Dorsal hippocampal CREB is both necessary and sufficient for spatial memory

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SUPPLEMENTARY MATERIAL

Supplementary results

Increasing CREB produces robust spatial memory in WT mice given weak training that does not normally support spatial memory formation: training data

To examine whether increasing CREB function in the dorsal hippocampus is sufficient to produce spatial memory using sub-threshold training conditions that do not normally support the formation of spatial memory, we microinjected WT mice with CREB, mCREB or Control vector prior to weak training in the watermaze (one block of 3 trials per day for 3 days). We chose to examine memory in the watermaze because it is a fairly complex cognitive task in which subjects determine their present position and orientation (self-localization), desired position (goal determination), and the optimal route between the two (path integration)(Redish and Touretzky, 1998). The neural mechanisms mediating this type of visuospatial memory in mice likely contribute to general human cognitive processing.

Over the 3 training blocks, all groups required progressively less time to locate the platform, although this decrease was more pronounced in mice with CREB vector (Supplementary Fig. 1). The results of a Vector x Block ANOVA supports this conclusion, showing a significant Vector x Block interaction ($F_{4,56} = 3.56$, $P < .001$) as well as a significant Block effect ($F_{2,56} = 7.55$, $P < .001$), but no significant effect of Vector ($F_{2,28} = 3.11$, $P > .05$)]. Post-hoc Newman-Keuls comparisons showed that the escape latencies on the first block of trials were similar in all groups, but that mice with CREB vector showed progressively shorter escape latencies over the blocks than mice with Control or mCREB vector, which did not differ. Importantly, all groups showed similar swim speeds ($P > .05$). Therefore, under-trained WT mice with increased CREB levels in the dorsal hippocampus show faster escape latencies over the course of watermaze training. This is consistent with the finding that only mice with CREB vector acquired spatial memory, as assessed in a probe trial (in which the platform was removed) conducted after training (Fig. 1B).

Increasing CREB enhances spatial memory produced by strong training in WT mice: training data

We also examined the effects of acutely increasing CREB function in the dorsal hippocampus in WT mice trained with a strong protocol (2 blocks of 3 trials per day, for 3 days, or double the amount of training in the weak protocol)(Supplementary Fig. 2). WT mice were microinjected with CREB or Control vector into the dorsal hippocampus prior to training. Over the 6 training blocks, both groups required progressively shorter times to locate the hidden platform, but these escape latencies declined more rapidly in mice with CREB vector (Supplementary Fig. 2A). The results of a Vector x Block ANOVA supports this conclusion, showing a significant Vector x Block interaction ($F_{5,95}$

= 3.44, $P < .001$) as well as a significant effect of Block ($F_{5,95} = 36.89$, $P < .001$]. Post-hoc Newman-Keuls analysis revealed that on the first training block both groups required the same time to locate the platform but, over training blocks, mice with CREB vector located the platform progressively faster than mice with the Control vector. There was no difference in swim speed between the groups ($P > .05$). Therefore, both groups showed decreased escape latencies over the course of training, and mice with CREB vector showed a more pronounced decrease. These data suggest that, over the course of training, mice with CREB vector outperformed mice with Control vector.

Increasing CREB enhances spatial memory produced by strong training in WT mice: probe data

To examine if WT mice with CREB vector showed enhanced spatial memory following strong training, we tested mice with CREB and Control vectors in a probe test in which the platform was removed from the pool. Mice with CREB vector showed enhanced spatial memory during the probe test; these mice spent more time than mice with Control vector in the target area of the pool where the platform was located during training (Supplementary Fig. 2C, D). We first quantified this spatial bias by comparing the amount of time mice spent in the target zone (20 cm radius circular zone centered on the former platform location; 11% of the pool surface) versus the average time spent in equivalent zones in the other three quadrants of the pool (Fig. 2B). An ANOVA with a between-subjects variable Vector and within-subjects variable Zone (Target, Others) revealed a significant interaction ($F_{1,19} = 7.42$, $P < .05$) as well as a significant main effect of Zone ($F_{1,19} = 68.21$, $P < .001$). Post-hoc comparisons on the significant interaction showed that both groups searched selectively in the target zone, with CREB vector mice showing a stronger preference for the target zone. This was confirmed by an ANOVA comparing the time spent in target zone between the two groups confirmed this ($F_{1,19} = 5.71$, $P < .05$). Therefore, acutely increasing CREB further enhances the formation of spatial memory in strongly-trained WT mice.

