Supplementary data

Bistability is present in Purkinje cells of anesthetized mice

To confirm the occurrence of bistability of Purkinje cells, which has so far only been shown in anesthetized rats and guinea pigs\(^1\), we performed whole-cell patch recordings in vivo in mice under isoflurane or ketamine/xylazine anesthesia. Under isoflurane all Purkinje cells \((n = 6)\) showed a bistable or multistable membrane potential (Supplementary Fig. 1a). The upstate of the membrane potential (on average \(-52 \pm 1\) mV, mean ± SEM) always occurred in conjunction with continuous firing of action potentials (mean firing frequency overall 40 ± 7 Hz; upstate 80 ± 22 Hz), whereas the downstate \((-62 \pm 3\) mV) was completely silent except for complex spikes. In 5 out of 6 recorded Purkinje cells we observed a third state, which was even more depolarized \((-37 \pm 1\) mV). The third state, hereafter referred to as the “overstate”, was quiescent but it occasionally displayed small spikelets (~3-4 mV). While the upstate usually occurred after a downstate, the overstate always occurred after the upstate. Shifts from a particular state to a more depolarized state were often associated with the occurrence of a complex spike (complex spikes preceded a transition from down- to upstate in 57 ± 16 % of the cases and from upstate to overstate in 57 ± 17 %; as opposed to 13 ± 8 % from up- to downstate and 17 ± 17 % from overstate to either upstate or downstate). Most of the time (68 ± 12 %) the cells were in the upstate; downstate and overstate covered 25 ± 12 % and 7 ± 5 % of the time, while their average duration was 1.1 ± 0.3 s and 0.8 ± 0.5 s, respectively.

Under ketamine/xylazine anesthesia 6 out of 10 Purkinje cells showed a bistable or multistable membrane potential (Supplementary Fig. 1b). Here too, the upstate of the membrane potential (on average \(-51 \pm 1\) mV) was correlated with
continuous firing of action potentials (mean firing frequency overall 68 ± 9 Hz, same for upstate), while the downstate (–61 ± 2 mV) and overstate (–39 ± 3 mV) were silent. Moreover, the shifts showed the same characteristics as described for the experiments under isoflurane in that the higher states usually occurred after a gradual stepwise elevation and that these elevations were relatively often associated with complex spike activities (down- to upstate 45 ± 14 % and up- to overstate 86 ± 11%). The average duration of individual downstates (1.2 ± 0.5 s) and individual overstates (1.1 ± 0.8 s) did not differ from those found under isoflurane (P = 0.91 and P = 0.74, resp.). However, the percentage of time that the membrane potential was in the downstate (3 ± 2 %) as well as the percentage of cells displaying an overstate (20%) were both significantly lower (P = 0.022 and P = 0.035, respectively) than those under isoflurane (25 ± 12 % and 83%, respectively).

These data show that Purkinje cells in mice can show multiple stable states under various forms of anesthesia, that the transitions to down- and overstate are more prominent under isoflurane than ketamine/xylazine, and that under both forms of anesthesia the complex spikes can elicit simple spike firing by shifting the membrane potential from down- to upstate.

**Action potentials of intracellular recordings correspond to simple spikes of extracellular recordings**

While it is well established that the complex spike is an all or none response of the Purkinje cell to climbing fiber activation, much less is known about how well extracellularly recorded events correspond to the sub- and suprathreshold potentials that can be observed with the use of the whole-cell recordings such as those described above. We therefore recorded simultaneously both intracellularly and extracellularly
the activity of single Purkinje cells in vivo under ketamine/xylazine anesthesia (n = 2) using double electrode recordings (extracellular recording electrodes were attached to patch electrodes that reached 10 µm deeper) (Supplementary Fig. 2a-b). The recording traces showed a perfect match in that every simple and complex spike recorded intracellularly corresponded to a single spike recorded extracellularly and vice versa (resulting in a 100% match at a time resolution of 1.0 ms; Supplementary Fig. 2c-d). Thus, during the downstate silent periods that were identified with the use of intracellular data, no extracellularly recorded simple spikes were observed. Moreover, the prolonged silent periods observed in the extracellular recordings corresponded exclusively to down- or overstate periods of the intracellular recordings. To exclude the possibility that cross-talk between the two electrodes caused the perfect match, we gave short negative current pulses similar in size to the spikes recorded intracellularly. These current injections resulted in a hyperpolarization in the intracellular recording, while no deflection occurred in the extracellular recording (Supplementary Fig. 2e). The longest inter-simple spike interval (ISSI) in any intracellularly recorded upstate was 158 ms (median 92 ms). In contrast, the maximum duration of an ISSI during a downstate or overstate period reached a value of over 5 s (median 1079 ms), and the vast majority (> 90 %) of all downstate and overstate ISSIs were greater than 200 ms. Thus, any silent period observed in an extracellular recording that is longer than 158 ms, most likely reflects a downstate or overstate.

Together these data indicate that simple spikes of extracellular recordings always correspond to action potentials of intracellular recordings, and that the state of the membrane potential of a Purkinje cell can be relatively reliably deduced from the
temporal patterns of simple spike activities that have been recorded with extracellular methods.

