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Impaired odour discrimination on desynchronization of odour-encoding neural assemblies

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Stimulus-evoked oscillatory synchronization of neural assemblies has been described in the olfactory[1–5] and visual[6–8] systems of several vertebrates and invertebrates. In locusts, information about odour identity is contained in the timing of action potentials in an oscillatory population response[9–11], suggesting that oscillations may reflect a common reference for messages encoded in time. Although the stimulus-evoked oscillatory phenomenon is reliable, its roles in sensation, perception, memory formation and pattern recognition remain to be demonstrated—a task requiring a behavioural paradigm. Using honeybees, we now demonstrate that odour encoding involves, as it does in locusts, the oscillatory synchronization of assemblies of projection neurons and that this synchronization is also selectively abolished by picrotoxin, an antagonist of the $GABA_A$ (γ-aminobutyric acid) receptor. By using a behavioural learning paradigm, we show that picrotoxin-induced desynchronization impairs the discrimination of molecularly similar odorants, but not that of dissimilar odorants. It appears, therefore, that oscillatory synchronization of neuronal assemblies is functionally relevant, and essential for fine sensory discrimination. This suggests that oscillatory synchronization and the kind of temporal encoding it affords provide an additional dimension by which the brain could segment spatially overlapping stimulus representations.

Investigation of olfactory processing in the locust antennal lobe—a functional and morphological analogue of the vertebrate olfactory bulb—has indicated that both monomolecular and complex odours are represented there combinatorially by dynamical assemblies of projection neurons[5,9–11]. Each neuron in an odour-coding assembly responds with an odour-specific temporal firing pattern consisting of periods of activity and silence[5,9]. Any two neurons responding to the same odour are usually co-active only during a fraction of the population response. The spikes of co-activated neurons are generally synchronized[5,9,10] by the distributed action of GABA-ergic local neurons[12], resulting in large-amplitude, 20–35 Hz local field potential (LFP) oscillations in their target area, the calyx of the mushroom body[5]. Each successive cycle of the odour-evoked oscillatory LFP can therefore be characterized by a co-active subset of projection neurons, and an odour is thus represented by a specific succession of synchronized assemblies[10,11]. This representation thus comprises three main features—the identity of the odour-activated neurons, the temporal evolution of the ensemble, and oscillatory synchronization—whose importance to the animal for learning and recognition needs to be examined.

We have previously shown that picrotoxin (PCT) applied to the locust antennal lobe selectively blocks the fast inhibitory synapse between local and projection neurons and abolishes their oscillatory synchronization: this manipulation altered neither the response profiles of projection neurons to odours, nor their odour specificity[12]. We have now made use of this pharmacological tool to assess whether oscillatory synchronization plays a role in odour learning and discrimination, an experiment that requires a behavioural assay. We therefore used honeybees, which can be trained to extend their mouth parts (proboscis) in response to specific odours after a few associative forward pairings of these odours with a sucrose reinforcement (proboscis-extension (PE) conditioning)[13–15]. First, we demonstrated that odour representation in the honeybee includes the same three features as those discovered in the locust; second, we tested the importance of oscillatory synchronization for odour learning and discrimination.

Odours, but not air alone, puffed onto an antenna of a honeybee evoked bouts of ~30 Hz LFP oscillations in the calyx of the ipsilateral mushroom body (for example, mint; Fig. 1a). These oscillations lasted for ~0.5–1 s in response to a 1-s long odour puff. Sliding-window autocorrelations of these LFPs revealed the sustained periodic structure of the odour-evoked responses (Fig. 1a). Simultaneous intracellular recordings from antennal lobe neurons showed that, as in locusts[5], individual antennal lobe neurons responded selectively to certain odours with prominent membrane-potential oscillations (Figs 1b, 3a; $n = 21$ neurons in 16 animals) which are locked to the mushroom body LFP (Fig. 1c, d). Mushroom body LFP oscillations lagged behind those in antennal lobe neurons (phase, $-53° \pm 5$; mean ± s.e.m.; $n = 290$ cycles, where 0° is defined as the peak of the LFP; Fig. 1c). This is consistent with our findings in locusts, in which LFP oscillations in the mushroom body result, at least in part, from the coherent input

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of synchronized and convergent antennal lobe projection neurons[5,12]. This was directly confirmed with paired intracellular recordings from antennal lobe neurons, showing synchronized membrane-potential oscillations in response to specific odours (Fig. 1e; $n = 2$ animals).

