzed by Agilent 2100 Bioanalyzer (Agilent, USA). A total amount of 1 μg RNA per sample was used for polyA mRNA preparation by using NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB, USA) and sequencing libraries were generated by using NEBNext Ultra II RNA Library Prep kit (NEB, USA) according to the manufacturer's instructions. Index codes were added to attribute sequences to each sample by using NEBNext Multiplex Oligos for Illumina (Index Primers Set 1; NEB, USA). The libraries were sequenced, and single-end reads were generated on an Illumina Hiseq 3000 platform (UCLA Technology Center for Genomics & Bioinformatics). N only reads were filtered using cutadapt (v1.18)[67] and uploaded to galaxy (usegalaxy.org). Reads with a phred score of 20 or higher (across at least 50% of the nucleotides) were retained and aligned to a polyA database.[66,68] Aligned reads were discarded and unaligned reads longer than 25 nt were mapped to the mm10 genome using Tophat (v2.1.1),[69] with at most 2 mismatches allowed. HTSeq (htseq-count Galaxy version 0.9.1)[70] was used to obtain counts per gene and DESeq2(v1.18.1)[71–73] was used to obtain normalized counts per gene and log2fold change. All source data for RNA-seq Figures are shown in Tables S1, S2, S3, S4, S5, S6, and S7. RNA-seq was carried out in dorsal hippocampi of 4–5-month-old male *Mbnl2*$^{+/+}$ and *Mbnl2*$^{\Delta E2/\Delta E2}$ mice, dissected on day 1 of the NORT prior to (Not exposed to the empty chamber: house controls; n = 3/genotype) and immediately after exploration of the empty chamber for 10 minutes (Exposed to the empty chamber: trained; n = 3/genotype).

RT-qPCR

Total RNA was prepared using Trizol (Invitrogen, USA) according to the manufacturer's protocol. 5 μg of total RNA was used to synthesize cDNA using Maxima H minus First strand cDNA kit (ThermoFisher Scientific, USA). Real time quantitative PCR was performed using the CFX96 Real-time PCR Detection System (Bio-Rad, USA) with the PefeCTa SYBR Green SuperMix (Quanta, USA). The specificity of the amplification reactions was monitored by a melting curve analysis. The threshold cycle (Ct) value for each gene was normalized to the Ct value for *Gapdh*. The primers and conditions used for qPCR are as listed in Table S15. Source data for qPCR analysis is shown in Table S16.

QUANTIFICATION AND STATISTICAL ANALYSIS

Behavior

Statistical analysis of the behavioral data was performed with SPSS using Analysis of Variance (ANOVA) with between subject factors of genotype. Shapiro-Wilk Test was used to confirm a normal distribution and Levene's Test of equality of error variances was used to confirm homogeneity of variance. For the object recognition and social approach assays repeated measures ANOVA with object as a within-subject factor was performed within *Mbnl2*$^{+/+}$ and *Mbnl2*$^{\Delta E2/\Delta E2}$ groups to assess for preferential investigation of the novel object and novel mouse, respectively. For Fear Conditioning acquisition day freezing was analyzed with within-subject factor of genotype and between subject factor of time bins across training: baseline, Tone 1, Post-Tone 1, etc). Locomotion across Days 1 and 2 of exposure to the open field was analyzed in a similar manner. F-stats, p values and degrees of freedom (which indicates the number of subjects) are shown in the main text. Significance level was set at $p < 0.05$ and all tests are two-tailed.