**Extracellular recordings show low level of bistability in spontaneous awake state**

Whereas whole-cell recordings in awake behaving animals impose technical problems, stable extracellular recordings of Purkinje cells are quite feasible in the awake state\(^4\). Thus, since the temporal pattern of simple spike activities provides, as explained above, information about the state of the membrane potential, extracellular recordings offer a means to compare the occurrence of bistability in awake animals with that in anesthetized animals. Therefore we conducted extracellular recordings of Purkinje cell activity in awake mice (\(n = 36\) cells) as well as in mice anesthetized with either isoflurane (\(n = 24\)) or ketamine/xylazine (\(n = 21\)). As mentioned above, silent states will introduce ISSIs that are up to several orders longer in duration than ISSIs during the upstate. These long ISSIs affect the upper tail of the ISSI distribution. We examined the shape of the ISSI distribution using skewness and kurtosis as indicators of asymmetry and peakedness, respectively. We expected additional silent state ISSIs to positively skew the distribution. Because the variance of silent state ISSIs scales with their mean duration, we used the logarithm of the ISSIs. In addition, as a measure of spike train regularity, we calculated the coefficient of variation (CV), and since multiple firing states can lead to a bimodal log-ISSI distribution, we tested for unimodality using a dip test\(^5\).

While the simple spike firing frequencies and climbing fiber pauses did not vary significantly between Purkinje cells of awake mice and mice under both types of anesthetics (both \(P > 0.50\) and both \(P > 0.07\), respectively), the regularity of simple spike activities in awake mice was much greater than that found in anesthetized mice.
(Fig. 1a-c). Their CV, skewness, and kurtosis were all significantly smaller (Fig. 1d). Furthermore, the dip test indicated that the percentages of cells with bi-/multimodal ISSIs (Supplementary Fig. 4b) and bi-/multimodal firing frequency distributions (data not shown), both strong indicators of multiple firing states, were significantly increased in anaesthetized mice. If bistability were present in awake animals, one would expect to find a significant number of ISSI pauses with a duration exceeding 158 ms, as seen in anesthetized mice. However, pauses longer than 158 ms hardly occurred in awake mice. Both the total percentage of time spent in a pause state and the total percentage of cells that showed one or more pauses greater than 200 ms were substantially lower in awake animals than in those anesthetized (Fig. 1e-f). All values (i.e. CV, skewness and kurtosis) were compared for Purkinje cells in the floccular and non-floccular areas and no differences were observed (data not shown). In addition, we found little evidence for the toggling switch phenomenon mediated by the complex spikes in awake animals. When we calculated the percentage of complex spikes that occurred before or after a pause in simple spike activities of at least 100 ms, we found that complex spikes rarely triggered a change in simple spike activities from silent to upstate (0.3 ± 0.1 %) and in a limited number of cases from upstate to silent (2.9 ± 1.6 %). These percentages were significantly lower than those obtained under isoflurane (silent to upstate 9.3 ± 3.0 %, \( P < 0.001 \); upstate to silent 7.7 ± 2.2 %, \( P = 0.026 \)) or ketamine/xylazine (silent to upstate 2.5 ± 1.4 %, \( P = 0.046 \)). The occasional triggering from upstate to silent by a complex spike in awake animals was, in contrast to that in anesthetized animals, found to be directly related to the simple spike firing frequency, in that signs of toggling were only seen in cells with a relatively low simple spike firing frequency. This low simple spike firing frequency strongly increases the chance of finding a pause of 100ms in simple spike firing
before or after a complex spike. Therefore these occasions are most likely not actual
downstates but merely the result of low firing frequency.

Finally, if the use of anesthesia increases bistability, cessation of such
application should bring the same Purkinje cells to a stable upstate level resulting in
regular simple spike firing without pauses or inter-spike intervals longer than 158 ms
and without signs of toggling by the complex spikes. We therefore investigated the
extracellular activities of individual Purkinje cells (n = 2) both during and after
application of isoflurane, which can be administered by respiration and stopped
abruptly at will. **Figure 1g-i and Supplementary Figure 3** show an example of the
recordings of a Purkinje cell in the paramedian lobule. While the regular pauses and
signs of complex spike toggling described above were abundantly observed during
application of isoflurane, they disappeared within one minute after stopping it (**Fig. 1g-h**). To verify that the animal switched from an anesthetized state to an awake state
after this minute, we attempted to evoke compensatory eye movements during the
entire experiment. These recordings showed that sinusoidal optokinetic stimulation
indeed resulted in visual reflexes approximately one minute after application of
isoflurane was stopped (**Fig. 1i**).

Together, the extracellular recordings described above indicate that anesthetics
can dramatically induce bistability in Purkinje cells and that this process only occurs
infrequently in the awake state.

**Modulation of afferents to Purkinje cells does not alter their stability in awake state**

While the data above demonstrate that pauses, as a sign of bistability, hardly occur in
spontaneously active Purkinje cells in awake animals, they do not exclude the
possibility that bistability may be enhanced by sensory modulation or learning paradigms. We therefore compared the temporal patterns of the simple and complex spike activities of Purkinje cell recordings from awake mice without stimulation \((n = 36)\) with those during optokinetic stimulation (OKR modulation; \(n = 24\)) and visuovestibular training (VVT modulation; \(n = 15\)). Since optokinetic stimulation modulates the ISSIs independent from the presence of complex spikes\(^6\) and thereby alters the ISSI distribution, we corrected each ISSI for the average firing rate during the part of the stimulus cycle encompassed by that ISSI (Fig. 2a). This rescaling procedure removes the linear effect of the stimulus on the firing rate. The skewness and kurtosis values of the rescaled ISSI distributions recorded during both optokinetic stimulation and visuovestibular training did not differ from those during spontaneous activity in awake mice (all \(P\)-values > 0.25, Fig. 2b). Moreover, the time that the cells were in pause and percentage of cells that showed pauses also did not differ significantly among the groups (all \(P\)-values > 0.16, Fig. 2c-d). These results indicate that bistability of Purkinje cells in awake mice is neither enhanced by the sensory stimulation that modulates these cells nor by the motor performance or motor learning that is controlled by these cells.
Supplementary reference list for results section


