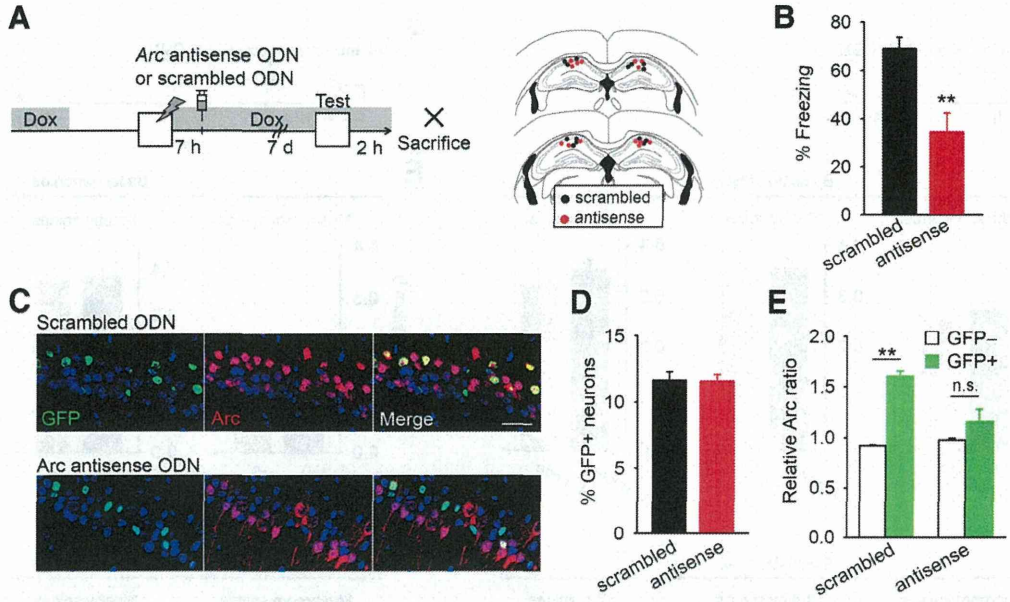

Figure 8. Late Arc expression is required for reactivation of neurons that were activated during learning. ***A***, Fos-H2BGFP mice were subjected to FC in the absence of Dox and received infusions of *Arc* antisense or scrambled ODN into the dorsal hippocampus 7 h later. Memory retention was tested 7 d after FC and killed 2 h later. ***B***, Mice with *Arc* antisense ODN infusions ($n = 7$ mice) showed less freezing behavior than mice with scrambled ODN infusions ($n = 7$ mice). ***C***, Representative images of Arc and H2B-GFP immunostaining in hippocampal CA1 (scrambled, 660.0 ± 12.4 cells; antisense, 679.9 ± 17.0 cells). Scale bar, 50 μm. ***D***, No difference in proportion of CA1 neurons with H2B-GFP signals. ***E***, Normalized ratio of CA1 neurons with Arc signals in H2B-GFP$^-$ and H2B-GFP$^+$ neurons by proportion of overall Arc$^+$ neurons. H2B-GFP$^+$ neurons preferentially express Arc in mice given scrambled ODN but not *Arc* antisense ODN infusions. Error bars indicate mean ± SEM. $^{**}p < 0.01$. n.s., Not significant.

bled ODN into the dorsal hippocampus 19 h instead of 7 h after FC and removed the brains for spine analysis 7 d after conditioning (Fig. 7*D*). The density of all types of spines both on basal and apical dendrites was comparable between groups (all comparison, $p > 0.3$) (Fig. 7*E*,*F*). This result indicates that the effect of inhibiting Arc expression on spine reorganization is time-limited.

Late Arc expression is required for reactivation of neuronal ensembles activated during FC

Selective reactivation of neurons activated during learning is essential for memory recall (Reijmers et al., 2007; Han et al., 2009; Liu et al., 2012). Spine elimination potentially enhances signal-to-noise ratios to encode information during learning and then contributes to reactivation of neuronal ensembles that were established during learning (Grønli et al., 2013; Schacher and Hu, 2014). Thus, we speculated that late Arc expression is involved in reactivation of neuronal ensembles, possibly through spine elimination. To test this possibility, we examined reactivation of neuronal ensembles that were activated during learning in mice with inhibited late Arc expression. We subjected Fos-H2BGFP mice to FC in the absence of Dox (Fig. 8*A*). Then, we fed these mice with 1 g/kg Dox to rapidly inhibit H2B-GFP expression after conditioning. They received hippocampal infusions of *Arc* antisense or scrambled ODN 7 h later. They were exposed to the conditioning context as a memory retrieval session 7 d later and killed 2 h afterward. Consistent with the previous result (Fig. 4*D*), inhibiting late Arc expression disrupted freezing behavior at 7 d (Student's *t* test, $t_{(12)} = 3.8$, $p = 0.0027$) (Fig. 8*B*). This behavioral protocol allowed us to identify H2B-GFP$^+$ neurons as the neurons activated during FC and Arc$^+$ neurons as the neurons activated during the retrieval session. Arc and GFP immunostainings were obtained from the hippocampal CA1 region (Fig. 8*C*). The proportion of H2B-GFP$^+$ neurons was comparable between mice given *Arc* antisense and scrambled ODN infusions (Fig. 8*D*). In the mice given scrambled ODN infusions, H2B-GFP$^+$ neurons were more likely to be positive for Arc relative to H2B-GFP$^-$ neurons (repeated-measures ANOVA, $F_{(1,12)} = 10.8$, $p = 0.0065$; *post hoc* paired *t* test, GFP$^+$ vs GFP$^-$ in scrambled group, $p = 2.2 \times 10^{-5}$) (Fig. 8*E*), suggesting that the neurons activated during learning were preferentially activated during memory retrieval. This result is consistent with a previous study reporting that hippocampal CA1 neurons activated during contextual FC are reactivated during retrieval of a memory (Tayler et al., 2013). In the mice given *Arc* antisense ODN infusions, however, the preferential Arc expression in H2B-GFP$^+$ neurons was abolished (GFP$^+$ vs GFP$^-$ in antisense group, $p = 0.25$) (Fig. 8*E*). The total proportion of Arc$^+$ neurons was comparable between mice administered scrambled and *Arc* antisense ODN infusions (scrambled ODN, 35.6 ± 1.0%; *Arc* antisense ODN, 34.9 ± 0.85%; Student's *t* test, $t_{(12)} = 0.50$, $p = 0.63$). These results indicate that late Arc expression is required for the reactivation of neuronal ensembles that were activated during initial learning.

