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Second-order fear conditioning prevented by blocking NMDA receptors in amygdala

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Antagonists of NMDA (N-methyl-D-aspartate)-type glutamate receptors disrupt several forms of learning^{1–8}. Although this might indicate that NMDA-receptor-mediated processes are critical for synaptic plasticity, there may be other mechanisms by which NMDA-receptor antagonism could interfere with learning^{9–12}. For instance, fear conditioning would be blocked by microinfusion of the NMDA-receptor antagonist AP5 (D,L-2-amino-5-phosphonovalerate) into the basolateral amygdala^{6,13,14} if AP5 inhibited routine synaptic transmission, thereby reducing the ability of stimuli to activate amygdala neurons^{15,16}. In second-order fear conditioning^{17,18}, the reinforcer is a fear-eliciting conditioned stimulus rather than an unconditioned stimulus. Expression of conditioned fear is amygdala-dependent^{19,20} and so provides a behavioural assessment of the ability of the reinforcer to activate amygdala neurons in the presence of AP5. We report here that intra-amygdala AP5 actually enhances expression of conditioned fear to the conditioned stimulus that provides the reinforcement signal for second-order conditioning. Nevertheless, acquisition of second-order fear conditioning is completely blocked. Our findings strongly support the view that NMDA receptors are critically involved in synaptic plasticity.

Figure 1 Fear-potentiated startle to the second-order CS in ACSF (top) and AP5 (bottom) groups tested before second-order conditioning training, and after two (post-training 1), and three (post-training 2) sessions of second-order conditioning training. Fear-potentiated startle (hatched bars, $\pm$ s.e.m.) was calculated as the difference between startle amplitude in the presence (white bars, $\pm$ s.e.m.) and absence (black bars, $\pm$ s.e.m.) of the visual CS. No infusion was given immediately before tests. An analysis of variance (ANOVA) revealed a significant group $\times$ session interaction, $F(2, 34) = 3.8$, $P < 0.05$. In the ACSF group, fear-potentiated startle increased significantly relative to pretraining baseline in post-training tests 1 and 2; values of $t(8)$ were 2.4 and 2.6 respectively, $P < 0.05$. In the AP5 group, the CS actually tended to inhibit startle relative to the pretraining baseline in post-training tests 1 and 2, although this effect was not significant; $t(9)$ was 0.7 and 1.3, respectively.

Pavlovian fear conditioning seems to involve the convergence of inputs representing the conditioned stimulus (CS) and the unconditioned stimulus (US) in the basolateral amygdala²¹. Microinfusion of NMDA receptor antagonists into the amygdala blocks the acquisition of fear conditioning, measured using the fear-potentiated startle response^{6,13}. This is consistent with data from other behavioural models suggesting that NMDA receptor activation is critical for the synaptic plasticity underlying several forms of memory^{1–8}. Alternatively, NMDA receptor blockade might interfere with the ability of the CS or the US to activate cells in the amygdala^{15,16}. It is unlikely that the ability of the CS to activate amygdala neurons is impaired, because local infusion of NMDA antagonists, in contrast to AMPA antagonists¹⁹, does not interfere with the expression of fear conditioning previously acquired^{6,13}. However, the ability of the US (typically foot shock) to activate amygdala neurons is not behaviourally testable in this learning protocol.

In pavlovian second-order fear conditioning, a second-order CS acquires conditioned fear by being paired with a first-order CS that has been paired previously with foot shock. In this case, the ability of the reinforcer to activate the amygdala can be assessed by the degree to which the first-order CS elicits conditioned fear. Thus, second-order conditioning can be used to evaluate whether an NMDA-receptor antagonist blocks acquisition of conditioned fear or interferes with synaptic transmission of the reinforcement signal in the amygdala.