In locusts, the oscillatory synchronization of projection neurons—and thus the resulting odour-evoked mushroom body LFP oscillation—depends on inhibitory feedback from GABA-ergic local neurons[12]. This feedback is selectively abolished either by injection into the antennal lobe or by brain superfusion of 100 μM picrotoxin[12]. We therefore applied picrotoxin to the brains of bees and assessed its effect on odour-evoked projection neuron synchronization by assaying mushroom body LFPs (Fig. 2a). Power spectra calculated over three periods (each 1 s long, before, during and after the odour puff) of the LFP waveform showed a typical peak centred on 30 Hz only for the odour period (Fig. 2b, left). Eight minutes after application of picrotoxin, however, all odour-evoked power around 30 Hz had been abolished (Fig. 2b, right). This can also be seen from the raw LFP data (Fig. 2a) and from sliding-window autocorrelations of the LFP (Fig. 2c).

The absence of significance power at the stimulus-evoked oscillation frequency might be caused by a silencing, rather than desynchronization, of the antennal lobe projection neurons. We therefore repeated the picrotoxin experiments with intracellular recordings from antennal lobe projection and local neurons (Fig. 3). As in locusts, many antennal lobe neurons responded to odours with stimulus-specific slow temporal patterns superimposed on 30-Hz oscillations (Fig. 3A; $n = 8$); we have not yet examined the information content of individual spike times, as we did for locust projection neurons[10]. Picrotoxin changed neither the odour selectivity of these neurons, nor the slow temporal features of their responses ($n = 12$). The neurons shown in Fig. 3B, C, for example, retained their response patterns to odours 10 min after picrotoxin application, which did not significantly alter their average firing rate. We conclude that picrotoxin desynchronizes antennal lobe

