CREB Regulates Memory Allocation in the Insular Cortex

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Summary

The molecular and cellular mechanisms of memory storage have attracted a great deal of attention. By comparison, little is known about memory allocation, the process that determines which specific neurons in a neural network will store a given memory [1, 2]. Previous studies demonstrated that memory allocation is not random in the amygdala; these studies showed that amygdala neurons with higher levels of the cyclic-AMP-response-element-binding protein (CREB) are more likely to be recruited into encoding and storing fear memory [3–6]. To determine whether specific mechanisms also regulate memory allocation in other brain regions and whether CREB also has a role in this process, we studied insular cortical memory representations for conditioned taste aversion (CTA). In this task, an animal learns to associate a taste (conditioned stimulus [CS]) with the experience of malaise (such as that induced by LiCl; unconditioned stimulus [US]). The insular cortex is required for CTA memory formation and retrieval [7–12]. Previous studies demonstrated that CTA learning activates a subpopulation of neurons in this structure [13–15], and the insular cortex and the basolateral amygdala (BLA) interact during CTA formation [16, 17]. Here, we used a combination of approaches, including viral vector transfections of insular cortex, arc fluorescence in situ hybridization (FISH), and designer receptors exclusively activated by designer drugs (DREADD) system, to show that CREB levels determine which insular cortical neurons go on to encode a given conditioned taste memory.

Results and Discussion

Although CREB has a critical role in memory allocation in the amygdala, it is unclear whether cortical circuits share similar memory allocation mechanisms. To test the hypothesis that taste memory is preferentially allocated to a specific population of neurons expressing higher levels of CREB, we made a lentivirus vector to overexpress CREB tagged with GFP in a subpopulation of insular cortex neurons (vCREB neurons). The CREB vector we derived also coexpresses the inducible hM4Di designer receptors exclusively activated by designer drugs (DREADD) receptor tagged with hemagglutinin (HA) (also referred to as Gi-DREADD). The UbC promoter in the FG12 plasmid [18] was replaced by a 1.3 kb CaMK2a/calcmodulin-independent protein kinase (CaMK2a) promoter to direct expression to excitatory neurons. The CREB and hM4Di genes are expressed under the CaMK2a promoter and cloned on either side of a T2A self-processing viral peptide (CREB virus; Figure 1A). This allows them to be coexpressed in the same neurons [19]. The hM4Di receptor is specifically activated by CNO, which does not activate endogenous muscarinic acetylcholine receptor [20]. The activation of hM4Di turns on endogenous G-protein-coupled inwardly rectifying K+ (GIRK) channels, causing membrane hyperpolarization and decreased neuronal excitability. There is abundant expression of GIRK channels in the cortex [21], which allows the activity of infected neurons to be manipulated by systemic injection of CNO. As a control, we used a lentivirus that expresses dTomato-tagged GFP instead of enhanced GFP (EGFP)/CREB (control virus; Figure 1A).

We bilaterally microinjected these viruses into the insular cortex. Immunohistochemical studies with an antibody against GFP detected the expression of this viral gene in a region about 300 μm from the injection sites in the insular cortex (Figure 1B). We found that approximately 30% of the cells in the insular cortex expressed EGFP near the injection sites (CREB virus: 25.0% ± 2.1%; control virus: 31.2% ± 2.1%; n = 4 mice per group). The expression of the HA-tagged hM4Di receptor was detected using an HA tag antibody. As expected, we found that GFP and hM4Di were coexpressed in the same neurons (Figure 1C). To test whether CNO could selectively silence insular cortex neurons, we performed whole-cell patch-clamp recordings on both visually identified GFP-positive (hM4Di-positive) and GFP-negative neurons in brain slices from mice transfected with the CREB virus. Consistent with previous studies [20, 22, 23], we found that the resting membrane potential (RMP) and input resistance in GFP-positive (GFP+) neurons exposed to CNO were significantly more hyperpolarized and decreased than those in GFP-negative (GFP−) neurons (Figures 1D–1F; change in resting membrane potential [ΔVm]: 1.3 ± 1.0, n = 4, GFP−: −11.8 ± 5.8 mV, n = 3, GFP+: p < 0.05; change in input resistance: 99.7% ± 1.8%, n = 4, GFP−; 65.4% ± 8.2%, n = 3, GFP+: p < 0.01). We also observed that frequency of firing in insular cortex around GFP+ neurons was decreased after CNO injection in an awake mouse, whereas firing was unchanged in areas of the insular cortex not infected with the virus (Figure S1 available online).