Increasing CREB does not change the expression of a previously acquired spatial memory

To examine if increasing CREB levels in the dorsal hippocampus impacts the expression of a previously acquired spatial memory, we microinjected CREB or Control vectors *after* training in WT mice trained using the strong protocol (2 blocks of 3 trials per day, for 3 days, as above). As expected, before vector microinjection, both groups showed equally strong spatial memory following the strong watermaze training ($F_{1,13} = 0.18$, $P > .05$) (Supplementary Fig. 2E, pre-injection target). Increasing CREB levels in the dorsal hippocampus did not affect spatial memory expression: both mice microinjected with CREB and Control vectors after training showed similarly strong spatial memory ($F_{1,13} = 11.07$, $P > .05$) (Supplementary Fig. 2E, post-injection target). Therefore, acutely increasing CREB function before training enhances spatial memory formation whereas similarly increasing CREB function after training has no effect on the expression of an existing spatial memory.

Acutely increasing CREB levels in the dorsal hippocampus rescues the spatial memory deficit in CREB-deficient mice: training data

To examine whether acutely increasing CREB function in the dorsal hippocampus is sufficient to rescue the spatial memory deficit in CREB-deficient mice, we microinjected CREB or Control vector into the dorsal hippocampus of CREB-deficient or WT littermate control mice before strong training (2 blocks of 3 trials a day for 3 days). Over the course of training, WT littermate mice (with either CREB or Control vectors) showed the characteristic progressive decrease in the time required to locate the platform, while CREB-deficient mice with Control vector showed a relatively flat escape latency curve (Supplementary Fig. 3A). However, microinjection of CREB vector into the dorsal hippocampus completely restored the escape-latency curve in CREB-deficient mice; CREB-deficient mice with CREB vector showed escape latencies similar to WT mice. The results of a Group (CREB-deficient mice/CREB vector, CREB-deficient mice/Control vector, WT mice/CREB vector, WT mice/Control vector) x Block (6 levels) ANOVA supports this conclusion, showing a Group X Block interaction ($F_{15,210} = 1.84$, $P < .05$) and significant main effects of Group ($F_{3,42} = 5.34$, $P < .05$) and Block ($F_{5,210} = 22.80$, $P < .001$). Post-hoc analysis revealed that, in contrast to WT mice, CREB-deficient mice with Control vector failed to locate the platform more quickly over blocks. Importantly, this deficit in CREB-deficient mice was rescued by acutely increasing CREB in dorsal hippocampal CA1 neurons.

To more thoroughly characterize the spatial memory impairment observed in CREB-deficient mice, we examined the swim patterns of mice during training. These patterns are thought to reflect the search strategies adopted by mice during training. Typically, during the initial watermaze training trials, WT mice tend to swim near the wall of the pool, in an instinctive behavior known as thigmotaxis [the tendency to search in the outer regions of the pool (Wolfer et al., 1998)]. After about two trials, most WT mice gradually begin to adopt more efficient search strategies, proceeding to scanning, chaining, focal searching and finally to a spatial search strategy (Wolfer and Lipp, 2000). Accordingly, we examined the levels of thigmotaxis (time spent within 5 cm of the pool wall) during training in CREB-deficient and WT mice microinjected with CREB and Control vectors as an index of an inefficient search strategy. Consistent with the decreased escape latencies over the course of training, WT mice with CREB or Control vector showed a dramatic decline in the time spent in the periphery of the pool over training blocks, suggesting the adoption of a more effective spatial search strategy (Martin et al., 2005) (Supplementary Fig. 3B). In contrast, CREB-deficient mice with Control vector showed high levels of thigmotaxis throughout training. This finding is consistent with previous reports that several lines of mice with different genetic mutations that disrupt CREB function show longer path lengths or escape latencies (Pittenger et al., 2002; Balschun et al., 2003; Hebda-Bauer et al., 2005), as well as higher levels of thigmotaxis (Gass et al., 1998; Balschun et al., 2003)during training, than WT mice. Therefore, disrupting CREB function seems to induce an increase in thigmotaxic behavior, perhaps reflecting a failure to adopt a spatial search strategy.