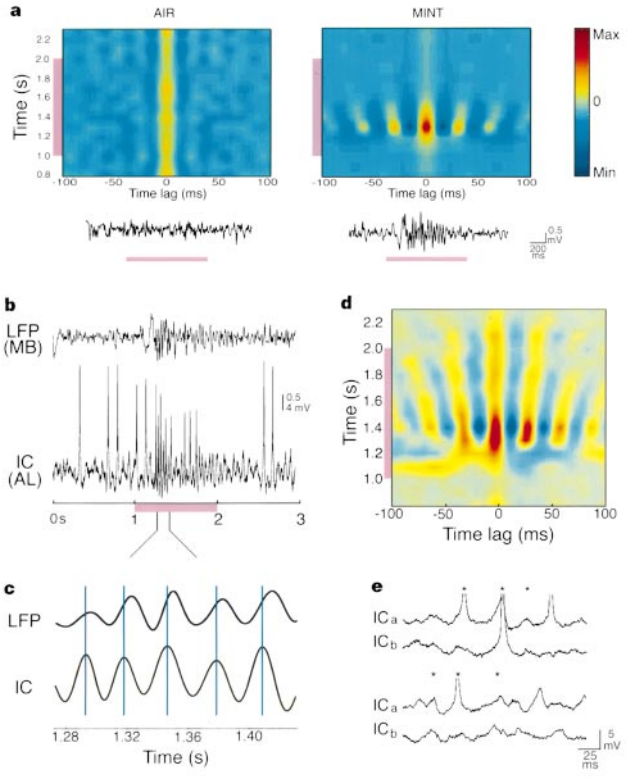

Figure 1 Odours elicit 30-Hz synchronous oscillatory activity in the local field potential (LFP) recorded in the calyx of the mushroom bodies and in antennal lobe neurons. **a**, Mint, but not air, elicits regular oscillations in the LFP (bottom traces), evident also in the repeating banding patterns in sliding-window autocorrelograms[5] (means of 6 consecutive odour presentations presented at 10-s intervals). Air and mint autocorrelograms are represented on the same scale, indicated by the colour-coded bar. Pink bars indicate odour presentations. **b**–**e**, Synchronous odour-elicited oscillations were observed in the mushroom body (MB) LFP and in intracellularly (IC) recorded neurons in the ipsilateral antennal lobe (AL). **b**, Mint presentation elicits oscillations in both the neuron and the LFP. The oscillatory LFP indicates rhythmic and synchronized firing of many antennal lobe neurons during the odour response. **c**, Detail of the example in **b** (both records band-pass-filtered at 5–55 Hz; vertical blue lines indicate peaks in intracellular membrane-potential waveform), showing phase locking of the two waveforms with a consistent phase lag. **d**, Sliding-window cross-correlogram between AL neuron membrane potential and mushroom body LFP (average of 6 consecutive odour presentations at 0.1 Hz) reveals consistency, regularity, synchrony and phase locking of oscillations in the two recordings. **e**, Paired intracellular recordings from AL neurons (ICa, ICb) responsive to apple odour, showing transient oscillatory synchronization of their membrane-potential waveforms (asterisks). Spikes are truncated.

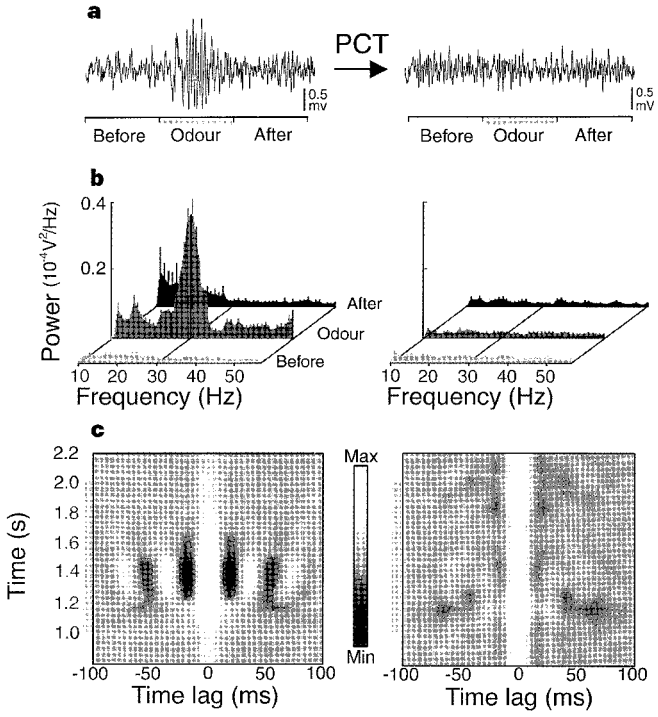

Figure 2 Picrotoxin abolishes the odour-elicited 30-Hz oscillations recorded in the LFP. **a**, Odour (mint) presentation elicits regular, large-amplitude oscillations before (left) but not 8 min after (right) superfusion with PCT. **b**, Power spectra (average of 10 trials at 0.1 Hz; vertical lines indicate s.e.m.) for 1-s periods before, during and after odour presentation, before (left) and 8 min after (right) PCT superfusion. The odorant uniquely and consistently elicits strong 30-Hz LFP oscillations that are abolished by PCT. **c**, Sliding-window autocorrelograms of odour-response periods (mean of 10 consecutive trials at 0.1 Hz) reveal the oscillatory odour response (left) and its suppression after PCT superfusion (right).

projection neurons in response to odours without otherwise altering their individual response patterns, even when these patterns include periods of reduced firing, as observed in locusts[12]. Our results establish picrotoxin as a selective pharmacological tool for testing the role of oscillatory synchronization in odour learning and recognition.