Discussion

In the present study, we characterized late Arc expression in the hippocampus following associative fear learning. We discovered that Arc is upregulated 12 h after contextual FC preferentially in CA1 neurons activated during conditioning in a BDNF-dependent manner. We also found that inhibiting late Arc expression impairs delayed elimination of dendritic spines, reactivation of neurons activated during conditioning, and expression of conditioned fear 7 d after initial FC. These findings suggest that BDNF-dependent late Arc expression eliminates dendritic spines and stabilizes neuronal ensembles to prolong long-term memories.

Intracellular molecular cascades are likely to be involved in late Arc expression. CA1 neurons activated during FC preferen-

tially expressed late Arc compared with those not activated during conditioning. In addition, TTX infusions did not affect late Arc expression, suggesting that action potential-dependent neurotransmission, at least shortly before late Arc expression, is not essential for Arc expression. In contrast, function-blocking anti-BDNF antibody inhibited late Arc expression. This result suggests that BDNF triggers late Arc expression. Indeed, previous studies have reported that BDNF is induced in the hippocampus and amygdala 8–12 h after contextual FC, inhibitory avoidance, and conditioned taste aversion tasks (Bekinschtein et al., 2007; Ou et al., 2010; Ma et al., 2011). This late-phase expression of BDNF is regulated via autoregulatory feedback loop cooperated with CCAAT-enhancer binding protein β expression (Bambah-Mukku et al., 2014). Moreover, applying BDNF to cultured neurons is sufficient to induce Arc expression (Yin et al., 2002). Together, molecular cascades that are initiated by learning presumably lead to late-phase expression of BDNF, and then BDNF is likely to trigger Arc expression in an autocrine manner (Kokaia et al., 1993; Zakharenko et al., 2003).

Memory recall requires stable reactivation of neuronal ensembles formed by learning (Han et al., 2007, 2009; Liu et al., 2012; Kim et al., 2014). Indeed, CA1 neurons activated during contextual FC are reactivated during memory retrieval (Tayler et al., 2013). This reactivation occurs at 2 and 14 d after conditioning, indicating that stable neuronal ensembles in CA1 established by learning persist for at least 14 d. In this study, we have shown that inhibiting late Arc expression disrupted reactivation of neuronal ensembles, as well as freezing behavior, 7 d after FC. These findings indicate that late Arc expression is essential for stabilization of neuronal ensembles formed by learning.

At the cellular level, Arc regulates morphological remodeling of dendritic spines and is essential for shaping functional circuits. A previous study using $Arc^{-/-}$ mice has shown that Arc loss leads to an increase in the proportion of mushroom spines on CA1 neurons with a decrease in the proportion of thin spines and increased epileptic-like network hyperexcitability (Peebles et al., 2010). Overall Arc expression may therefore regulate spine morphology and density, as well as stabilize network activity. In our current study, we focused on the role of learning-induced Arc in learning-related spine reorganization and network activity. We found that late Arc expression is required for spine elimination observed 7 d after FC and for persistence of neuronal ensembles that were established during learning. Because the strengthening of specific synaptic connections underlies a memory trace, elimination of redundant synapses could refine functional circuits for memory. Thus, late Arc-dependent spine pruning might be associated with persistence of neuronal ensembles established by learning. Interestingly, separate analyses of small and large mushroom spines revealed that small mushroom spines are selectively decreased 7 d after FC. Because learning *in vivo* and synaptic potentiation *in vitro* are tightly associated with spine enlargement (Matsuzaki et al., 2004; Roberts et al., 2010), it is possible that large mushroom spines, which are not eliminated 7 d after FC, are involved in a fear memory trace.

De novo spine formation has been proposed as a structural basis for memory traces (Bailey and Kandel, 1993); in our study, however, we did not detect an increase in spine density 7 d after contextual FC. Similarly, no changes in spine density 1–3 d after contextual FC have been reported in another study using DiI for visualizing dendritic spines (Matsuo et al., 2008). Presumably, spine elimination may equal spine formation, preserving overall density. Moreover, in some conditions, spine formation may not make a central contribution to a memory trace (Lai et al., 2012; Sanders et al., 2012). Indeed, a recent time-lapse imaging study of the frontal association cortex found that, after FC, spine elimination is preferentially observed relative to spine formation (Lai et al., 2012).

Spine reduction following FC is selective for mushroom spines on basal dendrites. Selective reduction of mushroom spines might reflect thinning of spine heads, converting originally small mushroom spines into thin spines and eliminating small thin spines altogether. Indeed, Arc overexpression in hippocampal neurons reduces spine thickness (Peebles et al., 2010). A possible explanation for preferential spine reduction on basal but not apical dendrites is that cholinergic modulation enhances synaptic plasticity on basal dendrites. More cholinergic input arrives in the striatum oriens where CA1 basal dendrites are located rather than in the striatum radiatum (Schäfer et al., 1998). Because acetylcholine lowers the threshold for synaptic changes of CA1 neurons (Ovsepian et al., 2004), dendritic spines on basal dendrites could be more susceptible than those on apical dendrites (Perez-Cruz et al., 2011). Indeed, selective spine remodeling on basal dendrites has been previously reported (Moser et al., 1997; Leuner et al., 2003; Santos et al., 2004).

