

Figure 2 Absorbance spectra for *B. thermydron* visual pigments. Coloured curves are Stavenga-type rhodopsin templates²². Top, the zoeal visual pigment is fitted best by a template with $\lambda_{\max} = 447$ nm ($n = 6$ scans). Middle, the megalopa, the last instar before the juvenile crab, possesses a visual pigment with $\lambda_{\max} = 479$ nm ($n = 3$ scans). Bottom, adult eyes contain a red-shifted rhodopsin-like pigment with $\lambda_{\max} = 489$ nm ($n = 18$ scans).

rhodopsin-like photopigment in adult eyes preserved by rapid freezing suggests that the rhabdoms of *B. thermydron* beyond the first juvenile crab stage may be resistant to the chemical fixation used for anatomical analysis. Rapid freezing of crabs collected remotely under low light conditions at infrequently visited vents may be required to determine the *in situ* state of adult and late-juvenile eyes.

Because related shallow-water brachyuran crabs do not undergo visual metamorphosis^{12,21}, the metamorphosis of the eyes of *B. thermydron* described here appears to be a specific adaptation for life at the vents. □

Methods

Animals

Specimens were collected live under artificial illumination from DSV *Alvin*. Megalopae, collected at the vents, were identified by morphological and genetic analysis as described elsewhere¹³. Zoea larvae were hatched in darkness on board ship from a freshly collected ovigerous *B. thermydron*. Larvae were transported back to land in darkness, sorted briefly under ambient lighting, and returned to darkness before use.

Microscopy

Upon *Alvin's* return to the surface (under natural light), whole juvenile crabs and excised adult eyes were submerged in fixative (5% paraformaldehyde w/v, 0.8% glutaraldehyde v/v, 4.5% sucrose w/v, 3% NaCl w/v in 0.1 M phosphate buffer; pH 7.2) under ambient illumination aboard RV *Atlantis*. Larvae were fixed by immersion under ambient lighting in Lancaster, Pennsylvania, 17 d after hatching. Adults and juveniles were stored in fixative at 4 °C in darkness until they were processed for microscopy on land by using standard methods¹⁰. Sample sizes: zoea ($n = 7$ eyes from 7 animals); juvenile crab stage 1 ($n = 6$ eyes from 3 animals); juvenile crab stage 3 ($n = 4$ eyes from 2 animals); adult ($n = 4$ eyes from 2 animals).

Microspectrophotometry

Animals were kept in darkness for several days before being used. Adult eyes were dissected out, quick-frozen using cryogenic spray, and sectioned at 14 μ m. Larvae and juveniles were frozen whole, but otherwise treated like the adults. Sections were collected on coverslips, mounted in marine crustacean Ringer solution containing 2.5% glutaraldehyde v/v (to enhance photobleaching), and placed in the microspectrophotometer. Photoreceptors were selected for scanning in dim, red light, and scanned using a beam (1.5–5 μ m) placed in each rhabdom. Rhabdoms were scanned twice: first when fully dark-adapted, and subsequently after being photobleached for several minutes with bright, white light. The difference between the scans was taken to be the spectrum of the visual pigment. Between 3 and 32 individual rhabdoms (depending on the developmental stage under study and the quality of the material) were scanned, bleached, and averaged for spectral characterization. Averaged scans were fitted mathematically with Stavenga templates²², using a least-squares procedure, to determine their characteristic wavelengths of maximum absorption.

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Neurons in medial prefrontal cortex signal memory for fear extinction

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Conditioned fear responses to a tone previously paired with a shock diminish if the tone is repeatedly presented without the shock, a process known as extinction. Since Pavlov¹ it has been hypothesized that extinction does not erase conditioning, but forms a new memory. Destruction of the ventral medial prefrontal cortex, which consists of infralimbic and prelimbic cortices, blocks recall of fear extinction^{2,3}, indicating that medial prefrontal cortex might store long-term extinction memory.

Here we show that infralimbic neurons recorded during fear conditioning and extinction fire to the tone only when rats are recalling extinction on the following day. Rats that froze the least showed the greatest increase in infralimbic tone responses. We also show that conditioned tones paired with brief electrical stimulation of infralimbic cortex elicit low freezing in rats that had not been extinguished. Thus, stimulation resembling extinction-induced infralimbic tone responses is able to simulate extinction memory. We suggest that consolidation of extinction learning potentiates infralimbic activity, which inhibits fear during subsequent encounters with fear stimuli.