We used a proboscis-extension conditioning assay to test whether picrotoxin could disrupt olfactory discrimination[14,16–18]. When forager honeybees experience forward pairing of an odour (conditioned stimulus) with sucrose reinforcement, their PE response to that odour increases dramatically for 48 h or longer. This increase is due to associative learning mechanisms[17]. Typically, the conditioned response generalizes to some extent to odours that are structurally similar to the conditioned odorant[16]; for example, once conditioned to an aliphatic alcohol (such as 1-hexanol), bees show a heightened response to structurally similar alcohols (such as 1-octanol). This generalization response is never as strong as the response to the conditioned stimulus itself, but it is higher than the generalization response to structurally dissimilar odorants (such as terpenes).

If oscillatory synchronization plays a role in odour learning or discrimination, application of picrotoxin to the antennal lobe should diminish the ability of animals to discriminate between odours; hence, these animals should show stronger generalization to odours not experienced during conditioning. Bees were divided into a control group (saline-treated, $n = 36$) and a test group (PCT-treated, $n = 33$) (see Methods). They were individually treated with saline or picrotoxin and then conditioned with C, an aliphatic alcohol, after a recovery interval $t_1 = 10$ min (Fig. 4a). Training and testing were conducted blind. Bees in both PCT- and saline-treated groups learned the conditioned-stimulus sucrose pairing equally well, showing a maximum response by conditioning trial 5 or 6 (Fig. 4b). After a retention interval $t_2 = 90$ min, the two groups were tested with the conditioned stimulus (C), a similar odour (S, an aliphatic alcohol of different chain length) and a dissimilar odour (D, the terpene geraniol). The percentage of animals in each group that responded with a proboscis extension to C, S or D was then measured. Saline-treated bees responded significantly more often to C than they did to S ($P < 0.05$), indicating that they could discriminate between the two related odours (Fig. 4c). PCT-treated bees, by contrast, failed to differentiate C from S (NS; see Fig. 4c). When tested with odour D, animals from both groups performed equally well (that is, each group had an equally low probability of proboscis extension in response to D relative to C; $P < 0.01$; Fig. 4c), indicating that the picrotoxin-injected bees did not have a non-specific learning, memory or performance deficit. Rather, picrotoxin selectively impaired discrimination between C and S. To

Table 1 Percentage of bees in saline- and PCT-injected groups showing PE response

	$t_1 = 45$ min ($n = 40$ per group)		$t_1 = 45$ min ($n = 40$ per group)		$t_1 = 60$ min ($n = 35$ per group)	
	Saline	PCT	Saline	PCT	Saline	PCT
Conditioned stimulus (C)	53	40	29	20	26	26
Similar odour (S)	23*	30 (NS)	9*	14 (NS)	11*	9*
Dissimilar odour (D)	ND	ND	6*	3*	0**	3**

The percentage is shown of animals in saline- and PCT-injected groups that gave a PE response during tests with C, S and D, $t_2 = 60$ min after conditioning. Saline or PCT were administered by antennal lobe injection t_1 min before conditioning (see Methods).
*, ** Significantly different from test with *C* at $P < 0.05$ or $P < 0.01$, respectively; one-tailed test criteria. ND, not determined; NS, not significant.