In conclusion, we found that late Arc expression depends on BDNF, but not neuronal activity, and is critical for delayed elimination of dendritic spines and the stable reactivation of neuronal ensembles. Late Arc expression seems to slowly refine functional circuits to prolong long-term fear memories. The precise timing of gene expression is thus crucial to both structural and functional activity-dependent changes underlying learning and animal behavior.

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Report

Frontal Association Cortex Is Engaged in Stimulus Integration during Associative Learning

Daisuke Nakayama,[1] Zohal Baraki,[1] Kousuke Onoue,[1] Yuji Ikegaya,[1,2] Norio Matsuki,[1] and Hiroshi Nomura[1,*]
[1]Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan
[2]Center for Information and Neural Networks, Suita City, Osaka 565-0871, Japan

Summary

The frontal association cortex (FrA) is implicated in higher brain function [1]. Aberrant FrA activity is likely to be involved in dementia pathology [2–4]. However, the functional circuits both within the FrA and with other regions are unclear. A recent study showed that inactivation of the FrA impairs memory consolidation of an auditory fear conditioning in young mice [5]. In addition, dendritic spine remodeling of FrA neurons is sensitive to paired sensory stimuli that produce associative memory [5]. These findings suggest that the FrA is engaged in neural processes critical to associative learning. Here we characterize stimulus integration in the mouse FrA during associative learning. We experimentally separated contextual fear conditioning into context exposure and shock, and found that memory formation requires protein synthesis associated with both context exposure and shock in the FrA. Both context exposure and shock trigger *Arc*, an activity-dependent immediate-early gene, expression in the FrA, and a subset of FrA neurons was dually activated by both stimuli. In addition, we found that the FrA receives projections from the perirhinal (PRh) and insular (IC) cortices and basolateral amygdala (BLA), which are implicated in context and shock encoding [6–8]. PRh and IC neurons projecting to the FrA were activated by context exposure and shock, respectively. Arc expression in the FrA associated with context exposure and shock depended on PRh activity and both IC and BLA activities, respectively. These findings indicate that the FrA is engaged in stimulus integration and contributes to memory formation in associative learning.

Results

The Frontal Association Cortex Is Required for Memory Formation in Contextual Fear Conditioning

As a model for associative learning, we used a contextual fear-conditioning task, which establishes an association between context and shock. To test whether the frontal association cortex (FrA) is involved in memory formation in contextual fear conditioning, we infused (2*R*)-amino-5-phosphonovaleric acid (APV), an *N*-methyl-D-aspartate (NMDA) receptor antagonist, anisomycin, a protein synthesis inhibitor, or vehicle into the FrA. APV and anisomycin were infused 30 min prior to, or immediately after, contextual fear conditioning, respectively (experiment 1; Figures 1A and 1B). Contextual fear memory was assessed by measuring the percentage of freezing time in the conditioning context 1 day after conditioning. Both APV and anisomycin infusions disrupted freezing behavior. When anisomycin infusions were administered into the dorsomedial prefrontal cortex, which is close to the FrA (experiment 2; Figure S1A available online), freezing behavior was comparable to that of mice administered vehicle infusions (Figure S1B). These results indicate that NMDA receptor activation and protein synthesis in the FrA are required for contextual fear conditioning.

Protein Synthesis in the FrA Is Required for Encoding Both Context and Shock

We aimed to determine whether the FrA encodes context, shock, or both. To this end, we separated 10 min of context exposure and immediate shock by a 1-day interval and infused anisomycin into the FrA after either context exposure or shock (experiment 3; Figures 1C and 1D). Anisomycin infusions into the FrA immediately after both context exposure and immediate shock disrupted freezing behavior during the test. However, when anisomycin infusions were administered into the FrA 6 hr after context exposure (Figure 1E), freezing behavior was comparable to that of mice administered vehicle infusions. These results indicate that protein synthesis in the FrA is required for encoding both context and shock.

FrA Neurons Receive Convergent Information Regarding Context and Shock during Fear Conditioning

Because protein synthesis in the FrA is required for encoding both context and shock, we hypothesized that paired stimuli converge in a subset of FrA neurons to potentially contribute to the memory trace. To visualize stimulus convergence, we analyzed the temporal dynamics of nuclear versus cytoplasmic *Arc* localization by fluorescent in situ hybridization [9]. *Arc* is an activity-dependent immediate-early gene that is essential for synaptic plasticity and long-term memory [10–13]. Transcribed *Arc* mRNA first appears in neuronal nuclei, and processed *Arc* mRNA then accumulates in the cytoplasm. Thus, an analysis of the subcellular localization of *Arc* enabled us to identify active neuronal ensembles during two behavioral tasks [9, 14, 15]. We first examined the time course of the nuclear and cytoplasmic *Arc* signal after neural activity in the FrA. Mice were exposed to a context for 5 min and sacrificed either immediately or 30 min later (experiment 4; Figures S2A and S2B). We observed more nuclear Arc^+ neurons and more cytoplasmic Arc^+ neurons in the FrA immediately and 30 min after context exposure, respectively (Figure S2C). Thus, in the following analysis, we identified neurons that were activated ~30 min before and immediately before sacrifice based on the cytoplasmic *Arc* and nuclear *Arc*, respectively.