To first determine whether CREB has a role in memory formation in the mouse insular cortex, we used the lentiviruses described above to test the impact of CREB overexpression on memory strength. Previous results showed that overexpression of CREB in either the amygdala or hippocampus leads to enhancements in memory tasks that depend on these structures [3, 6, 24]. Accordingly, our results demonstrated that overexpression of CREB in the insular cortex results in enhancements in conditioned taste aversion (CTA) memory, a finding that is consistent with a role for this transcription factor in learning and memory in this structure (Figures S2A–S2C).

Selective Silencing of vCREB Neurons Impairs CTA Memory Retrieval

Next, we determined whether silencing the subpopulation of insular cortical neurons that express vCREB disrupts the
retrieval of a long-term memory for CTA. If vCREB preferentially recruits the neuronal ensemble encoding CTA in the insular cortex, then silencing these neurons should impair recall when compared to mice transfected with the control virus. To test this hypothesis, CREB and control viruses were bilaterally microinjected into the insular cortex 3 weeks before training. During training, mice were given 150 mM LiCl, which tastes salty (conditioned stimulus [CS]) and simultaneously induces malaise (unconditioned stimulus [US]) [25]. Mice were then administered saline or CNO prior to the test. In this test, mice were presented with two choices: water and 150 mM NaCl. Trained mice show a clear avoidance of NaCl during the test. We utilized this CTA protocol to avoid any stress associated with an intraperitoneal injection during training. To evaluate CTA memory, we used a learning index (LI) along with an aversion index that is commonly used in the CTA literature [7]. The advantage of the learning index is that, for each animal tested, it compares avoidance of the salty taste after training with that measured before training (see details in Experimental Procedures). This comparison is important because not all mice show the same preference for salty tastes before training and differences between mice could bias performance after training.

In support of the hypothesis that CREB modulates memory allocation in the insular cortex, memory retrieval was impaired by silencing vCREB-positive neurons (Figure 2A: LI: 100.0 ± 11.8, n = 8, CREB/saline; 53.3 ± 15.3, n = 9, CREB/CNO; p < 0.05; Figure S2D: aversion index: 77.0 ± 6.7, CREB/saline; 57.0 ± 6.2, CREB/CNO; p < 0.05). Importantly, silencing control virus-positive neurons in the insular cortex did not result in impairments of memory retrieval (Figure 2B: n = 9 per group; LI: 100.0 ± 8.7, control/saline; 86.4 ± 7.6, control/CNO; p = 0.25; Figure S2E: aversion index: 73.9 ± 5.3, control/saline; 66.4 ± 4.7, control/CNO; p = 0.30). This result suggests that vCREB-positive neurons are preferentially chosen to encode taste memory in the insular cortex because inactivation of a similar population of
insular cortical neurons with normal CREB levels did not affect CTA. Taken together, these data suggest that the insular cortex neurons expressing higher levels of CREB are preferentially involved in memory for CTA. Interestingly, Han and colleagues showed that disruption of CREB function in the lateral amygdala before tone fear training did not impair subsequent memory formation, due to the likely exclusion of those neurons from memory allocation, whereas enhancing CREB function in a similar population of neurons before tone fear training biases them toward allocation [3, 4]. These findings suggest that CREB regulates memory allocation during training/memory encoding.