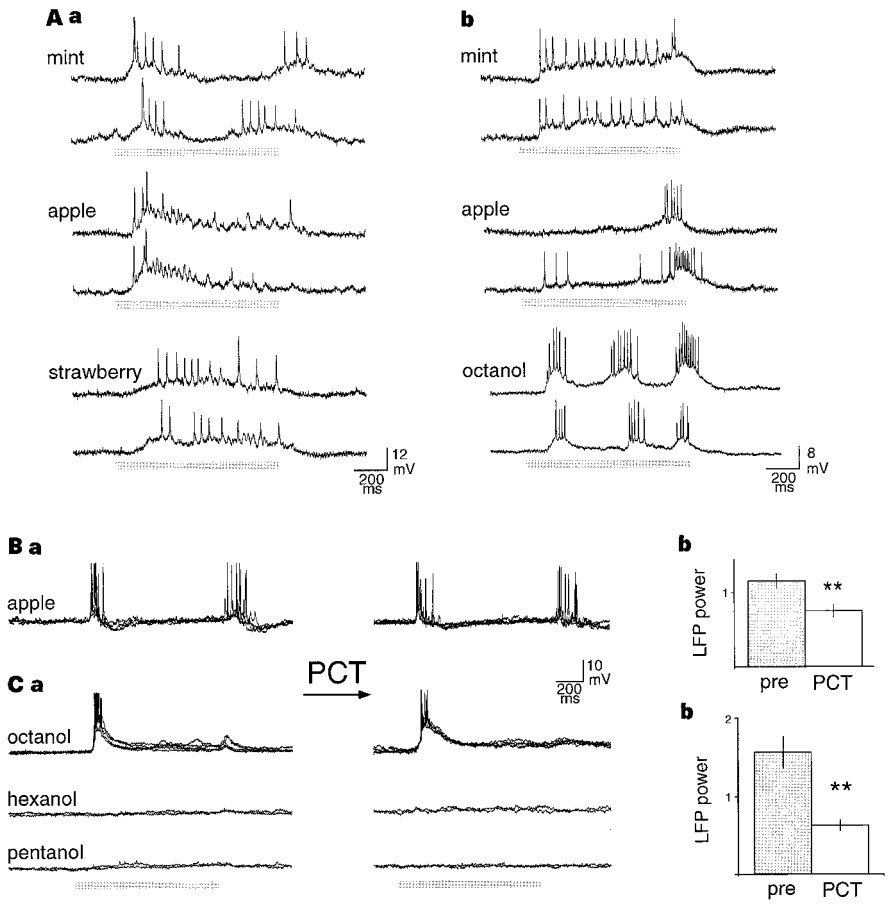

Figure 3 Many AL neurons display odour-specific temporal response patterns. Picrotoxin spares these slow temporal characteristics while abolishing 30-Hz oscillations, as revealed by simultaneous LFP and intracellular recordings. **A, a** and **b**, Examples of slow temporal response patterns evoked by different odours in two representative antennal lobe neurons (two trials shown for each odour). Subthreshold oscillatory activity is immediately evident in some neurons (**a**) but not in others (**b**). Note the pattern differences across odours and consistency across trials for each odour. **B**, Response pattern of a third neuron to apple odour (**a**, 4 superimposed traces) remains unaffected by PCT application, although LFP power at 32–37 Hz is greatly reduced (**b**, $t = 3.122$, $P < 0.005$). **C**, Response pattern and odour selectivity of a fourth neuron remains unaffected by PCT application (**a**), although LFP power at 31–36 Hz is greatly reduced (**b**; $t = 4.312$, $P < 0.0007$). There are five superimposed traces for octanol and two for hexanol and pentanol, respectively; LFP power is given as in $10^{-4}\,V^2\,Hz^{-1}$. Some spikes are truncated in **B**, **C**.

determine the time course of picrotoxin activity, we carried out experiments with three other groups ($n = 220$ animals), using different intervals t_1 and t_2 (Table 1). These experiments confirmed the results shown in Fig. 4 and indicated that the behavioural effects of picrotoxin wore off as time elapsed between drug injection and conditioning; for $t_1 = 60$ min and $t_2 = 60$ min, for example, C and S could again be discriminated ($P < 0.05$; Table 1). This recovery time course indicates that the effect of picrotoxin in the experiment shown in Fig. 4c was limited to the conditioning period, the time of specific odour-reinforcement association.

