Olfactory behavior and physiology are disrupted in prion protein knockout mice

Claire E. Le Pichon¹, Matthew T. Valley¹, Magdalini Polymenidou², Alexander T. Chesler¹, Botir T. Sagdullaev¹*, Adriano Aguzzi², and Stuart Firestein¹

Supplementary information
**Supplementary Figure 1** Controls for the cookie finding test. *(a-b)* Metabolism controls: average daily food consumption *(a)* and body weight *(b)* of B6129 (filled circles, n=9), ZI PrP<sup>−/−</sup> (empty circles, n=10), and NSE-PrP (filled grey circles, n=9) mice was similar regardless of strain. Thus, metabolic differences can be ruled out as factoring into the cookie finding phenotype of the PrP<sup>−/−</sup> mice. The animals shown here are the same B6129, PrP<sup>−/−</sup> and NSE-PrP mice as represented in Figs. 2 and 4. Black lines represent median values. *(c)* Visible cookie test: B6129 (filled black circles, n=10), ZI PrP<sup>−/−</sup> (empty circles, n=8), and NSE-PrP (filled grey circles, n=9) mice were subjected to a modified version of the cookie finding test where the olfactory cookie stimulus was placed on the surface of the bedding. Latencies to find the cookie were scored. Black lines denote median latencies. All mice approach the cookie within ~10 s. The PrP<sup>−/−</sup> mice are clearly able to locomote towards the cookie in a similar time range as the controls. Interestingly, the NSE-PrP are slightly faster than the other 2 strains (*p*<0.05, Dunn's test).
Supplementary Figure 2  EOG recordings from PrP−/− mice are normal. Electro-olfactogram (EOG) recordings from the olfactory epithelia of wild type B6129 (top row) and ZI PrP−/− (bottom row) mice in response to a panel of 7 odorants. To account for variability between individuals, all responses were normalized to the first response (amyl acetate). PrP−/− epithelium gave rise to robust and normal looking responses to the panel of odorants.
Supplementary Figure 3  Breath-centered spectrograms showing the change in LFP oscillations after an odor pulse. (a) Example LFP and breathing trace from a B6129 mouse over the duration of spectral analysis. Each strain’s series of spectrograms represents the averaged spectrum for the entire cohort. (b) B6129 (n = 11), (c) PrP$^{+/-}$ (n = 10), and (d) NSE-PrP (n = 5). To correct for variability in the rate and regularity of breathing over successive trials, spectral analysis of LFPs was chunked into 500 ms windows around each breath (see methods). Decibel values represent the log-change above the average baseline spectral power, calculated per frequency. Dotted outlines indicate breaths taken for further analysis in Figure 7.
Supplemental Methods

Cookie finding behavior test

In this test, a cookie is buried under the cage bedding so as to offer a purely olfactory cue, and the time taken by a mouse to retrieve the cookie is recorded. All animals tested were male, between 7 and 25 weeks old, maintained on a 12:12 light/dark cycle, and tested in the light phase. Mice were food deprived overnight with ad libitum access to water. The next morning, each mouse was placed in a novel test cage in which a ~3 g piece of cookie (Nutter Butter, Nabisco) had been buried under fresh bedding. The latency of the animal to retrieve the cookie was recorded. Retrieving the cookie was defined as digging it up with forepaws and snout so the cookie became visible to the experimenter. Each individual was given two trials about an hour apart. Trial 1 lasted 10 minutes and Trial 2 either 5 or 10 minutes depending on the experiment (10 min in the case of Fig. 2 and Fig. 3g). The position of the cookie was changed in the second trial so the animal could not rely on spatial cues to find it. Prior to the first trial, all animals were naïve to the test. For purposes of statistical analysis, if an animal failed to find the cookie within the test time, it was given the conservative score of the total test time (either 600 s in the 10-minute tests or 300 s in the 5-minute tests). The average improvement factor was calculated as: \( \Sigma (T_1/T_2)/n \).

For the experiments shown in all except Figure 2, the cage size was 425 x 266 x 155 mm and the bedding consisted of wood shavings. In the experiments shown in Figure 2, the
cage size was 266 x 166 x 120 mm and the bedding was corncob-based (Bed-o’-cobs, The Andersons, PO Box 119, Maumee, Ohio 43537).