RNA-seq

The R package DESeq2 was used to calculate the log2FoldChange and the corresponding (Wald test) p values and p-adjusted values.[71] In this study, we are using transcriptome analysis of the dorsal hippocampus as a means to test if *Mbnl2*$^{\Delta E2/\Delta E2}$ mice learn and form long term memories of the novel context of the empty chamber in the NORT. As opposed to the identification of mRNA level changes in a single gene or a biomarker initiated by novel context exploration, a majority of mRNA alterations must be analyzed by IPA to accurately test if cellular and functional pathways associated with learning and memory are significantly enriched in *Mbnl2*$^{\Delta E2/\Delta E2}$ mice with novel context exploration. Thus, for this study, missing a large proportion of the genes that show bona fide alterations initiated by novel context exploration (false negatives or Type II errors) are of equal concern as the identification of a subset of genes as altered when they are not (false positives or Type I errors) that can occur with multiple comparisons. In a previous analysis of >200 transcriptome data sets, Mudge and colleagues demonstrate that Type II errors are high with the Benjamini-Hochberg (B-H) adjusted p values when compared to unadjusted p values, for differential expression prior probability ranging from 0.5 to 0.1 and critical effect sizes ranging from of 1 to 4 standard deviations.[76]

To test the relative severity of Type I and Type II errors in our data, we selected 8 genes, semi randomly (using both genes that are upregulated and down regulated during novel context exploration in *Mbnl2*$^{+/+}$ mice) whose levels in the house control and the trained *Mbnl2*$^{+/+}$ dorsal hippocampus were computed as significantly different by the unadjusted p value ($p < 0.02$). mRNA level differences between these samples were measured by qPCR analysis (Figure 2C). We surmised that if Type I errors were high in the unadjusted p values, we would observe non-significant results for several of the 8 genes tested. All 8 genes showed significant differences in the qPCR assay (students two tailed t-test; n = 3/mice/condition). Next, we compared the significance of these 8 gene alterations as computed using the B-H adjusted p value. 7 of the 8 gene alterations, validated by qPCR, were not found to have significant B-H adjusted p values (Table S2). Thus, consistent with the analysis of Mudge et al.[76] Type II errors appeared to be quite severe in the p-adjusted values (B-H correction) in our data set.

Severe type II errors can be a great liability in IPA analysis as they curtail the inclusion of bona fide changes in the test samples leading to inaccurate results. Although the qPCR validated gene number of 8 is small, these results and the analysis of Mudge and colleagues,[76] demonstrate that the use of unadjusted p values rather than B-H p-adjusted values can lead to fewer Type II errors and possibly fewer overall Type I and Type II errors in our data sets. Both adjusted and non-adjusted p values are shown in Tables S1, S2, S3, S4, S5, S9, and S11. Adjusted p values are useful in identifying a few highly significant mRNA level changes in genes, which can be used as markers to measure therapeutic efficacy of novel treatment regimens. Unadjusted p values allow insights into group behavior as they allow a greater number of bona fide changes to be included and analyzed in the test samples. Therefore, in these data sets the unadjusted p value was utilized to compute significance and genes with $p < 0.05$ were used for IPA analysis. All p values for the IPA analysis are corrected for multiple comparisons (B-H correction) as noted below.

qPCR

Validation of RNA-seq data was carried out by qPCR analysis. Statistical significance of the difference between the means of steady state mRNA levels was determined by unpaired two-tailed Student's *t* test using GraphPad Prism.

Ingenuity pathway analysis

Z-scores are a statistical measure of how closely the expression (or other measurement type) pattern of dataset molecules compares to the pattern that is expected based on literature evidence. *Z* score values $\geq +2.0$ mean that the pathway is predicted to be activated, whereas *Z* score values ≤ -2.0 mean that the pathway is predicted to be inhibited. A right-tailed Fisher's Exact Test was used to calculate a p value determining the probability that the association between the genes in the dataset and the function or pathway is explained by chance alone.[54] IPA settings for tissues & cell lines included Cell (Astrocytes), Neurons (All), Stem cells (All), Nervous system (All), CNS cell lines (All) and Neuroblastoma cell lines (All). All IPA analyses were corrected for multiple comparisons and q values (B-H multiple testing correction p values) are shown.

Replicates

For the behavioral studies, all experiments were performed once. Replication was not attempted. For qPCR experiments, all data points are independent biological data points from individual animals and not technical replicates. For RNA-seq, RNA was analyzed from independent animals.

Randomization

For all studies genotyping determined allocation to the experimental group.

Blinding

All behavioral studies were conducted blind to genotype/experimental condition. For the behavioral studies, one *Mbnl2*$^{+/+}$ animal that had discrepant genotyping was removed from the study.