Because picrotoxin affected the behavioural discrimination of similar alcohols, this effect could simply be due to a picrotoxin-induced loss of odour specificity (rather than desynchronization) of the projection neurons representing these alcohols[19]. We therefore tested the 'tuning' of antennal lobe neurons to three alcohols (pentanol, hexanol and octanol) before and after picrotoxin application ($n = 9$ animals): in no case did picrotoxin alter the specificity of neuronal tuning, despite significantly reducing odour-induced LFP power around 30 Hz (Fig. 3c).

We have shown that odours evoke the oscillatory synchronization of groups of projection neurons in the honeybee antennal lobe, and that picrotoxin can, as in locusts, abolish oscillatory synchronization while sparing neural response and odour selectivity. Behavioural experiments combining picrotoxin-induced desynchronization of the projection neurons during proboscis-extension conditioning with odour discrimination tests showed that picrotoxin-treated animals failed to discriminate between similar odorants (1-hexanol and 1-octanol) although they could still discriminate between structurally dissimilar ones (that is, either alcohol from the terpene geraniol). These results indicate that neural synchronization—and thus the ability it affords to encode stimulus features in time[10,11]—plays a role in fine sensory discrimination tasks which require the separation of stimuli whose neural representations spatially overlap. Synchronization appears to be unimportant, however, for the discrimination of unrelated stimuli (ones less likely to have overlapping neural representations, as suggested by imaging experiments[20]). Because the effect of PCT was limited to the conditioning period, and because discrimination of dissimilar odours was possible 60–90 min after conditioning, we conclude that PCT did not impair odour learning *per se*. Rather, PCT (and thus desynchronization) appeared to impair the separation of the neural representations of two related odour stimuli, of which one was stored in memory in a form that lacked its natural periodicity and synchronization features. Synchronization of neuronal assemblies therefore appears to be important to help reduce the overlap between the neural representations of related stimuli, possibly by using the temporal aspects of their representations[10,11] as separable features. It is tempting to speculate that neural oscillatory synchronization might play a similarly important role for refined stimulus encoding and recognition in the other sensory systems and animals where oscillations occur[1,3,6,8,21,22]. □

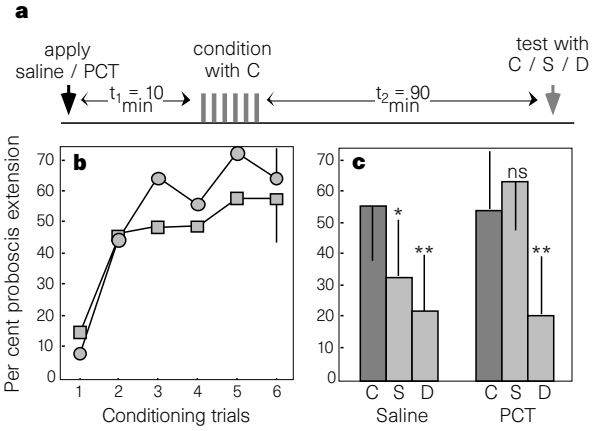

Figure 4 Application of picrotoxin, but not saline, to the antennal lobes impairs discrimination of structurally similar simple odours but not of dissimilar ones. **a**, Experimental protocol. **b**, Acquisition for groups treated with saline (circles; 36 animals) or PCT (squares; 33 animals) was equally rapid and reached asymptote by trial 5. The ordinate represents the percentage of animals that responded with a proboscis extension on each conditioning trial. Vertical lines in **b**, **c** represent 95% confidence intervals. To avoid clutter, we show only the limit (upper or lower) that is relevant for hypothesis testing. **c**, Discrimination of odour C (dark bars) from S differed in groups treated with saline and PCT (same animals as in **b**). Significantly more saline-treated bees responded to C than to S ($\chi^2 = 3.7$, $P < 0.05$; single asterisk). By contrast, PCT-treated bees gave statistically comparable responses to C and S ($\chi^2 = 0.6$; NS). The response levels to C in either group did not differ significantly from one another ($\chi^2 = 0.4$; NS). Discrimination of C from the dissimilar odour (D), however, was possible even in the PCT-treated group (saline group: $\chi^2 = 8.6$, $P < 0.01$, double asterisks; PCT group: $\chi^2 = 8.0$, $P < 0.01$).