To separately visualize neuronal ensembles that transcribe *Arc* in conjunction with context exposure and shock, we divided contextual fear conditioning into 5 min of context exposure and immediate shock with an interval of 25 min (experiment 5; Figure 2A). Mice that were preexposed to the conditioning context on the previous day received both context exposure and shock with an interval of 25 min on the

*Correspondence: nomura@mol.f.u-tokyo.ac.jp

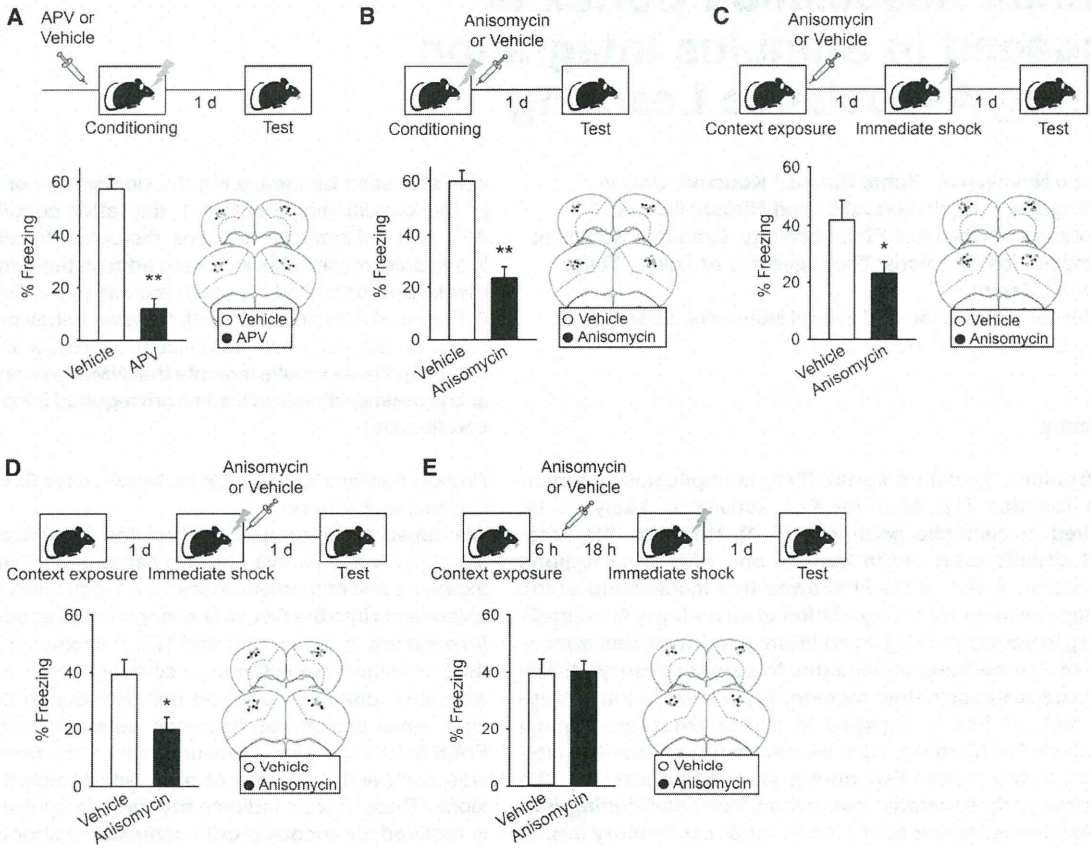

Figure 1. The FrA Contributes to Memory Consolidation of Context and Footshock

(A) APV infusions into the FrA before fear conditioning impaired freezing behavior in the memory test (vehicle, n = 8; APV, n = 8; Student's t test, $t_{(14)} = 8.4$, $p = 8.2 \times 10^{-7}$, $^{**}p < 0.01$).

(B) Anisomycin infusions into the FrA immediately after fear conditioning impaired freezing behavior in the memory test (vehicle, n = 9; anisomycin, n = 8; $t_{(15)} = 6.1$, $p = 2.1 \times 10^{-5}$, $^{**}p < 0.01$).

(C and D) Mice underwent 10 min of context exposure on day 1 and immediate shock on day 2. Anisomycin infusions into the FrA after either 10 min of context exposure (C) or immediate shock (D) decreased freezing in the memory test (C: vehicle, n = 9; anisomycin, n = 9; $t_{(15)} = 2.4$, $p = 0.031$, $^{*}p < 0.05$) (D: vehicle, n = 8; anisomycin, n = 8; $t_{(14)} = 2.8$, $p = 0.014$, $^{*}p < 0.05$).

(E) Anisomycin infusions into the FrA 6 hr after 10 min of context exposure had no effect on freezing behavior in the memory test (vehicle, n = 7; anisomycin, n = 7; $t_{(12)} = 0.12$, $p = 0.90$).

Data are represented as mean ± SEM. See also Figure S1.

conditioning day. They showed a higher freezing level on the test 1 day later, compared with those that underwent either context exposure or shock (Figure 2B).

To analyze *Arc* expression associated with context exposure and shock, we prepared different mice that were preexposed to the conditioning context on the previous day (experiment 6). The mice underwent either no behavioral task, only context exposure, only an immediate shock session, or both context exposure and an immediate shock session on the conditioning day (Figures 2C and 2D). We demonstrate that context exposure increased the proportion of cytoplasmic *Arc*$^+$ neurons and that shock presentation increased the proportion of nuclear *Arc*$^+$ neurons (Figure 2E). These results suggest that both context exposure and shock were effective in induction of *Arc* transcription in FrA neurons. Furthermore, we asked whether the same FrA neurons are dually activated by context exposure and shock by measuring the proportion of cytoplasmic and nuclear double *Arc*$^+$ neurons. The proportion of double *Arc*$^+$ neurons in the fear-conditioning (FC) group was higher relative to chance (Figure 2F). This result suggests that a subset of FrA neurons preferentially receives convergent context and shock information during contextual fear learning.