Disrupting CREB function may produce high levels of thigmotaxis during training by interfering with the natural progression to a spatial search strategy or by non-specifically interfering with watermaze performance, perhaps by increasing anxiety. We

observed that the high levels of thigmotaxis in CREB-deficient mice were rescued by microinjecting the CREB vector into the dorsal hippocampus of CREB-deficient mice [significant main effects of Group ($F_{3,42}$ = 7.58, P < .001) and Block ($F_{5,210}$ = 40.22, P < .001) but no Group by Block interaction ($F_{15,210}$ = 0.96, P > .05)]. Swim speeds between the 4 groups of mice were comparable [P > .05]. Because thigmotaxic behavior was rescued by increasing CREB in the dorsal hippocampus, it suggests that the high levels of thigmotaxis observed in CREB-deficient mice during training are due to the failure of CREB-deficient mice to adopt a spatial-based search strategy in the watermaze (to progress from the thigmotaxic strategy to a spatial strategy) and not a reflection of poor overall performance or increased anxiety.

We also examined the level of thigmotaxis during the probe test. CREB-deficient mice with Control vector spent more time searching in the periphery of the pool than WT mice with either CREB or Control vectors ($F_{3,42}$ = 6.48, P < .001) (Fig. 2D). Importantly, CREB-deficient mice with CREB vector showed similar low levels of thigmotaxis as WT mice (post-hoc comparisons showed that thigmotaxis was greatest in CREB-deficient mice with Control vector, while the other groups did not differ). Therefore, on two different measures (time spent in the target zone and level of thigmotaxis during the probe test), CREB-deficient mice showed poor spatial memory, but acutely increasing CREB function just in the dorsal hippocampus rescues both of these measures of spatial memory. Because a brain-wide deficit in CREB function was rescued by locally increasing CREB levels, these results indicate that the formation of spatial memory requires intact CREB function only in CA1 neurons of the dorsal hippocampus.

CREB-deficient mice have a chronic disruption in CREB function. Although this disruption may produce changes in the developing brain (such as alterations in neurogenesis, cell migration and neuronal connectivity (Lonze and Ginty, 2002)) that may account for the spatial memory deficits observed in adulthood, we found that acutely increasing CREB in CA1 neurons in the dorsal hippocampus in adult mice was sufficient to completely reverse the spatial memory deficit. This finding highlights the importance of acute CREB-mediated transcription in spatial memory formation and furthermore, shows the spatial memory deficit observed in these CREB-deficient mice cannot be attributed to developmental deficits.

In the present experiments, we have examined the effects of increasing CREB levels and function in the CA1 region of the dorsal hippocampus on spatial learning as assessed in the watermaze. A failure to find a spatial preference for the target zone during the probe trial could be due to impairment in several processes, including spatial memory formation and procedural learning. It would be interesting to examine the effects of increasing CREB function in other types of spatial memory.

Supplementary figure legends

Supplementary Figure 1. Increasing CREB in the CA1 region of the dorsal hippocampus is sufficient to induce spatial memory in WT mice trained with a weak protocol that normally does not support spatial memory formation. WT mice with CREB (n = 10), mCREB (n=8) or Control (n = 13) vector were trained using a weak protocol (3

trials a day for 3 days). Mean (± SEM) time to locate the platform over training decreased in all groups, but this decrease was more pronounced in mice with CREB vector. The escape latencies of mice with mCREB and Control vectors did not differ.