Methods

Our results represent experiments conducted with 739 animals (*Apis mellifera*) (450 for physiology, 289 for behaviour).

Preparation for physiology. Foraging worker bees from a colony established by a multiply mated queen were collected as they returned to the hive and were immobilized in a moulded wax holder. The first antennal segment was held forward with a small drop of epoxy resin on its proximal joint. To stabilize the brain for intracellular recording, the oesophagus was gently retracted through a small window cut open between the antennae and the mouth. The oesophagus was held taut and the window was sealed with a drop of wax[23]. A second, larger window was then opened posterior to the antennae. Glandular and sheath material were gently removed as the brain was superfused with oxygen-saturated saline (in mM: 140 NaCl, 5 KCl, 5 $CaCl_2$, 4 $NaHCO_3$, 1 $MgCl_2$, 6.3 HEPES, pH 7.0).

Odour stimulation. Controlled puffs of odorant (1 s duration, 0.3 l min^{-1}) were delivered by 15 stainless steel nozzles placed 21 mm in front of the antenna. The circularly arrayed nozzles were angled inwards to converge on the antenna. Clean, dry 'background' air was delivered constantly (0.3 l min^{-1}). Each odour (3–10 μl apple, strawberry (Gilberties), cherry (Bell Flavors and Fragrances), spearmint (Flavco), eugenol, geraniol, 1-pentanol, 1-hexanol, 1-octanol (Sigma), cineole, isoamylacetate, citral (Aldrich)) was carried in a separate nozzle; odorants were then vented through an exhaust funnel or a fume hood.

Electrophysiology. Local field potentials were recorded using saline-filled blunt glass micropipettes (tip, ~10 μm) and were amplified with a d.c. amplifier (NPI, Adams-List). Recordings from antennal lobe neurons were made intracellularly using sharp glass micropipettes (120 MΩ) filled with 0.5 M potassium acetate, and were amplified with a separate d.c. amplifier (Axon). Micropipettes were stretched by a horizontal puller (Sutter). Data were stored on digital tape (Micro Data) and analysed off-line using National Instruments NBMI16L hardware and LabVIEW (National Instruments) and MatLab (The MathWorks) software. Non-phase shifting, band-pass filtering (5–55 Hz, 5-pole; Butterworth) was accomplished by using a software algorithm. Picrotoxin (PCT, Sigma; 100 μM in oxygen-saturated saline[12]) was superfused over the brain. Cross-correlation analysis and display were carried out as described[5,9]. Sliding-window correlations were averages of correlations calculated for single trials. Statistical comparisons were made by unpaired two-tailed *t*-tests.

Proboscis extension (PE) conditioning. Bees were individually collected in vials at the entrance of a colony as they returned from or departed on a foraging trip. Vials were immediately placed in an ice–water bath to anaesthetize the animals. Each bee was placed in a harness made of a small metal tube and a strip of tape was inserted dorsally between its head and thorax[15]. Details of the PE conditioning protocol are described elsewhere[24,25]. After a recovery period, bees were tested for motivation by touching one antenna with a droplet of sucrose. Bees that vigorously extended their probosci to this stimulus were selected for treatment and conditioning. PCT or saline was applied in two ways. In the first method (providing the best learning performance; group 1, Fig. 4), a 3-nl drop of saline or of 100 μM PCT in saline was topically applied to the dorsal anterior