In the experiment above, we transferred mice to the conditioning context when we administered shocks to the mice. Although the time spent in the context was just 6 s, it can be argued that nuclear *Arc* expression could be attributed to this translocation, but not to footshock. Therefore, we prepared additional behavioral groups (experiment 7; Figure 2G). Mice in the 35′ context group were exposed to the context for 35 min until they were sacrificed. Mice in the 35′ context + shock group were exposed to the context, given footshock 30 min later, and sacrificed 5 min later. We found a higher proportion of cytoplasmic *Arc*$^+$ neurons but a lower proportion of nuclear *Arc*$^+$ neurons in the 35′ context group (Figure 2H), suggesting that *Arc* transcription responsive to context exposure decreases over time. In the 35′ context + shock group, the proportion of nuclear *Arc*$^+$ neurons was higher than that in the 35′ context group (Figure 2H). The proportion of double *Arc*$^+$ neurons in the 35′ context + shock group was also higher

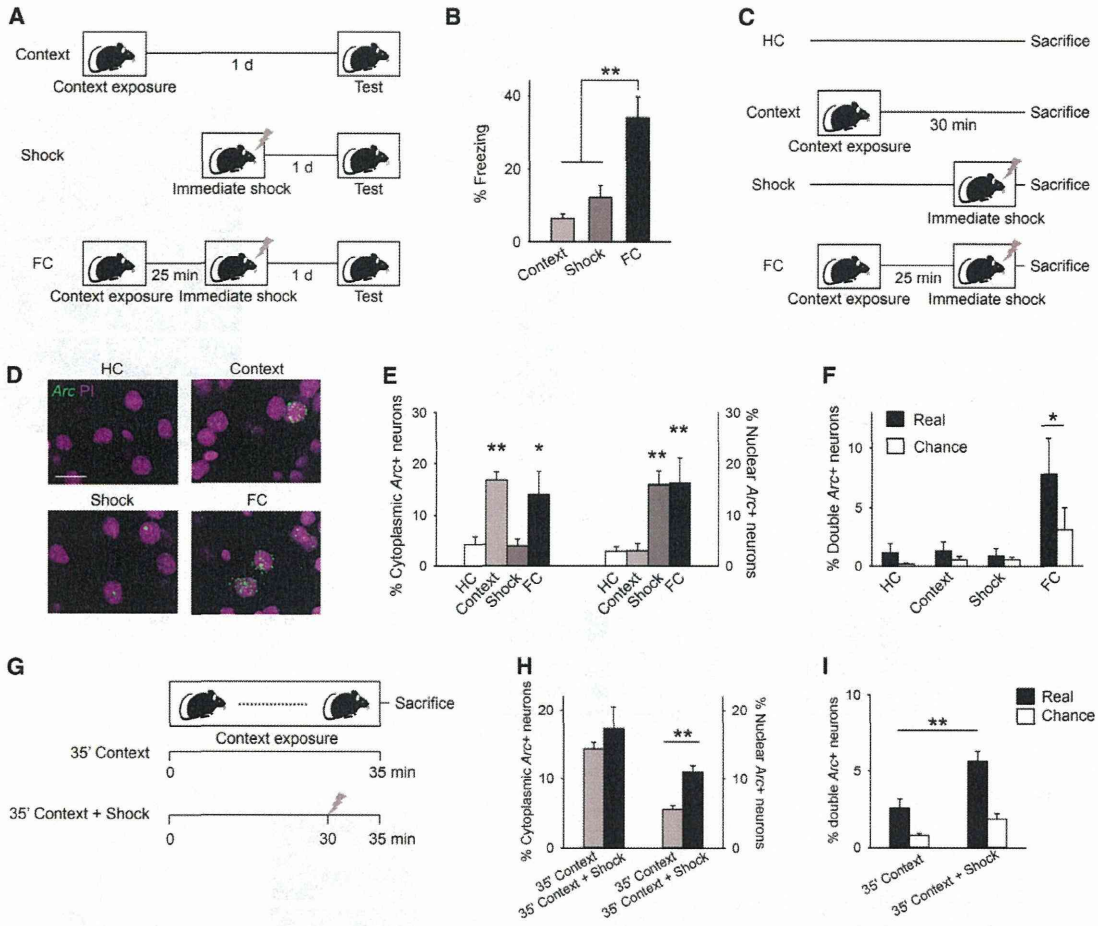

Figure 2. Context Exposure and Shock Activate *Arc* Transcription in Overlapping Neurons in the FrA

(A) Behavioral procedure for (B) (context, n = 6; shock, n = 7; FC, n = 6).

(B) Mice in the FC group showed greater freezing behavior relative to the context and shock groups (one-way ANOVA, $F_{(2,16)} = 14.0$, $p = 0.00031$; Tukey's test, context versus FC, $p = 0.00037$; shock versus FC, $p = 0.0024$).

(C) Behavioral procedure for (D)–(F) (HC, n = 7; context, n = 4; shock, n = 4; FC, n = 5).

(D) Representative images of *Arc* RNA expression in the FrA. PI, propidium iodide. The scale bar represents 20 μm.

(E) Context exposure increased the proportion of cytoplasmic *Arc*$^+$ neurons ($F_{(3,16)} = 7.3$, $p = 0.0026$; context versus HC, $p = 0.0078$; FC versus HC, $p = 0.025$). Shock increased the proportion of nuclear *Arc*$^+$ neurons ($F_{(3,16)} = 7.9$, $p = 0.0019$; shock versus HC, $p = 0.014$; FC versus HC, $p = 0.0072$).

(F) The proportion of cytoplasmic and nuclear double *Arc*$^+$ neurons was higher than the chance level in the FC group (repeated-measures ANOVA, $F_{(3,16)} = 6.0$, $p = 0.0062$; paired t test, $t_{(4)} = 3.4$, $p = 0.028$).

(G) Behavioral procedure for (H) and (I) (35′ context, n = 5; 35′ context + shock, n = 5).

(H) Shock increased the proportion of nuclear *Arc*$^+$ neurons (Student's t test, $t_{(8)} = 4.6$, $p = 0.00090$).

(I) Shock increased the proportion of cytoplasmic and nuclear double *Arc*$^+$ neurons (35′ context versus 35′ context + shock, $t_{(8)} = 3.5$, $p = 0.0083$).