The control version of this test (visible cookie finding test) was performed on a separate cohort of animals in exactly the same manner except that the cookie was placed on the bedding’s surface instead of being buried underneath it.

**Habituation-dishabituation test**

Naïve mice were habituated to a fresh test cage 4-5 hours prior to testing. Testing occurred during the dark phase under red lights. During the test, each odor presentation lasted 2 minutes and the time (in seconds) spent investigating the odor was recorded. Investigation time was defined as the cumulative time the mice spent actively sniffing the odor source within a 2 cm radius, snout oriented towards the source, during the 2-minute presentation period. For each animal, all investigation times were normalized to the maximal investigation time for that animal and plotted as a fraction of this maximal time.

The first odor (peanut butter or PB) was presented four consecutive times (PB 1, PB 2, PB 3, PB 4). The initial interest in the odor is expected to decrease with each successive presentation as the animal habituates to the odor. On the 5th presentation, a novel odor is presented. The novelty of the odor should induce an increase in the animal’s investigation time, and this is interpreted as an ability to discriminate the difference between odors 1 and 2. For odor 2, we used a mixture of PB (odor 1) and a new odor (vanilla) as a difficult discrimination. On the 6th presentation, we used a very different odorant, amyl acetate, to which we knew the PrP^{−/−} mice respond with normal EOG responses.
Odor presentation: odors were pipetted onto the fiber plug of an upturned 5 ml serological pipette (BD Falcon) which was attached to the top of the cage such that the extremity containing the odor extended down into the center of the cage at animal head level. The fiber plug onto which the odors were pipetted was situated ~2 cm inside the end of the pipette such that the animals could not directly contact the odor source, but could sniff up the tube.

Odors were (1) PB (peanut butter): 200 µl of 10% w/v creamy Skippy peanut butter diluted in mineral oil; (2) PB + vanilla mix: 100 µl of the PB solution mixed with 100 µl of 10% vanilla essence diluted in water; (3) amyl acetate: 100 µl of 10 mM amyl acetate (Sigma Aldrich) diluted in mineral oil.

In situ hybridization

Plasmid for Prnp probe, designed such that it recognized the wild type Prnp allele but not the knockout allele, was kindly provided by O. Giger, University Hospital of Zürich. Mice were transcardially perfused in 4% PFA in DEPC-treated PBS, then postfixed overnight at 4°C. Brains were transferred to 30% sucrose in DEPC-treated PBS overnight at 4°C for cryoprotection. The olfactory epithelium was first decalcified in 0.5 M DEPC-treated EDTA for 3 days, followed by cryoprotection. 10-12 µm cryosections were cut then warmed at 55°C. They were fixed in 4%PFA, washed in DEPC-PBS, treated with 10 µg/ml proteinase K at 37°C, fixed once more in 4% PFA, washed, then incubated in 0.1 M triethanolamine with acetic anhydride (0.03%), and washed. Slides were dehydrated in 60%, 80%, 95%, and 100% ethanol, and then hybridized overnight with 1 µg/ml DIG-labeled probe in hybridization solution (50% formamide, 10 mM
TrisCl (pH 8.0), 200 µg/ml yeast tRNA, 10% dextran sulfate, 1X Denhardt’s solution, 600 mM NaCl, 0.25% SDS, 1 mM EDTA (pH 8.0)). The next day, slides were washed in 50% formamide 2X SSC (sodium chloride sodium citrate), treated with 20 µg/ml RNaseA at 37ºC to remove any unhybridized probe, washed in 2X SSC and then 0.2X SSC. The slides were blocked in 0.5% NEN buffer (Perkin Elmer) then incubated overnight at 4ºC in alkaline phosphatase-conjugated anti-DIG (1:1000, Roche). Slides were incubated in HNPP/Fast Red (Roche) for 45 min. If the transcript level was low (e.g. detection of Prnp in Lck-PrP and CD19-PrP brains), this step was repeated twice. Images were obtained using a Leica DMR microscope and a CCD Spot camera (Diagnostic Instruments). Images were not modified except using Photoshop to adjust brightness and contrast to the entire image.