Supplementary Figure 2. Acutely increasing CREB levels in the CA1 region of the dorsal hippocampus enhances the formation of spatial memory in WT mice given strong watermaze training. (**A**) WT mice microinjected with CREB (n = 10) or Control (n = 11) vectors were trained using a strong protocol (3 blocks of 2 trials a day for 3 days) in the watermaze. Mean (± SEM) time to locate the platform over training blocks decreased in both groups, but this decrease was more pronounced in mice with CREB vector. (**B**) Schematic drawing of the watermaze. During training, the platform (10 cm diam) is located in the lower right quadrant (gray). A variety of distal (extra-maze) cues surround the pool. However, in Fig. 1D these cues were obscured by a curtain (in the "no cues" condition) to examine whether the apparent enhancement in performance relied on these distal cues, reflecting spatial memory. During the probe test conducted after training, the platform is removed from the pool and the amount of time spent in the target zone (20 cm radius) versus the equally-sized other zones (20 cm radius) is compared. (**C**) Spatial memory, as evaluated in a probe test conducted at the end of training, was greater in mice with CREB vector than in mice with Control vector trained with a strong protocol. Both groups of mice spent more time searching in the target zone compared with the other equally sized-zones of the pool (**B**), although CREB vector mice showed a stronger preference for the target zone**.** (**D**) Density plot for the grouped data showing where mice with CREB or Control vector concentrated their searches in the probe test. Color scale represents the number of visits per animal per 5 x 5 cm^2 area of the pool. (**E**) Increasing CREB in the dorsal hippocampus does not affect expression of a previously acquired spatial memory. WT mice microinjected with CREB (n=8) or Control (n=7) vector after (rather than before) strong training showed similarly strong spatial memory both before and after vector injection (both groups spent equal amounts of time in the target zone in probe trials conducted pre-injection and post-injection). Therefore, increasing CREB after spatial memory formation does not affect expression of a previously formed spatial memory.

Supplementary Figure 3. Acutely increasing CREB in the dorsal hippocampus rescues the spatial memory deficits of CREB-deficient mice with disrupted CREB function throughout the brain. (**A**) CREB-deficient (MUT) mice with Control (n=12) or CREB (n=10) vector and WT mice with CREB (n=10) or Control (n=14) vector were trained in the watermaze (strong training, 2 blocks of 3 trials per day for 3 days). WT mice, with Control or CREB vector, showed a decrease in escape latency over training blocks. This characteristic decrease in escape latency over training was not observed in CREB-deficient mice with Control vector. However, CREB-deficient mice with CREB vector performed similarly to WT mice, thereby rescuing the spatial memory deficit observed in CREB-deficient mice. (**B**) The high levels of thigmotaxis (time spent in pool periphery) decreased over training in WT mice. However, CREB-deficient mice with Control vector continued to show high levels of thigmotaxis throughout training. This effect was

reversed by microinjecting the CREB vector into the dorsal hippocampus of CREB-deficient mice.

Supplementary methods

Mice

Adult F1 hybrid (C57Bl/6NTac x 129S6/SvEvTac) female mice were used for experiments depicted in Fig. 1 (comparing all WT mice). The $CREB^{\alpha\delta}$ mutation (Hummler et al., 1994) was backcrossed to inbred mouse strains C57Bl/6NTac (N10) and 129S6/SvEvTac (N11). Experimental mice for the experiment depicted in Fig. 2 (both male and female WT and homozygotes) were the F1 cross of mice heterozygote for the CREB mutation ($CREB^{\alpha\delta-/+}$). Therefore, in all cases the genetic background of the experimental mice was 50% C57Bl/6NTac and 50% 129S6/SvEvTac. Mice were group housed (3-5 mice per cage) on a 12 h light/dark cycle. Behavioral experiments were conducted during the light-phase. Food and water were available *ad libitum*. Procedures were approved by the Hospital for Sick Children Animal Care and Use Committee.

HSV Vectors

Genes of interest (CREB, mCREB, LacZ) were cloned into the HSV amplicon (HSV-PrpUC) and packaged using a replication-defective helper virus as previously described (Han et al., 2007; Han et al., 2008; Han et al., 2009). To visualize transgene expression, eGFP was fused to the 5' end of CREB, mCREB and LacZ cDNA. Previous studies established that tagging CREB with eGFP does not interfere with its functional activity (Chao et al., 2002). Transgene expression was regulated by the constitutive promoter for the HSV immediate-early gene IE 4/5. Virus was purified on a sucrose gradient, pelleted and resuspended in 10% sucrose. The average titer of the recombinant virus stocks was typically 4.0×10^7 infectious units/ml. Previous studies indicate that microinjection of this CREB vector increased CREB levels and function while microinjection of the dominant negative CREB vector (mCREB) decreased CREB function (Barrot et al., 2002; Olson et al., 2005).