Rats were given auditory fear conditioning in a 2-day experiment^{2,4}. The conditioned stimulus was a 30-s tone, and the unconditioned stimulus was a 0.5-s foot-shock that terminated simultaneously with the tone. On day 1, rats received five tones (habituation phase) followed immediately by five tones paired with foot-shock (conditioning phase). After 1 h, rats received 20 tones without foot-shock (extinction phase). On day 2, an additional 15 tones were given to test for recall of extinction learning. Freezing to the tone increased to 75% during the conditioning phase, and decreased to 10% by the end of the extinction phase (Fig. 1b). Twenty-four hours later, rats showed 35% freezing, which reflects good recall of extinction learning compared with the 80% freezing in animals that were not extinguished (data shown in Fig. 4b).

A total of 74 neurons were recorded from the medial prefrontal cortex (mPFC) across the 2 days. Cells were located in the infralimbic cortex (IL, $n = 31$, see Fig. 1a), prelimbic cortex (PL, $n = 25$) or medial orbital cortex (MO, $n = 18$). The initial spontaneous firing rate of all cells was 4.6, 5.5 and 4.2 Hz for IL, PL and MO, respectively. Spontaneous rates did not change significantly across the different phases of the experiment ($P > 0.3$, one-way analyses of

variance (ANOVAs) with repeated measures). We therefore focused on the tone-elicited activity of neurons. Figure 1c shows an example of a tone-responsive neuron in IL. No tone-elicited activity was observed during habituation, and cells remained unresponsive to tones during the conditioning phase. This agrees with previous studies showing that lesions of mPFC do not prevent acquisition of fear conditioning^{2,3,5}. Thus, unlike more dorsal parts of mPFC⁶, IL does not signal the acquisition phase of fear conditioning.

IL cells continued to be unresponsive to tones during extinction training on day 1. By day 2, however, robust tone-elicited activity in IL was visible from the start of the extinction phase. The increase in the neuronal tone response from day 1 to day 2 was inversely correlated with the spontaneous recovery of freezing on day 2 ($r = -0.73$, $P < 0.01$; Fig. 2a). Rats with the largest increase in IL tone responses showed the least freezing. To investigate further the relationship between IL activity and freezing, we divided the rats into two groups: those showing less than 50% spontaneous recovery of freezing and those showing more than 50% spontaneous recovery. As shown in Fig. 2c, IL activity was significantly increased 100–400 ms after tone onset in low-recovery rats but not in high-recovery rats. IL tone responses on day 2 are unlikely to reflect recall of conditioning because they were lowest in animals that showed the highest freezing. A more parsimonious explanation is that IL tone responses reflect extinction memory. Extinction-induced tone responses were not observed in nearby PL or MO (see Fig. 2c), indicating a high degree of anatomical specificity in the ability of mPFC to signal extinction memory.

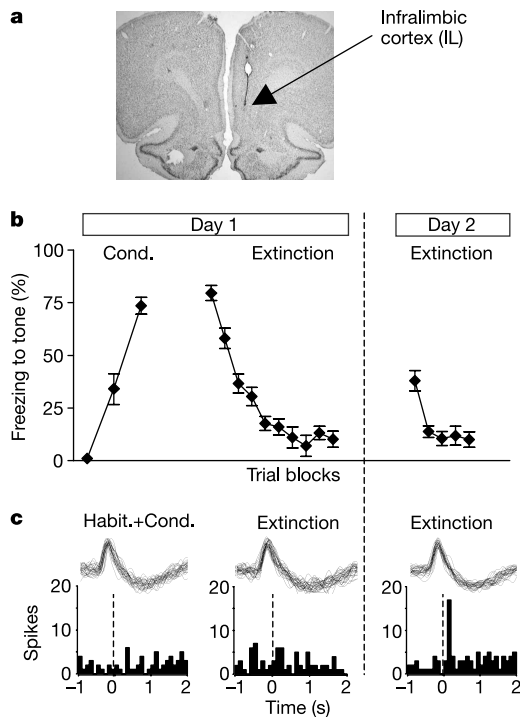


Figure 1 Tone response of a representative neuron in medial prefrontal cortex, (infralimbic area, IL) during acquisition and extinction of conditioned fear. **a**, Unit-recording electrode in IL. **b**, Freezing to the tone shown in blocks of two trials for 24 rats. Freezing was low on day 2, indicating good recall of extinction learning. **c**, Waveforms and post-stimulus time histograms (PSTHs) showing the tone-elicited activity of a representative IL neuron in each phase of the experiment (10 trials each, bin = 100 ms). Dashed line indicates tone onset. IL neurons only signalled the tone 24 h after extinction training. Cond, conditioning; habit., habituation.