To test this, male albino rats were implanted with bilateral cannulae aimed at the basolateral amygdala. In four sessions of

Figure 2 a, Fear-potentiated startle to the first-order auditory CS when rats were tested with no drug infused at the time of test (post-training 2), and when AP5 or ACSF vehicle were infused into the amygdala immediately before the test (post-training 3). The final test (post-training 3) was conducted after an additional session of first-order conditioning training. Fear-potentiated startle (hatched bars, $\pm$ s.e.m.) was calculated as the difference in amplitude of the startle response elicited at the start of the test session in the absence of the auditory CS (black bars, $\pm$ s.e.m.), and the startle response elicited in the presence of the auditory CS (white bars, $\pm$ s.e.m.). Intra-amygdala AP5 infusion significantly enhanced fear-potentiated startle in the final test. This was confirmed by a significant group $\times$ test session interaction $F(1, 9) = 5.2$, $P < 0.05$. Individual t -tests revealed that fear-potentiated startle was greater in the group infused with AP5 than in the group infused with ACSF in post-training test 3, $t(9) = 3.0$, $P < 0.01$, whereas the same groups did not differ significantly in the previous test, $t(9) = 1.0$. Furthermore, there was a tendency for fear-potentiated startle to be greater in the AP5 group in post-training test 3 (that is, in the presence of AP5) than in post-training test 2, $t(5) = 2.4$, $P = 0.06$. **b**, Fear-potentiated startle in the first and second halves of the final test session. There was no reduction of fear-potentiated startle in either group within the session, $F = 1.2$, and no group $\times$ session half interaction, $F < 1$, indicating that the enhancement of fear-potentiated startle by AP5 was not due to a blockade of extinction²³.

first-order training, an auditory CS was paired with foot shock. On the basis of a test conducted between the third and fourth training sessions, subjects were assigned to two groups with equivalent levels of fear-potentiated startle to the auditory CS. Subjects then received three sessions of second-order training, in which a visual CS was paired with the trained auditory CS. No auditory CS–shock pairings were given during these sessions. However, to reduce extinction of first-order conditioning, a fifth first-order training session was included between the second and third second-order training sessions. Immediately before each second-order training session, one group ($n = 10$) was infused with 12.5 nmol AP5 (the minimal dose effective in blocking acquisition of first-order conditioning^{6,13,14}) dissolved in artificial cerebrospinal fluid (ACSF), and a control group ($n = 9$) with the same volume of ACSF vehicle alone. Fear-potentiated startle was tested in separate sessions (without drug infusion) conducted after the second and third second-order training sessions.

The control subjects infused with ACSF alone showed acquisition of second-order conditioning, indicated by an increase in fear-potentiated startle across test sessions (Fig. 1). In contrast, subjects infused with AP5 showed no evidence of acquisition of second-order condition. In fact, the second-order visual CS produced a moderate inhibition of startle, which appeared to increase across sessions. These data indicate that AP5 blocked the acquisition of conditioned fear to the second-order visual CS.

The increase in inhibition of startle by the visual CS across test sessions seen in the AP5 group might indicate the acquisition of some form of inhibitory learning that is not blocked by intra-amygdala AP5. Alternatively, this inhibition may be related to the elevation in baseline startle across test sessions, which is somewhat greater in the absence than in the presence of the visual CS (for example, because of external inhibition¹⁷). This increase, although nonsignificant, $F(2, 18) = 2.6$, $P < 0.1$, may reflect an enhancement of conditioned fear to contextual cues²² learned during first-order conditioning sessions when AP5 was not given. Extinction of contextual fear in the shock-free second-order conditioning training sessions, an effect that would be blocked by intra-amygdala AP5

given on these sessions²³, could account for the fact that the same elevation in baseline startle was not seen in the ACSF group.

To assess the effect of infusion of AP5 on the expression of first-order conditioning, fear-potentiated startle to the auditory CS was tested after a further first-order conditioning session. Immediately before test, some subjects were infused with AP5 ($n = 6$) or ACSF vehicle ($n = 5$), retaining the group assignments used earlier in second-order training. As seen in Fig. 2a, both groups showed first-order conditioning, $F(1, 4) = 9.7$, $P < 0.05$ for the ACSF group, and $F(1, 5) = 28.7$, $P < 0.01$ for the AP5 group. Indeed, fear-potentiated startle was actually greater in the group infused with AP5, even though the two groups had shown equivalent levels of fear-potentiated startle in the previous test session, $F(1, 9) = 5.2$, $P < 0.05$. As seen in Fig. 2b, this enhancement was not attributable to a blockade of extinction of conditioned fear during the test session²³ because AP5 enhanced fear-potentiated startle equally in the first and second halves of the test session, and neither group showed extinction under these test conditions, $F = 1.2$. This enhancement of fear-potentiated startle is consistent with previous reports using both visual and auditory CSs^{6,13}. In contrast, the expression of conditioned freezing acquired to contextual cues is blocked by intra-amygdala AP5 (ref. 16). This suggests that amygdala pathways mediating explicit and contextual CS information are differentially dependent on NMDA receptor-mediated glutamatergic transmission in the amygdala.