Data are represented as mean ± SEM. $^{**}p < 0.01$, $^{*}p < 0.05$. See also Figure S2.

than that in the 35′ context group (Figure 2I). Our results indicate that footshock induces *Arc* transcription in the FrA and that a subset of FrA neurons preferentially receives convergent context and shock information during context fear learning.

FrA Neurons Receive Contextual Information from the Perirhinal Cortex and Shock Information from the Insular Cortex

FrA neurons were activated in response to both context and shock, suggesting that the FrA receives projections from brain regions that encode sensory stimuli. However, neural circuits that project to the FrA and are involved in fear learning are poorly understood. To investigate the brain regions projecting to the FrA, we infused Alexa Fluor conjugates of cholera toxin subunit B (CTB) [16] into the FrA (Figures 3A and 3B; Figures S3A and S3B). Seven days after CTB infusion, mice were sacrificed either immediately after removal from their home cages (HC group), 90 min after immediate shock (shock group), 90 min after 10 min of context exposure (context group), or 90 min after contextual fear conditioning (FC group) (experiment 8; Figure 3A). We found robust CTB retrograde signals in the insular cortex (IC), perirhinal cortex (PRh), and basolateral amygdala (BLA) (Figure 3C).

To test whether FrA-projecting IC and PRh neurons are activated during fear conditioning, we subjected brain slices including the IC and PRh to c-Fos immunohistochemistry (Figure 3D). c-Fos is widely used as a neural activity marker [17]. The proportion of c-Fos$^+$ neurons in CTB$^+$ IC neurons in the shock and FC groups was higher compared with the HC group

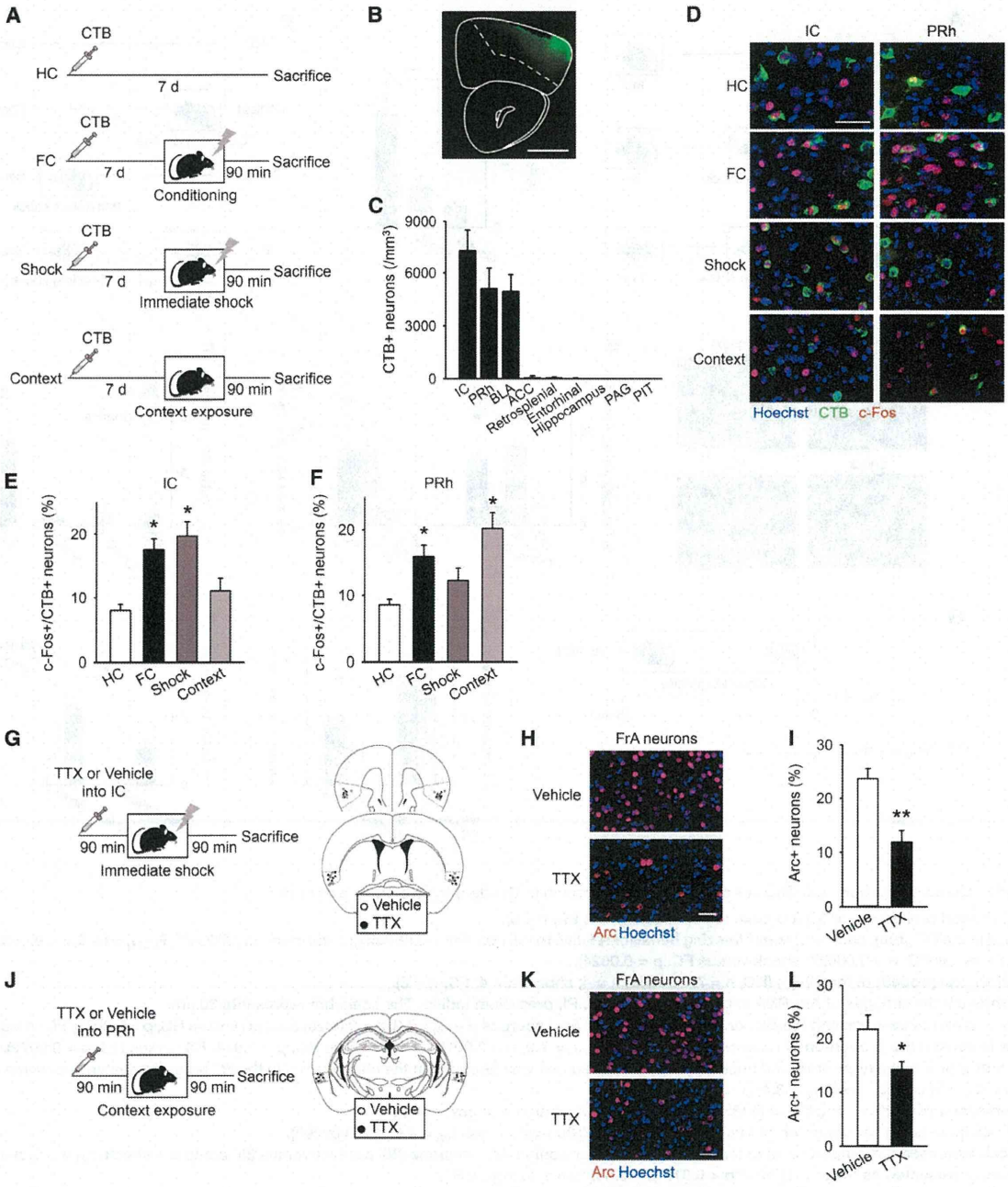

Figure 3. FrA Neurons Receive Contextual Information from the PRh and Shock Information from the IC

(A) Behavioral procedures for (B)–(F) (HC, n = 14; FC, n = 5; context, n = 5; shock, n = 5).

(B) Representative images of CTB diffusion within the FrA. The dashed line indicates the border between the FrA and its neighboring regions. The scale bar represents 1 mm.