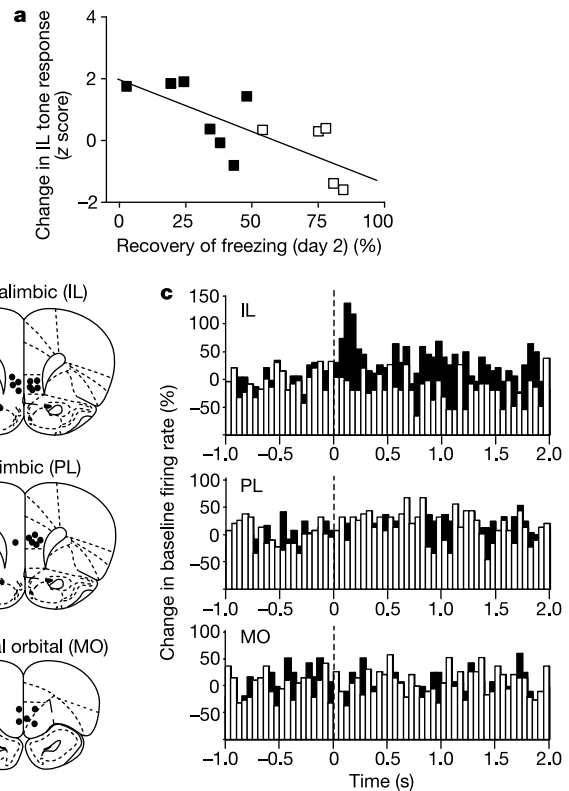


Figure 2 IL tone responses are correlated with spontaneous recovery of freezing after extinction. **a**, Scatter plot showing the change in IL tone response across days versus the percentage recovery of freezing on day 2. Firing rate 0–400 ms after tone onset was compared to pre-tone baseline rate with z-score. Each point represents the averaged response of all recorded neurons in each rat. Filled squares, low-recovery group (<50% 7 rats, 19 cells); open squares, high-recovery group (>50% 5 rats, 12 cells). $r = -0.73$; $P < 0.01$. **b**, Recording sites in IL, PL and MO. **c**, Group PSTHs showing tone responses of neurons from high-recovery (IL, 12 cells; PL, 12 cells; MO, 9 cells) and low-recovery (IL, 19 cells; PL, 13 cells; MO, 9 cells) groups on day 2. The bin size was 50 ms.

Figure 3 shows the average tone response of all IL neurons in each phase of the experiment. The difference in tone-elicited IL activity between high-recovery and low-recovery groups was significant on day 2 (Student's *t*-test, $t = 3.88$, degrees of freedom (d.f.) = 29, $P < 0.01$, Bonferroni corrected). On day 1 there was no relationship between IL activity and freezing. Cells in both groups were unresponsive to the tones despite large increases and decreases in freezing. This pattern of IL activity bears a striking resemblance to the effect of mPFC lesions in this task² (but see ref. 7). Lesions of the ventral mPFC with more than 70% destruction of IL had no effect on conditioning or extinction on day 1. However, 24 hours later, lesioned rats acted as though they had never received extinction training, spontaneously recovering high levels of freezing². Collectively, these data point towards a post-training consolidation process for extinction in IL. If this process fails to occur, either naturally or because of a mPFC lesion, extinction is not recalled.

The correlation that we observed between IL activity and freezing on day 2 indicates that IL tone responses are responsible for the suppression of freezing after extinction. However, correlations do not imply causality. Enhanced IL tone responses could be an epiphenomenon or a purely cognitive process that is not reflected in behaviour⁸. To determine whether tone-elicited activity in IL actually decreases freezing, we electrically stimulated IL during tones on day 2 in rats that were conditioned but not extinguished on day 1, to simulate a post-extinction state. Tones were paired with low-intensity IL stimulation delivered 100–400 ms after tone onset to model IL tone responses (see Fig. 4a). Control groups received either IL stimulation unpaired with tones, or no stimulation.