The results of this study reduce the number of viable interpretations of the effects of NMDA receptor antagonists on fear conditioning. First, expression of fear conditioning using an auditory CS was enhanced, not impaired, at a dose of AP5 that blocked acquisition of fear conditioning⁶. This argues against the possibility that NMDA antagonists block acquisition of fear conditioning by reducing general levels of cell excitability in the amygdala¹⁶. Second, the same dose of AP5 that enhanced expression of first-order conditioning totally blocked acquisition of second-order conditioning. This indicates that the blockade of second-order conditioning was not caused by a reduction in the effectiveness of the reinforcer in activating amygdala neurons. If AP5 had no influence on synaptic

plasticity, an enhancement of first-order conditioned fear would be expected actually to support greater acquisition of second-order conditioning, because the strength of second-order conditioning depends on the strength of first-order conditioning previously established^{17,18}. Third, the enhancement of the expression of first-order conditioning by AP5 makes it unlikely that the blockade of the acquisition of second-order conditioning was a state-dependent effect. Hence, our results strongly support the view that NMDA receptors in the amygdala are critically involved in the synaptic plasticity underlying fear conditioning.

The blockade of second-order fear conditioning has further implications regarding the specific nature of the involvement of the basolateral amygdala in learning and memory. First, in contrast to foot-shock information, the pathways carrying second-order reinforcer (auditory CS) information to the basolateral amygdala^{24,25}, and their relative dependence on NMDA-receptor activation²⁶, have been well characterized. Intra-amygdala application of AP5 blocks activation of neurons in the lateral nucleus of the amygdala normally produced by stimulation of the auditory thalamus^{15,26}. In contrast, neuronal activation through stimulation of auditory cortex, a second route by which an auditory CS could access the amygdala, is not disrupted by AP5 (ref. 26). Given that expression of first-order fear conditioning, which serves as the reinforcer for second-order fear conditioning, is not disrupted by AP5, input of auditory information critical for both functions may well activate neurons of the basolateral amygdala via cortico-amygdala rather than thalamo-amygdala projections.

Our results also suggest a role of NMDA receptors in the basolateral amygdala in fear conditioning based on two different forms of learning. Whereas first-order conditioning involves the formation of an association between representations of the stimuli paired during training (that is, the CS and US, 'stimulus-stimulus' learning), the parameters used to produce second-order conditioning in this study promote the formation of associations between the second-order CS and the conditioned response (fear) elicited by the first-order CS (that is, 'stimulus-response' learning)¹⁸. The blockade of acquisition of both first-^{6,13} and second-order fear conditioning suggests that a common neural mechanism—namely, activation of NMDA receptors in the amygdala—is critically involved in the plasticity that underlies these two distinct forms of fear conditioning.

In contrast, in conditioning situations other than fear conditioning, NMDA receptors in the basolateral amygdala may be essential for acquisition based on secondary reinforcement, but not for acquisition based on primary reinforcement. Second-order appetitive conditioning is blocked by excitotoxic lesions of the amygdala, whereas, in contrast to fear conditioning²⁰, first-order appetitive conditioning is left intact²⁷. Similarly, local infusion of AP5 into the basolateral amygdala blocks acquisition of taste-potentiated odour conditioning, but not acquisition of a first-order taste aversion². Thus, the basolateral amygdala seems to be critical for learning that involves a reinforcer with conditioned motivational value, irrespective of whether that reinforcer signals impending reward or punishment^{28,29}. Outside the laboratory, stimuli with intrinsic reinforcing value, such as a noxious US, are likely to be experienced less frequently than stimuli with conditioned reinforcing value. Hence, the blockade of higher-order fear conditioning by AP5 suggests a broader involvement of amygdala NMDA receptor-mediated processes in emotional learning than would be suggested by the blockade of first-order conditioning alone. □

Methods

Surgery. Under sodium pentobarbital anaesthesia, 22 male Sprague Dawley rats were stereotactically implanted with 22-gauge cannulae aimed bilaterally at the basolateral amygdala (BLA). The cannulae were secured with dental acrylic anchored to four jeweller's screws fixed in the skull. Cannula patency was maintained using internal stylets that protruded 1 mm beyond cannula tips.