**vCREB Neurons Are Preferentially Activated during CTA Memory Retrieval**

We next imaged the insular cortex following CTA retrieval to further test whether vCREB neurons are preferentially incorporated into neurocircuits encoding CTA. To visualize these neurocircuits, we used the activity-dependent gene arc (activity-regulated cytoskeleton-associated protein; also termed arg3.1) [26]. Neuronal activity induces a rapid but transient increase in arc transcription, such that arc RNA expression serves as a molecular signature of a recently (5–15 min) active neuron [26]. CREB and control viruses were bilaterally microinjected into the insular cortex 3 weeks before CTA training. Five minutes after CTA memory retrieval, we harvested the brain and performed fluorescence in situ hybridization (FISH) to analyze coexpression of arc and gfp mRNA (denoting viral transfection) in insular cortex neurons. If increased CREB expression biases memory allocation, then vCREB-positive neurons should be more likely to express arc mRNA than control virus-positive neurons. Consistent with this hypothesis, the probability of arc mRNA expression induced by memory retrieval in vCREB-positive neurons was higher than in control virus-infected neurons (Figures 3A and 3B; n = 4 mice per group; control virus, 32.9% ± 1.9%; CREB virus, 50.3% ± 4.9%; p < 0.05). There were no differences between the number of arc+ neurons between the mice transfected with either CREB or control viruses (Figure 3C). To further test whether arc expression is induced by vCREB expression independently of memory retrieval, we also measured the probability of arc expression in vCREB and control neurons in home cage mice. Our results show that there is no difference between these two groups (Figure 3D; n = 4 mice per group; control virus, 4.9% ± 1.5%; CREB virus, 4.7% ± 1.5%; p = 0.92). This result is consistent with a similar previous study in which CREB was overexpressed in the amygdala using the herpes simplex virus vector [3]. To further test whether vCREB neurons make a critical contribution to memory retrieval, we analyzed arc expression...
following CTA memory retrieval in vCREB-positive neurons after CNO or saline treatment. Consistent with the behavioral result (Figure 2A), the probability of arc expression in vCREB neurons during retrieval was significantly decreased by silencing these neurons (Figure 3E; saline group, 46.8% ± 5.1%, n = 6; CNO group, 20.9% ± 5.4%, n = 7; p < 0.01). These results further support the hypothesis that CREB levels bias which neurons store memory for CTA in the insular cortex.

The results presented here demonstrate that specific mechanisms regulate memory allocation outside of the amygdala. They showed that CREB plays a critical role in the allocation of CTA memory in the insular cortex. Because CREB plays a role in memory in two very different structures in the mouse brain, it is reasonable to propose that CREB may have a general role in memory allocation in the mammalian brain. By implication, these results also suggest that memory allocation is a general, highly regulated process in the mammalian brain.

We used two independent strategies to determine whether CREB regulates memory allocation in the insular cortex: with the DREADD neuronal-inactivation system, we showed that CTA memory retrieval was impaired by selectively silencing vCREB-positive neurons. Additionally, we used FISH to analyze the expression of the immediate early gene arc and showed that insular cortical neurons with virally transduced CREB are preferentially activated after memory retrieval. Arc is required for memory formation, and arc transcription is turned on in neurons involved in learning and memory [28–29]. All together, these findings indicate that, just as in the amygdala [3–6], taste memory is preferentially allocated into insular cortex neurons with higher levels of CREB. Therefore, these results suggest that CREB is a key component of a general mechanism of memory allocation across brain regions.

Our findings suggest that both the insular cortex and the amygdala use common mechanisms for memory allocation. In the amygdala, the CS and US are thought to converge on the amygdala [3–6], taste memory is preferentially allocated into insular cortex neurons with higher levels of CREB. Therefore, these results suggest that CREB is a key component of a general mechanism of memory allocation across brain regions.

In summary, the findings presented here demonstrate that specific mechanisms regulate memory allocation outside of the amygdala and that CREB in the insular cortex plays a critical role in the allocation of CTA memory.
References