As shown in Fig. 4b, rats that received tones paired with IL stimulation showed markedly less freezing than controls on day 2. This effect was evident from the very first trial. Freezing levels in trial 1 were 82%, 90% and 60% in unstimulated, unpaired-stimulated and paired-stimulated rats, respectively. One-way ANOVA revealed a significant main effect of group ($F_{(2,29)} = 7.09$, $P < 0.01$), and post-hoc analysis showed that the stimulated-paired group was significantly lower than both controls ($P < 0.05$). The significant decrease in freezing in trial 1 is consistent with the simulation of extinction memory by IL stimulation.

IL stimulation also accelerated extinction learning. Across all four

trial blocks, average freezing levels were 70%, 73% and 28%, in unstimulated, unpaired-stimulated and paired-stimulated rats, respectively. ANOVA showed a highly significant main effect of group ($F_{(2,29)} = 16.38$, $P < 0.001$) and trial block ($F_{(4,116)} = 25.62$, $P < 0.001$), as well as a significant interaction ($F_{(8,116)} = 3.06$, $P < 0.01$). Freezing in the paired-stimulated group was significantly lower than controls in all blocks ($P < 0.01$). In contrast to IL, paired stimulation of PL ($n = 10$) had no effect (PL, 76%; unstimulated, 70%; $t = 0.71$, d.f. = 17, $P > 0.40$). Thus, similar to our unit-recording findings, the effect of stimulation was specific to IL. On day 3, IL-stimulated rats continued to show low freezing to the tone in the absence of stimulation (post-hoc test: $P < 0.05$; see Fig. 4b), providing further evidence of facilitated extinction learning. Enhanced extinction learning could be mediated directly by stimulation (for example, inducing extinction-related plasticity in IL or downstream structures) or indirectly through decreased freezing. A decrease in freezing in early trials might accelerate extinction by removing the behavioural feedback that maintains freezing on subsequent trials. Either way, this raises the interesting possibility that increased IL activity during recall might serve to strengthen extinction memory.

IL stimulation did not decrease freezing through a non-specific increase in locomotion. Rats stimulated during the inter-tone interval (unpaired group) showed no decrease in freezing during subsequent tones, or during the 30-s period immediately after stimulation (post-stimulation, 33% freezing; pre-stimulation 31%; $t = 0.15$, d.f. = 22, $P > 0.80$). Another possible explanation for the decreased freezing during the tone is that IL stimulation was rewarding⁹. This is unlikely because rewarding stimulation would also be expected to decrease freezing during the inter-tone interval. To address this issue further, we trained a separate group of rats ($n = 5$) to press a bar for food and then replaced food reward with press-elicited stimulation of IL, using the same stimulus parameters as above. For food, rats pressed at a rate of $20 \pm 2.0 \text{ min}^{-1}$ (mean \pm s.e.). Switching to IL stimulation caused the press rate to drop to $10 \pm 1.5 \text{ min}^{-1}$ after 1 day and $0.3 \pm 0.06 \text{ min}^{-1}$ after 7 days. Thus the stimulation used in our experiment did not sustain self-stimulation behaviour, arguing against a reward mechanism of action.

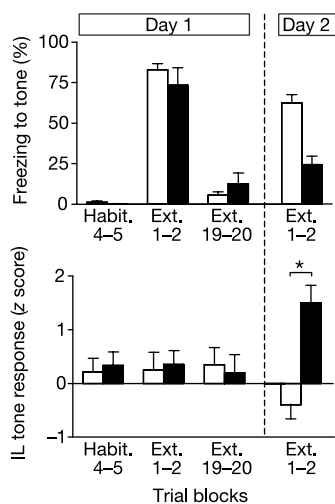


Figure 3 Average freezing (top) and IL tone response (bottom) in each phase of training. Numbers indicate trials. Despite increases and decreases in freezing during conditioning and extinction (ext.) on day 1, IL cells showed no tone responses. On day 2, however, neurons in low-recovery rats (filled squares) showed significantly larger tone responses than in high-recovery rats (open squares), indicating that IL neurons selectively signal recall of fear extinction. The asterisk indicates a statistically significant ($P < 0.01$) difference. Habit., habituation.