Three subjects were excluded from data analysis, either because a cannula was clogged, or because histological examination indicated that cannula placement was further than 1 mm from the BLA.

Apparatus and stimuli. For measurement of the startle reflex and delivery of all stimuli, subjects were individually housed in a stabilimeter device³⁰. The startle-eliciting stimuli were 95 dB, 50-ms bursts of white noise (rise-decay time: 5 ms), against a background white noise of 55 dB SPL (A scale). The auditory CS was a 6.7-s, 70-dB white noise that was band-pass filtered, with the low and high passes both set at 2,000 Hz. A 3.7-s visual CS was provided by an 8-W fluorescent light bulb (rise-decay time, 100 μ s; 800 foot-lamberts intensity). Scrambled, 0.5-s, 1.2-mA foot shocks were delivered through the floor bars of the stabilimeter.

Behavioural procedures. On first-order training sessions, subjects were presented with 10 initial startle stimuli, followed by 20 presentations of the auditory CS. On 16 presentations, the interval between onset of the auditory CS and foot shock was equally often 0.2, 2.2, 4.2 or 6.2 s. On the remaining 4 occasions, the CS was presented without foot shock. On second-order sessions, following the initial startle stimuli, subjects were presented with 10 pairings of auditory and visual CSs. The onset of the auditory CS occurred 3.5 s after the onset of the visual CS. In both first- and second-order sessions, the mean interval between successive presentations of the auditory CS was 150 s (range: 90–210 s).

In test sessions, following 20 initial presentations of the startle stimulus to habituate the startle reflex, startle was further elicited in the presence and absence of the CSs. Startle stimulus onset occurred at the same times after CS onset at which reinforcement had occurred in training: that is 3.5 s after visual CS onset, and 0.2, 2.2, 4.2 and 6.2 s after auditory CS onset. The interval between successive startle stimuli was 30 s. Fear-potentiated startle was calculated by subtracting mean startle amplitude in the absence of the CS from mean startle amplitude in the presence of the CS. Startle in the presence of the auditory CS was calculated as the mean amplitude of startle elicited 2.2, 4.2 and 6.2 s following CS onset.

Drug. Bilateral amygdala infusions were made with 28-gauge cannula injectors connected by polyethylene tubing to 10- μ l Hamilton syringes fitted to a Harvard apparatus infusion pump. 12.5 nmol D,L-2-amino-5-phosphonovale- rate (AP5) dissolved in 0.3 μ l ACSF was infused through each injector at a rate of 0.1 μ l min⁻¹. Injectors were left in place for 2 min following infusion.

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Transport, docking and exocytosis of single secretory granules in live chromaffin cells

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Neurons maintain a limited pool of synaptic vesicles which are docked at active zones and are awaiting exocytosis^{1–4}. By contrast, endocrine cells releasing large, dense-core secretory granules have no active zones, and there is disagreement about the size⁵ and even the existence⁶ of the docked pool. It is not known how, and how rapidly, secretory vesicles are replaced at exocytic sites in either neurons or endocrine cells. By using electron microscopy, we have now been able to identify a pool of docked granules in chromaffin cells that is selectively depleted when cells secrete. With evanescent-wave fluorescence microscopy⁷, we observed single granules undergoing exocytosis and leaving behind patches of bare plasmalemma. Fresh granules travelled to the plasmalemma at a top speed of 114 nm s⁻¹, taking an average of 6 min to arrive. On arrival, their motility diminished 4-fold, probably as a result of docking. Some granules detached and returned to the cytosol. We conclude that a large pool of docked granules turns over slowly, that granules move actively to their docking sites, that docking is reversible, and that the 'rapidly releasable pool' measured electrophysiologically represents a small subset of docked granules.