surface of the antennal lobes through a small window cut in the head cuticle; these experiments were done without the experimenter knowing whether the drop contained PCT or saline. In the second method (group 2, Table 1), 0.1 nl saline or picrotoxin (100 μM–1 mM in saline) was injected directly into the antennal lobes through a small window in the head just above the base of each antenna using a Picospritzer (General Valve)[26]. Injections gave the same results as topical applications, although PE response rates were reduced, as commonly observed after extensive surgery. After a time t_1 (10, 45, 60 or 90 min) for recovery, animals were trained by using the following protocol[13,14]: 6 paired presentations of odorant (4-s pulse into a vented air stream) and sucrose (0.4 μl of 1.25 M solution for group 1, 2 μl of 2 M solution for group 2, presented to the antenna and the proboscis 3 s after odorant pulse onset), every 2 min (group 1) or 30 s (group 2). Animals showing a PE response in each trial were selected to receive 2 or 3 extinction (odour only) trials (one with each of the 2 or 3 test odours; see below) 90 min (group 1) or 60 min (group 2) after conditioning. The odorants used for conditioning were 1-hexanol or 1-octanol. Groups were counterbalanced to contain roughly equal numbers of bees trained with either alcohol. The odours used for testing (1-octanol, 1-hexanol, geraniol) were presented to each animal in a randomized order. Generalization between the alcohols and geraniol is typically low[16]. We used the percentage of subjects that responded to an extinction test as the response measure. Results were compared with χ^2 statistics because behavioural data were categorical (PE or no PE). Statistical values are one-tailed because generalization responses were not expected to exceed the response levels to conditioned stimuli.

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Prion (PrP^{Sc})-specific epitope defined by a monoclonal antibody

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Prions are infectious particles causing transmissible spongiform encephalopathies (TSEs). They consist, at least in part, of an isoform (PrP^{Sc}) of the ubiquitous cellular prion protein (PrP^{C}). Conformational differences between PrP^{C} and PrP^{Sc} are evident from increased β-sheet content and protease resistance in PrP^{Sc} (refs 1–3). Here we describe a monoclonal antibody, 15B3, that can discriminate between the normal and disease-specific forms of PrP. Such an antibody has been long sought as it should be invaluable for characterizing the infectious particle as well as for diagnosis of TSEs such as bovine spongiform encephalopathy (BSE) or Creutzfeldt–Jakob disease (CJD) in humans. 15B3 specifically precipitates bovine, murine or human PrP^{Sc}, but not PrP^{C}, suggesting that it recognizes an epitope common to prions from different species. Using immobilized synthetic peptides, we mapped three polypeptide segments in PrP as the 15B3 epitope. In the NMR structure of recombinant mouse PrP, segments 2 and 3 of the 15B3 epitope are near neighbours in space, and segment 1 is located in a different part of the molecule. We discuss models for the PrP^{Sc}-specific epitope that ensure close spatial proximity of all three 15B3 segments, either by intermolecular contacts in oligomeric forms of the prion protein or by intramolecular rearrangement.

PrP-null mice were immunized with full-length recombinant bovine PrP. After fusion of spleen cells with myeloma cells, we selected ~50 hybridoma cells that produced monoclonal antibodies recognizing either native bovine PrP^{Sc} (PrP^{BSE}) immobilized on nitrocellulose or recombinant bovine PrP (rbPrP) in an enzyme-linked immunosorbent assay (ELISA). One of these antibodies (15B3) was selected for binding to protease-digested BSE brain homogenates; a second (6H4) efficiently recognized recombinant PrP. On western blots, 6H4 recognized rbPrP, as well as bovine, human, mouse and sheep PrP^{C}, whereas 15B3 did not react with any form of PrP (results not shown). To determine the reactivity of these antibodies with native PrP^{C} and PrP^{Sc}, we immunoprecipitated PrP from brain homogenates of normal and BSE-infected cattle. The precipitated proteins were then analysed on western blots using a rabbit polyclonal antiserum to rbPrP (Fig. 1). The 6H4 antibody precipitated PrP from BSE as well as from normal brain homogenates; 15B3 precipitated only PrP from brain homogenates of BSE-diagnosed cattle (Fig. 1a). Upon proteinase K treatment, normal PrP is completely digested, whereas the 33K–35K form of PrP^{Sc} is shortened to 27K–30K (PrP 27–30), probably as a result of