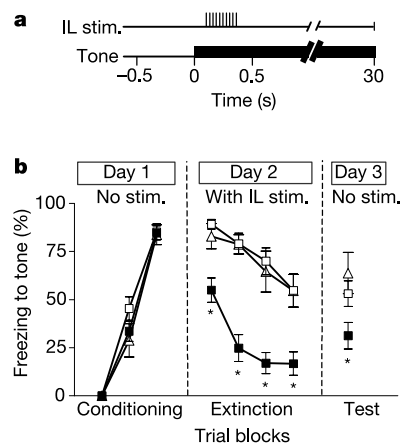


Figure 4 Brief IL stimulation (stim.) paired with tones simulates extinction memory. **a**, A train of pulses was delivered to IL 100–400 ms after tone onset on day 2 in non-extinguished rats, to model IL tone responses observed after extinction (see Fig. 2c). **b**, Percentage freezing to the tone for unstimulated ($n = 9$; open triangles), unpaired IL stimulated ($n = 11$; open squares) and paired IL stimulated ($n = 12$; filled squares) rats. The paired-stimulated group showed significantly lower freezing than controls at all points on day 2 ($P < 0.001$, asterisk). Paired-stimulated rats continued to show low freezing on day 3 ($P < 0.05$, asterisk) in the absence of stimulation. Data are shown in blocks of 2-trials.

Our observation that IL neurons are responsive to tones after extinction, but not during extinction itself, indicates that IL-dependent mechanisms are responsible for long-term, but not short-term, extinction memory. Separate extinction mechanisms are also indicated by pharmacological evidence: short-term memory for fear extinction seems to be independent of *N*-methyl-D-aspartate (NMDA) receptors^{4,10}, whereas long-term memory for extinction requires NMDA receptors^{4,11}. We suggest that post-training consolidation of extinction involves the potentiation of tone inputs to IL, perhaps by means of NMDA-dependent plasticity. The basolateral amygdala complex (BLA), which is crucial for the learning and expression of auditory fear conditioning^{12–14}, sends direct excitatory projections to IL^{15–17}. Microinjection of NMDA antagonists¹¹ or protein kinase inhibitors¹⁸ into BLA blocks the extinction of fear. Inputs to IL from BLA are therefore a likely candidate for potentiation during consolidation of extinction.

Once potentiated, how does IL influence freezing? The central nucleus of the amygdala modulates the expression of conditioned fear responses by means of projections to midbrain and hypothalamic sites that mediate fear responses¹⁹. IL sends robust projections to the capsular division of the central nucleus, which contains amygdaloid intercalated cells²⁰. Intercalated cells strongly inhibit output from the central nucleus²¹. When sufficiently depolarized, intercalated cells show sustained firing lasting tens of seconds in response to a brief suprathreshold stimulus²². This could account for the prolonged decrease in freezing that we observed with brief stimulation of IL. Thus, extinction-induced activation of IL neurons might decrease freezing by dampening the output of the amygdala. In support of this, prolonged stimulation of IL prevents increases in blood pressure and defensive behaviours elicited by amygdala stimulation²³.

Our data provide physiological evidence for the long-standing notion that extinction forms a new memory that inhibits the conditioned response^{1,24}. Failure to achieve an adequate level of potentiation in medial prefrontal cortex after extinction might lead to exaggerated fear responses²⁵. In support of this, patients with post-traumatic stress disorder exhibit depressed ventral mPFC activity correlated with increased autonomic arousal, when re-exposed to traumatic reminders^{26–28}. Pairing reminder stimuli with activation of ventral mPFC induced by repetitive transcranial magnetic stimulation²⁹ might serve to strengthen extinction of fear in a clinical setting. □

Methods

Subjects

Male Sprague–Dawley rats weighing 300–350 g were maintained on a restricted diet until they reached 85% of their original body weight. They were trained to press a bar for food on a variable-interval schedule of reinforcement (VI-90 s) to maintain a constant level of activity against which freezing responses could be reliably measured²⁴. Bar-pressing occurred in a standard operant chamber with dimensions 25 cm × 29 cm × 28 cm (Coulbourn Instruments).

Surgery and histology

The surgical procedures performed were similar to those previously described¹². Rats were anesthetized with Nembutal (50 mg kg⁻¹ intraperitoneally) after pretreatment with atropine (0.24 mg kg⁻¹ intraperitoneally). An eight-channel movable microelectrode was implanted in the ventral mPFC, with coordinates 2.9 mm anterior, 0.5 mm lateral and 3.9 mm ventral to bregma. Wires were arranged either individually or twisted in pairs to form four stereotrodes with a tip impedance of 1–3 MΩ. Rats were allowed to recover for 1 week before recording. At the conclusion of the experiment, recording sites were marked with electrolytic lesions before perfusion, and electrode locations were reconstructed with standard histological techniques.