Even in chromaffin cells, a population of docked dense-core granules can be morphologically identified if chemical fixation is avoided (Fig. 1a). We measured the closest distance between granules and the plasmalemma and plotted the results as a histogram (Fig. 1b). Granules populated the cytosol at a constant density, although those touching the plasmalemma were more numerous. In terms of granules per μm^2 (Fig. 1 legend), the cytosolic density of granules (dashed line) is only 5% of the density of those touching the plasmalemma. The remaining 95% must be bound to the plasmalemma, or docked. Their number in a resting cell was $1.68 \pm 0.11 \mu\text{m}^{-2}$ (5 experiments) or $1,010 \pm 80$ in a typical cell with a $600 \mu\text{m}^2$ surface area. When cells were stimulated by elevating the external potassium ion concentration ($[\text{K}^+]$), granules were lost by exocytosis (Fig. 1c). Granules touching the plasmalemma were lost selectively (Fig. 1d); $53 \pm 11\%$ of them were lost during 2 minutes of stimulation (5 experiments). Selective depletion of docked granules over 2 min is not expected if cytosolic and docked granules reach equilibrium over that time. For instance, the 'rapidly releasable pool' of secretory vesicles in chromaffin cells⁸ and neurons^{9,10} is replenished with time constants of only 10 s. In chromaffin cells, this evidently can happen without docking new granules.

The dynamics of these morphological changes was studied in living cells²⁴ by fluorescence microscopy. Cells were allowed to attach to glass coverslips, then stained briefly with acridine orange and washed thoroughly. For viewing single granules beneath the plasmalemma, confocal microscopy had insufficient depth resolution and, most importantly, could image single granules only at illumination levels that caused rapid bleaching and phototoxicity. To image a thin layer of cytosol with less intense illumination, we used a method not previously applied in neurobiology. Fluorescence was excited selectively in a 300-nm-thin layer of cytosol immediately adjacent to the coverslip, using the evanescent wave set up by a laser beam as it suffered total reflection at the glass/cell interface⁷. Typical images (Fig. 1e, f) show scattered fluorescent spots. Because granules are by far the most abundant organelle near the plasmalemma (Fig. 1a), most spots must represent granules close to the plasmalemma where it adheres to the coverslip.

Stimulation with high $[\text{K}^+]$ caused fluorescent spots to disappear (Fig. 1f) as exocytosing granules lost their dye. Granules were lost in patches, consistent with earlier reports of localized secretion¹¹. The patches did not arise because the plasmalemma buckled and moved out of the evanescent wave, because in simultaneous interference reflection images (Fig. 1g, h) the 'footprint' of the cell on the coverglass remained dark; the footprint would have brightened had the plasmalemma detached. During a 2-min exposure to high $[\text{K}^+]$, $34 \pm 6\%$ of the granules were lost (15 cells). This is less than the 53% lost in Fig. 1b, c, presumably because the evanescent wave also excites fluorescence in some granules that do not touch the plasmalemma and hence cannot perform exocytosis. Comparison of the two values suggests that in a resting cell, $34/53 = 64\%$ of the fluorescent spots are secretory granules touching the plasmalemma.

The cells we studied had 1.3 ± 0.4 fluorescent spots per μm^2 (24 cells). If only 64% represent granules touching the plasmalemma, we see fewer such granules (0.8 per μm^2) than in electron micrographs. Although plasmalemma adhering to glass may carry fewer granules, we believe that two other factors are also important: first, we tended to select cells with lower granule density (see Methods); second, granules in electron micrographs varied 2–3-fold in diameter and hence more than 8-fold in volume. If granules carry dye and fluoresce in proportion to their volume, small ones will be overlooked if they are next to larger ones.

Four findings indicate that most of the fluorescent granules in Fig. 1e, f were lost through exocytosis. First, the effect was not seen when Mg^{2+} replaced external Ca^{2+} and 0.1 mM Cd^{2+} was added ($99 \pm 4\%$ remaining after 2 min at high $[\text{K}^+]$; 5 cells). Second, fluorescent spots disappeared abruptly from one frame to the next