(C) Many neurons with CTB signals were observed in the perirhinal and insular cortices and basolateral amygdala. ACC, anterior cingulate cortex; PAG, periaqueductal gray; PIT, posterior intralaminar thalamic complex.

(D) Representative images of c-Fos immunostaining and CTB signals in the PRh and IC. The scale bar represents 50 μm.

(E) FC and shock groups showed a higher proportion of neurons with c-Fos signals in IC neurons projecting to the FrA (one-way ANOVA, $F_{(3,24)} = 13.4$, $p = 2.5 \times 10^{-5}$; Tukey's post hoc test, HC versus FC, $p = 8.4 \times 10^{-4}$; HC versus shock, $p = 7.0 \times 10^{-5}$).

(F) FC and context groups showed a higher proportion of neurons with c-Fos signals in PRh neurons projecting to the FrA ($F_{(3,26)} = 12.7$, $p = 2.7 \times 10^{-5}$; HC versus FC, $p = 0.0035$; HC versus context, $p = 3.2 \times 10^{-5}$).

(G) Mice received vehicle or TTX into the IC 90 min before an immediate shock session.

(H) Representative images of Arc immunostaining in the FrA. The scale bar represents 50 μm.

(I) TTX infusions decreased the proportion of Arc^+ neurons in the FrA (vehicle, n = 6; TTX, n = 6; Student's t test, $t_{(10)} = 4.1$, $p = 0.0021$).

(J) Mice received vehicle or TTX into the PRh 90 min before 10 min of context exposure.

(K) Representative images of Arc immunostaining in the FrA. The scale bar represents 50 μm.

(L) TTX infusions decreased the proportion of Arc^+ neurons in the FrA (vehicle, n = 6; TTX, n = 6; $t_{(10)} = 2.5$, $p = 0.029$).

Data are represented as mean ± SEM. $^{**}p < 0.01$, $^{*}p < 0.05$. See also Figures S3 and S4.

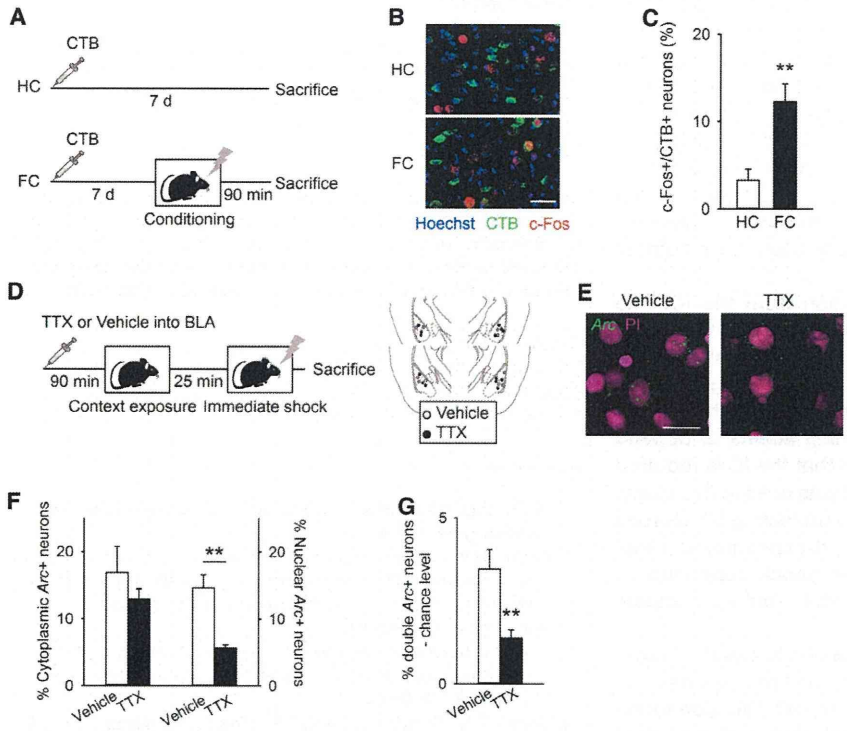

Figure 4. FrA-Projecting BLA Neurons Are Activated during Fear Conditioning, and FrA *Arc* Expression Responsive to Shock Depends on BLA Activity

(A) Behavioral procedures for (B) and (C) (HC, n = 5; FC, n = 5).

(B) Representative images of c-Fos immunostaining and CTB signals in the BLA. The scale bar represents 50 μm.

(C) Fear conditioning increased the proportion of neurons with c-Fos signals in the BLA neurons projecting to the FrA (Student's t test, $t_{(8)} = 5.3$, $p = 7.4 \times 10^{-4}$).

(D) Mice were placed in the context 90 min after infusions of vehicle or TTX into the BLA, given immediate shock 25 min later, returned to their home cages, and sacrificed.

(E) Representative images of *Arc* RNA expression in the FrA. The scale bar represents 20 μm.

(F) TTX infusions decreased the proportion of nuclear *Arc*$^+$ neurons, but not cytoplasmic *Arc*$^+$ neurons (vehicle, n = 5; TTX, n = 6; repeated-measures ANOVA, $F_{(1,9)} = 5.4$, $p = 0.045$; nuclear *Arc*$^+$ neurons, $p = 9.0 \times 10^{-4}$).

(G) TTX infusions decreased the proportion of cytoplasmic and nuclear double *Arc*$^+$ neurons ($t_{(9)} = 3.5$, $p = 0.0066$).

Data are represented as mean ± SEM. **$p < 0.01$.

(Figure 3E). In contrast, the proportion of c-Fos$^+$ neurons in CTB$^+$ PRh neurons in the context and FC groups was higher compared with the HC group (Figure 3F). These results indicate that FrA-projecting IC neurons are activated by shock and that FrA-projecting PRh neurons are activated by context exposure in fear conditioning. We do not exclude the possibility that pathways from the IC and PRh to other regions are also activated during fear conditioning, because the proportions of c-Fos$^+$ neurons in CTB$^+$ IC and PRh neurons in the FC group were comparable to those in overall IC and PRh neurons (IC, 19.0% ± 2.3%; PRh, 16.1% ± 3.9%).