Odor Delivery
A custom-made olfactometer was adapted from a previous design to deliver odors during electrophysiology. Compressed air was humidified and passed by the animal’s nose at a rate of 1 l/min (carrier stream). The odor amyl acetate, to which the animals were naïve, was dissolved in 1 ml DMSO to a final concentration of 0.5 mM, and a 20 ml vapor headspace was kept saturated with a cotton or paper wick to maximize liquid surface area. Amyl acetate was used as a representative odor because it evoked highly reproducible and stereotyped spectral-temporal patterns across multiply sampled positions within the granule cell layer (GCL). Odor puffs, 2 s in duration, were diverted into the carrier stream, and puffs were separated by a minimum of 40 s. To avoid pressure changes, a compensatory open air channel was switched off during odor presentation. No
decrease of odor response was measured in the LFPs after repeated stimulation over hours (unpublished observations). Odor concentration reported in the text does not take into account the dilution of the saturated headspace into the carrier stream, therefore final concentrations are much less. For every mouse, odor was delivered at least 7 times, spaced apart with pulses of solvent headspace.

**Electrophysiology**

Anesthesia was induced with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (7 mg/kg) in mammalian Ringer’s solution and supplemental injections were given every ~ 45 min. The animal was mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) and body temperature was maintained at 37°C with a feedback controlled heating pad (CT-1000, CWE, Ardmore, PA). The animal’s nose was inserted into an air-tight gas mask through which humidified air from the olfactometer passed at a rate of 1 l/min. Two small craniotomies were performed at the left hemisphere at stereotaxic coordinates corresponding to the region above the lateral olfactory tract (LOT), and to the central dorsal surface of the OB. A custom made tungsten recording electrode with an impedance of 0.7-2 MΩ was inserted 1 mm deep with a hydraulic microdrive into the granule cell layer of the MOB, and a custom made bipolar tungsten stimulating electrode was driven between 3.5-4.5 mm deep into the LOT. We recorded evoked field potentials (Fig. 8) from the same location in the granule cell layer as for the LFP recordings (Fig. 7; Supplementary Fig. 3). Correct placement of the stimulating electrode was confirmed by recording the antidromic evoked waveform
in the GCL. If the evoked GCL waveform did not contain three distinct phases corresponding to 1) back-propagating spikes, 2) field GC excitatory postsynaptic potentials (EPSPs), and 3) field MC inhibitory postsynaptic potentials (IPSPs), we assumed the stimulation electrode was reaching too many centrifugal afferents, and not enough LOT fibers. In this case the stimulating electrode was inserted to a new position. In some animals the position of the stimulating and recording electrode was confirmed histologically after burning the tip position, and visualized in 100 µm thick vibratome slices with cresyl violet.

Local field potentials were amplified (DP-301, Warner Instruments, Hamden, CT), filtered (1 Hz highpass, 3 kHz lowpass), and digitized at 5-1 kHz (Power 1401, CED, UK). Breathing was continuously monitored with a piezoelectric force-transducer (Stoelting, Wood Dale, IL) in contact with the animal’s back, and this signal was used to trigger odor delivery upon the transition of inhalation to exhalation (I/E transition). In the breathing trace, inhalation occurs during positive values; exhalation during negative values (see left inset in Fig. 6b). The average breath length did not significantly differ between groups (PrP+/− 511 ms; B6129 445 ms; NSE-PrP, 630 ms; ANOVA p>0.05). All on-line event detection and triggering was done using the software Spike2 (CED, UK). Similarly, LOT stimulation was triggered by the phase of the breathing cycle, and current was created using a pulse generator and stimulus isolation unit (Grass S88, W. Warwick, RI). For every mouse, the stimulus-intensity response relationship was recorded with the GCL recording electrode. All subsequent stimulation in the paired-pulse paradigm was given at saturating intensity (1-1.5 mA over 100 µs). Experiments that involved LOT stimulation were always performed after the completion of odor delivery protocols.
LFP signal processing and analysis

All signal processing was done off-line using custom written scripts in Spike2, and in Matlab using a combination of custom written scripts and the program eeglab 6.01b\(^{42}\). LFP spectra for Figure 6 were made using the multi-taper fft algorithm PMTM, applied to 1024 samples (205 ms) with a time-bandwidth product of 4, over 500 ms windows of data surrounding the inhalation/exhalation (I/E) point of every breath. To visualize the time-course of power over multiple breaths, we averaged the power within a band of frequencies, and for every animal expressed this as the fold-change from baseline. The baseline band-power is the average power of the first three breaths before odor exposure. The decay time of these normalized traces was measured as the time (in number of breaths) for the response to fall below 90% of its peak.