Fear conditioning

Fear conditioning took place in the same operant chamber as bar-pressing. The chamber was located in a sound-attenuating box, and food reward was available continuously on a VI-90 s schedule throughout the experiment. The conditioned stimulus was a tone (4 kHz, 80 dB sound pressure level, 30 s), and the unconditioned stimulus was a scrambled foot-shock (0.5 mA, 0.5 s) that terminated simultaneously with the tone. The inter-trial interval varied from 2 to 6 minutes (average 4 min) throughout. The behaviour of rats was recorded with a video camera for offline scoring of freezing.

Single-unit recording

Electrodes were connected to a headstage (SUNY-HSC Biomedical Engineering Services) containing eight unity-gain operational amplifiers. The headstage was connected to an eight-channel, computer-controlled, preamplifier (Lynx-8) via a rotating commutator (Crist Instruments). The electrode was advanced in 40-μm steps until well-isolated cells were obtained in at least two or three wires, at which time fear conditioning began. Signals exceeding a voltage threshold were digitized at 32 kHz and stored on a PC (DataWave Technologies). The occurrences of tone and shock onset were flagged in the recording files. Waveforms were sorted off-line by using waveform characteristics such as peak amplitude, valley amplitude and spike width, and a clustering algorithm (Autocut; DataWave Technologies). Individual cells were tracked from day 1 to day 2 by applying cluster boundaries from day 1 and checking manually for goodness of fit. Waveforms were considered constant across the two days when they fitted the boundaries from day 1 and showed similar waveform shapes. Of 100 cells recorded, 26 did not fit these criteria and were rejected.

Brain stimulation

Rats were surgically implanted with concentric bipolar stimulating electrodes (Rhodes Medical Instruments) 0.2 mm in diameter with an exposed tip length of 0.25 mm. Stimulating electrodes were implanted into the right IL (2.7 mm anterior, 1.0 mm lateral, and 4.9 mm ventral to bregma, angled 6° toward the midline). Rats were implanted unilaterally rather than bilaterally to minimize track-related damage to the dorsal mPFC, which has been shown to increase conditioned freezing³⁰. On day 1, rats were conditioned as described above, but not extinguished. On day 2, rats received eight extinction tones paired with IL stimulation. A pulse generator with constant current output (Grass Instruments) delivered a 300-ms train of square pulses (0.2 ms pulse width, 100 μA, 100 Hz). Trains were delivered one per tone, in either paired (100–400 ms after tone onset) or unpaired (middle of inter-trial interval) fashion. An unstimulated control group was implanted with stimulating electrodes but was never stimulated. A separate group of rats implanted with stimulating electrodes in IL were trained to press for food on a variable-interval schedule of reinforcement (VI-60 s). Food reward was then replaced with stimulation of IL on a VI-15 s schedule. Rats were allowed to press for IL stimulation during daily 30-min sessions for 7 days, following a previously used protocol for studying intracranial self-stimulation in mPFC⁹.

Data analysis

Spontaneous recovery of freezing on day 2 was measured as the freezing at the start of day 2 (extinction trials 1 and 2) divided by the peak acquired freezing on day 1 (extinction trials 1 and 2). Low spontaneous recovery indicates good recall of extinction learning from the previous day. To measure tone-induced activity of neurons, the firing rate in the first 400 ms after tone onset was compared with the firing rate of 20 pre-tone bins of equal duration using a *z*-score transformation. The change in tone-induced activity of neurons across days was determined by subtracting the *z*-score at the end of the extinction phase on day 1 (trials 19 and 20) from the *z*-score at the beginning of the extinction phase on day 2 (trials 1 and 2). Group post-stimulus time histograms were generated by summing the spike count of all cells per bin and normalizing to the average pre-tone firing rate. ANOVA or Student's *t*-tests were used to compare neural tone responses (*z*-scores), firing rates and spontaneous recovery of freezing. Post-hoc comparisons were made with Tukey's HSD ('Honestly Significantly Different') method.