To test whether the IC and PRh are required for the contextual fear conditioning used in this study, we infused tetrodotoxin (TTX), a sodium channel blocker, or vehicle into the IC or PRh 90 min before contextual fear conditioning (experiment 9). TTX infusions into both the IC and PRh prevented fear conditioning (Figure S4), indicating that the IC and PRh are involved in formation of contextual fear memory.

Based on the results above, we expected that the FrA receives shock-related inputs from the IC and context-related inputs from the PRh. To test this possibility, we examined the effect of IC or PRh inhibition on Arc expression in the FrA responsive to shock or context exposure, respectively. First, we infused TTX or vehicle into the IC 90 min before footshock (experiment 10; Figure 3G). The mice were sacrificed 90 min after footshock, and FrA slices were subjected to Arc immunohistochemistry (Figure 3H). TTX infusions into the IC decreased the proportion of Arc$^+$ FrA neurons (Figure 3I). Next, we prepared different mice and infused TTX or vehicle into the PRh 90 min before context exposure (experiment 11; Figure 3J). TTX infusions into the PRh decreased the proportion of Arc$^+$ FrA neurons (Figures 3K and 3L). These results indicate that Arc expression in the FrA in response to shock and context exposure depends on IC and PRh activities, respectively.

FrA-Projecting BLA Neurons Are Activated during Fear Conditioning, and FrA *Arc* Expression Responsive to Shock Depends on BLA Activity

Because the FrA also receives projections from the BLA, which is a key region for contextual fear learning [8], we also tested whether BLA neurons projecting to the FrA are activated during contextual fear learning (Figures 4A and 4B). Fear conditioning increased the proportion of c-Fos$^+$ neurons in CTB$^+$ BLA neurons (Figure 4C), indicating that FrA-projecting BLA neurons are activated during contextual fear conditioning.

To examine the contribution of BLA activity to context and shock encoding in the FrA, we infused TTX or vehicle into the BLA 90 min before mice were subjected to context exposure and shock (experiment 12; Figure 4D). Mice were sacrificed 5 min after footshocks, and FrA slices were subjected to FISH for *Arc* (Figure 4E). Intra-BLA TTX infusions decreased the proportion with nuclear, but not cytoplasmic, *Arc* signals (Figure 4F). Intra-BLA TTX infusions also decreased the proportion of neurons expressing both cytoplasmic and nuclear *Arc*, whereas a chance level was not different between the two groups ($t_{(9)} = 2.2$, $p = 0.06$) (Figure 4G). These data indicate that *Arc* expression, responsive to shock, depends on BLA activity.

Discussion

In this study, we show that the FrA is involved in associative fear learning and that it receives converging inputs from the PRh, IC, and BLA, integrates these stimuli, and encodes their association. Further, we specifically demonstrate that PRh and IC (along with BLA) neurons projecting to the FrA are specifically activated by context exposure and shock, respectively.

FrA neurons receive contextual information from the PRh. The PRh receives projections from sensory brain areas such as the visual, auditory, and piriform cortices, forms reciprocal connections with the hippocampal CA1 and entorhinal cortex, and then encodes contextual information [18]. Indeed, inhibition of the PRh impairs contextual fear conditioning [19]. Here we found that FrA-projecting PRh neurons were activated by context exposure and that Arc expression in the FrA responsive to shock depended on PRh activity. These results suggest that the PRh-to-FrA circuit is likely to participate in context encoding.

FrA neurons receive shock information from the IC. The IC receives convergent inputs from the somatosensory cortices, ventroposterior and posterior thalamic nuclei, posterior intralaminar nuclei, and midbrain parabrachial nucleus and can be involved in aversive pain sensation [6]. Although the involvement of the IC in fear conditioning seems to depend on the conditions [6, 20], we confirmed that the IC is required for the contextual fear conditioning that was used in this study. In the present study, we found that FrA-projecting IC neurons were activated by shock, but not context exposure, and that Arc expression in the FrA responsive to shock depended on IC activity. Therefore, the IC-to-FrA circuit could participate in shock encoding.

The BLA-to-FrA circuit is also likely to contribute to contextual fear conditioning. Arc expression in the FrA responsive to shock depends on the BLA as well as the IC. We also found that FrA-projecting BLA neurons are activated during contextual fear learning. These results suggest that the FrA receives shock information from the BLA. Alternatively, the FrA might receive associative information from the BLA, because the association between context and shock is produced in the BLA at the time of shock presentation [21].

A subset of FrA neurons receives multimodal information from the PRh, IC, and BLA. Because the proportion of neurons responsive to both context exposure and shock was higher than a chance level, a specific subset of FrA neurons may receive convergent information. Neurons that were activated by context exposure could be more likely to be activated by shock than the neighboring neurons that were not activated by context exposure. This allocation mechanism could contribute to associative learning. Further studies are needed to determine whether convergent activation in FrA neurons occurs only during associative learning and then whether such an allocation mechanism contributes to associative learning.

In conclusion, we found a novel form of stimulus integration, involved in associative learning, in the FrA, where convergent activity from context and shock might induce synaptic remodeling in FrA neurons [5] and contribute to memory formation. Because the frontal cortex is implicated in the planning and execution of complex cognitive behavior [22, 23], memory traces in this region could affect these functions. In fact, subjects with posttraumatic stress disorder show impairment of cognitive behavior, including executive function [24]. In addition, because the frontal cortex receives and integrates multimodal information, traces of different types of memory could affect each other in this region. This might explain an association of diverse memories.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.10.078.

Author Contributions

D.N., Z.B., K.O., and H.N. performed the experiments, and D.N. and H.N analyzed the data and wrote the manuscript. H.N. designed the study, and N.M. and Y.I. supervised the project.

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