Surgery

Mice were pre-treated with atropine sulfate (0.1 mg/kg, ip), anesthetized with chloral hydrate (400 mg/kg, ip) and placed in a stereotaxic frame. The skin was retracted and holes drilled in the skull bilaterally above the dorsal hippocampus (AP = -2.3, ML = ±1.6, V = -1.6 mm from bregma) according to (Paxinos and Franklin, 2001). Bilateral microinjections of the vectors (2.0 μl) were delivered over 20 min through glass micropipettes. Micropipettes were left in place an additional 10 min to ensure diffusion of the vector. Because transgene expression using this viral system peaks 3 d following surgery (Barrot et al., 2002), we trained mice 1 d following surgery for 3 d in all experiments except where specified. In the memory expression experiments (Fig. 1E, Supplementary Fig. 2E), mice were trained and given one probe test prior to surgery and an additional probe test 4 d following surgery. This delay was consistent with the delay between vector microinjection and probe testing in all other experiments, and ensures that

the level of transgene expression during the final probe test was equivalent across experiments.

Histology

Following the probe test, mice were perfused with 4% paraformaldehyde. Brains were sliced coronally (50 μm). Consistent with previous reports from many labs, infusion of HSV vectors (either CREB, mCREB or Control vector) produces robust localized transgene expression and minimal tissue damage around the site of microinjection (Fig. 1A). GFP expression did not differ between vectors.

Placement and extent of the viral infection for each mouse was determined using GFP-immunofluorescence and used to classify mice as "hits" or "misses" by an examiner unaware of the behavioral data or treatment condition. Only mice that were determined to be bilateral "hits" were included in subsequent data analysis. The number of neurons in the CA1 region of the dorsal hippocampus expressing GFP was counted manually using a fluorescent microscope (Nikon) at 40X magnification. We observed that a portion of dorsal CA1 neurons around the site of microinjection (typically encompassing a circular region of 1.6 mm in diam) were infected by HSV microinjection. Mice were classified as "hits" only if they had robust bilateral expression of GFP in over 2,000 neurons in the CA1 region of the dorsal hippocampus. All other mice were classified as "misses", including mice with weak transgene expression (less than 2,000 GFP-positive neurons per side) in the dorsal hippocampus. Although our microinjections were aimed at the CA1 region of the dorsal hippocampus, in some mice we observed GFP-positive neurons in other regions of the dorsal hippocampus (CA3 and dentate gyrus). There was no difference in the performance of mice with robust bilateral CA1 infection (CA1 only) and mice with robust bilateral CA1 infection as well as infection in other hippocampal regions (CA1 + CA3 and/or dentate gyrus) in any experiment. Therefore, we included all mice that showed robust bilateral expression of GFP in the CA1 region dorsal hippocampus ("hits") in the subsequent statistical analysis. The number of GFP-positive neurons in our "hit" groups did not correlate with spatial memory.

Immunohistochemistry

To examine CREB protein levels in Fig. 2, mice were perfused with 4% paraformaldehyde. Brains were sliced coronally (50 μm) and prepared for immunohistochemistry using anti-CREB primary mouse antibody (1:2500, Upstate Cell Signaling Solutions, NY). The CREB signal was visualized using an anti-mouse Alexa 568 secondary antibody (1:500, Invitrogen, Carlsbad, CA). Importantly, no staining was detected in the absence of the primary or secondary antibodies. GFP was visualized using endogenous GFP fluorescence.

Watermaze training

The circular watermaze tank (120 cm diam, 50 cm deep) was located in a dimly lit room [see (Teixeira et al., 2006)]. The pool was filled to a depth of 40 cm with water made opaque by nontoxic white paint. Water temperature was maintained at 28±1°C. A circular escape platform (10 cm diam) was submerged 0.5 cm below the water surface and located in a fixed position throughout training. The pool was surrounded by white

curtains painted with distinct cues, 1 m from the pool perimeter (except in the "no cues" experiment).