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p75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp

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In inhibiting neurite outgrowth, several myelin components, including the extracellular domain of Nogo-A (Nogo-66)¹, oligodendrocyte myelin glycoprotein (OMgp)² and myelin-associated glycoprotein (MAG)^{3,4}, exert their effects through the same Nogo receptor (NgR). The glycosyl phosphatidylinositol (GPI)-anchored nature of NgR indicates the requirement for additional transmembrane protein(s) to transduce the inhibitory signals into the interior of responding neurons. Here, we demonstrate that p75, a transmembrane protein known to be a receptor for the neurotrophin family of growth factors^{5,6}, specifically interacts with NgR. p75 is required for NgR-mediated signalling, as neurons from p75 knockout mice are no longer responsive to

myelin and to each of the known NgR ligands. Blocking the p75–NgR interaction also reduces the activities of these inhibitors. Moreover, a truncated p75 protein lacking the intracellular domain, when overexpressed in primary neurons, attenuates the same set of inhibitory activities, suggesting that p75 is a signal transducer of the NgR–p75 receptor complex. Thus, interfering with p75 and its downstream signalling pathways may allow lesioned axons to overcome most of the inhibitory activities associated with central nervous system myelin.

A recent study suggested that p75 does not interact directly with MAG, but is required for its activity in inhibiting neurite outgrowth⁷. As NgR is a functional receptor of myelin-associated inhibitors including MAG^{1–4}, we examined the possibility that p75 and NgR formed a receptor complex in mediating these inhibitory activities. We first overexpressed haemagglutinin (HA)-tagged rat full-length p75 in both Chinese hamster ovary (CHO) cells (data not shown) and CHO cells stably expressing Flag-tagged human NgR, and found that p75 could be immunoprecipitated together with NgR, but not with a control transmembrane protein plexin A3 (ref. 8; Fig. 1a). Similarly, endogenous p75 and NgR proteins in rat postnatal cerebellar granule neurons (CGNs) could be immunoprecipitated together (Fig. 1b). Treatment of both the NgR-express-

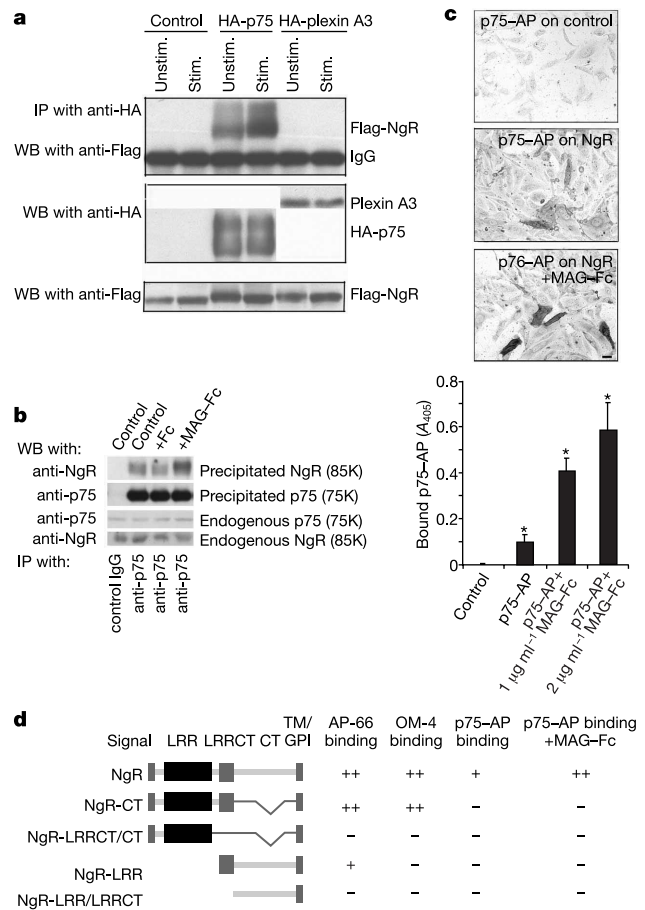


Figure 1 p75 and NgR form receptor complexes. **a**, Co-immunoprecipitation of p75–NgR from Flag-tagged, NgR-expressing cells transfected with vector, HA-p75 or HA-plexin A3, and mock-treated (Unstim.) or treated with MAG–Fc (Stim.). **b**, Co-immunoprecipitation of p75–NgR from CGNs treated with or without Fc or MAG–Fc. **c**, Visualization and quantification of p75–AP binding to NgR-expressing cells. Asterisk, $P < 0.05$ by Student's t -test comparing bound p75–AP under various conditions with control. Scale bar, 10 μm. **d**, Summary diagram of AP fusion proteins binding to cells expressing different truncations of NgR. Signal, signal peptide; TM/GPI, transmembrane domain/GPI anchor.