For Figure 7, we used wavelet analysis of the odor response, and analyzed 6-second epochs around the transition between inhalation and exhalation (I/E point) in every breath surrounding an odor pulse or a DMSO pulse. In this way, overlapping data epochs were made around 30 breaths, with an odor or DMSO pulse always triggered at the I/E point of breath 4. Analysis of the timing of spectral components relative to breathing was done on breath 1 (no odor) and breath 5 (+odor). No significant spectral contribution is observed in the DMSO containing pulses, indicating that observed spectral changes around odor stimulation are not due to artifacts from the stimulation apparatus. Wavelet transformations used the Morlet basis with an initial window size of 446 ms at 2 cycles, and tapered to 43 cycles. 400 analysis windows were placed within the middle 5.5 s of
each epoch, producing a time bin size of 14 ms. Spectral power at every frequency band (94 bands from 5 Hz to 120 Hz) was normalized to the average band-power contained in the baseline region (1.5 s after I/E of breath 1). Spectral power above baseline is expressed in decibels (10*log_{10}(signal/baseline)). In this way, each odor response was normalized to its own spectral baseline, and the normalized power of repeated odor stimulations for each animal (7-9 per animal) was averaged. Because of this normalization, wavelet powers will not reflect absolute difference in spectral power between strains and conditions, but rather the flux in spectral power over time. Additional normalization to bring the pre-stimulus baseline to 0 dB was done relative to the first three baseline breaths. Compound spectrograms (Supplementary Fig. 3) were made by averaging across all animals in a group after the data had been corrected to baseline and normalized. All spectrograms are colored using the same LUT (–12 dB to +12 dB).

**Paired-Pulse analysis**

For short inter-stimulus intervals (<100 ms), we observed that both the peak (GC fEPSP) and trough (MC fIPSP) of the second pulse occur on top of a moving baseline created by the first pulse. We assumed the two waveforms overlapped additively, and corrected for this bias by subtracting the value of a single pulse at the time of the event (fEPSP or fIPSP) in the second pulse. This bias factor was taken from an average of multiple single stimulations, and the baseline of these single stimulations was set to zero before averaging. Therefore in all paired stimulations with ISI<100 ms, it is necessary to compare the bias factor to the specific baseline preceding the first stimulus (baseline 1).
The formula for the paired pulse ratio of two fEPSPs for an ISI less than 100 ms was:

1) \( \frac{(\text{peak2} - (\text{bias} - \text{baseline1}))}{(\text{peak1} - \text{baseline1})} \)

where “peak” is the magnitude of the fEPSP peak, “bias” is the value of the average waveform at the time of the peak, and “baseline” is the value prior to the stimulus artifact.

For measurements of fIPSPs, trough1 was measured from the average trace that was already set to baseline prior to averaging, therefore it was not necessary to compare it to baseline 1. Thus, the formula for fIPSPs (ISI<100 ms) was:

2) \( \frac{(\text{trough2} - (\text{bias} - \text{baseline1}))}{(\text{trough1})} \)

For ISI greater than 100 ms, each peak and trough was set to their respective pre-stimulus baseline, so for fEPSPs this was calculated by the following formula:

3) \( \frac{(\text{peak2} - \text{baseline2})}{(\text{peak1} - \text{baseline1})} \)

fIPSP paired-pulse ratios (ISI>100 ms) were also calculated with formula 3.

**EOG recordings**

Field potential recordings were performed in the olfactory epithelium as described\(^{43}\). For each animal, all responses were normalized to the response to a standard odor, amyl acetate. The peak amplitude of the amyl acetate response was always given the value of 1, and all other responses are represented as a percentage of the amyl acetate response.
All odorants were obtained from Sigma Aldrich (St. Louis, MO). Care was taken to place the electrodes in a similar location between animals, so the OSNs sampled would be from a similar zone (the recordings shown in Supplementary Fig. 2 were from the 3rd most ventral turbinate, central area).

*Food intake / body weight*

Animals were group housed by strain in groups of 9-10 mice. Body weight and daily food intake per cage was recorded across 10 consecutive days. Every 24 hours, 10 g of food was provided per animal, the remaining food was weighed 24 hours later, and the weight of the food consumed per animal for this 24-hour period was calculated as an average.