Before training, mice were handled for 2 min per day for 1 week. For the weak training protocol, mice received 1 block of 3 trials per d, for 3 d. The intertrial interval was 30 s for both protocols and interblock interval for the strong training protocol was 30 min. Previous pilot work in our lab established that this under-training protocol is not sufficient to induce spatial memory in WT mice. For the strong training protocol, mice received 2 blocks of 3 trials per d, for 3 d. This protocol reliably produced robust spatial memory in our hands. Each trial lasted a maximum of 60 s. To begin each trial, mice were placed in the pool, facing the wall in one of four start locations (varied pseudorandomly). The trial was complete once the mouse found the platform or 60 s had elapsed. If the mouse failed to find the platform on any trial, the experimenter guided the mouse onto the platform. After each training trial, the mouse was allowed 15 s on top of the platform. Sixty min after the final training trial, spatial memory was assessed in a probe test during which the platform was removed from the pool and the mouse allowed to search for 60 s.

Behavioral data from the training and testing phases were acquired and analyzed using an automated tracking system (Actimetrics, Wilmette, IL). Using this software, we recorded a number of variables during training, including escape latency and swim speed. In probe tests we quantified spatial memory by measuring the amount of time mice spent searching in the target zone (20 cm radius, centered on the location of the platform during training; 11% of the pool surface) versus the average of three other equivalent zones in other areas of the pool (Moser et al., 1993; Moser and Moser, 1998; de Hoz et al., 2004; Maei et al., 2009). Thigmotaxic behavior during training or the probe test was quantified by calculating the amount of time mice spent in the peripheral region of the pool (within an area of 5 cm from the wall) (Martin et al., 2005).

Statistical analysis

For the training data, we analyzed the time required to reach the platform (escape latency) using a 2-way Analysis of Variance (ANOVA) with between-group factor Vector and within-group factor Block (3 levels for weak training, 6 levels for strong training). For the probe test, we first quantified spatial bias by comparing the amount of time mice spent in the target zone versus the average time spent in equivalent zones in the other three quadrants of the pool using an ANOVA with a between-subjects variable Vector and within-subjects variable Zone (Target, Others). Next, we analyzed the time spent in the target zone between groups using a one-way ANOVA with between-group factor Vector. Significant effects from all analyses were further analyzed using Newman-Keuls post-hoc tests.

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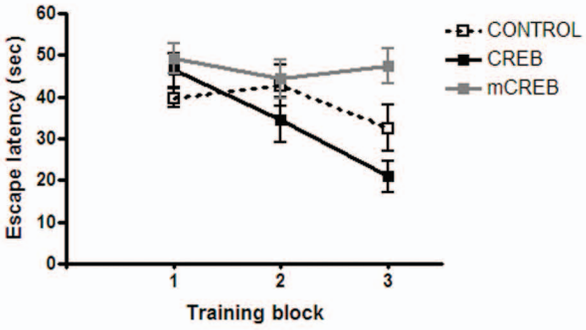
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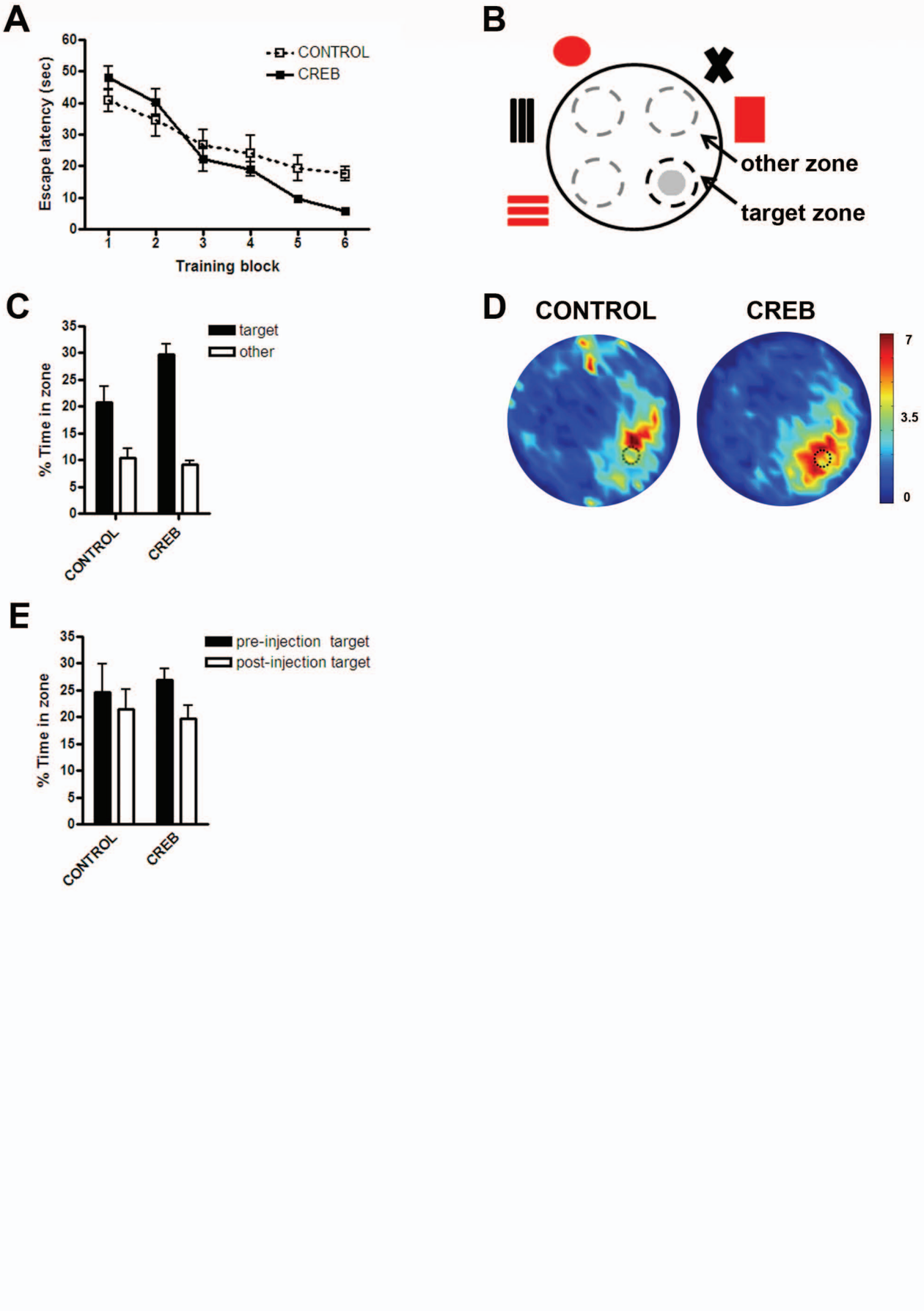
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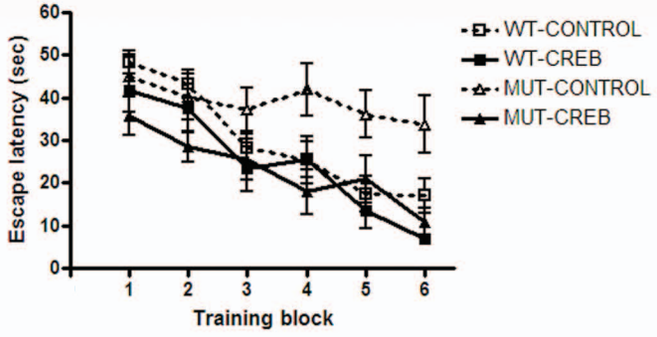
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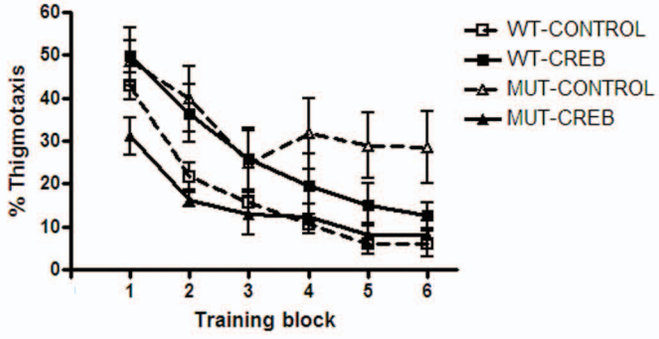
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