Purkinje cells in awake behaving animals operate at the upstate membrane potential

To the Editor:

Over the last decades, cellular bistability or multistable states of the membrane potential have been demonstrated both *in vitro* and *in vivo* for different types of neurons throughout the brain, and various functions have been proposed for this phenomenon[1,2]. Recently, Loewenstein *et al.*[3] proposed that bistability in Purkinje cells has a key role in the short-term processing and storage of sensorimotor information in the cerebellar cortex and that complex spikes may act as a toggle switch to control these processes. However, all intracellular recordings of bistability to date have been obtained either in slices or in anesthetized animals[4]. Because anesthetics can directly or indirectly affect the membrane potential[5], it remains to be seen whether the proposed functional roles of bistability are valid in normal behaving animals under physiological conditions.

To confirm the occurrence of bistability in Purkinje cells, we performed whole-cell patch recordings *in vivo* in mice under isoflurane or ketamine/xylazine anesthesia.

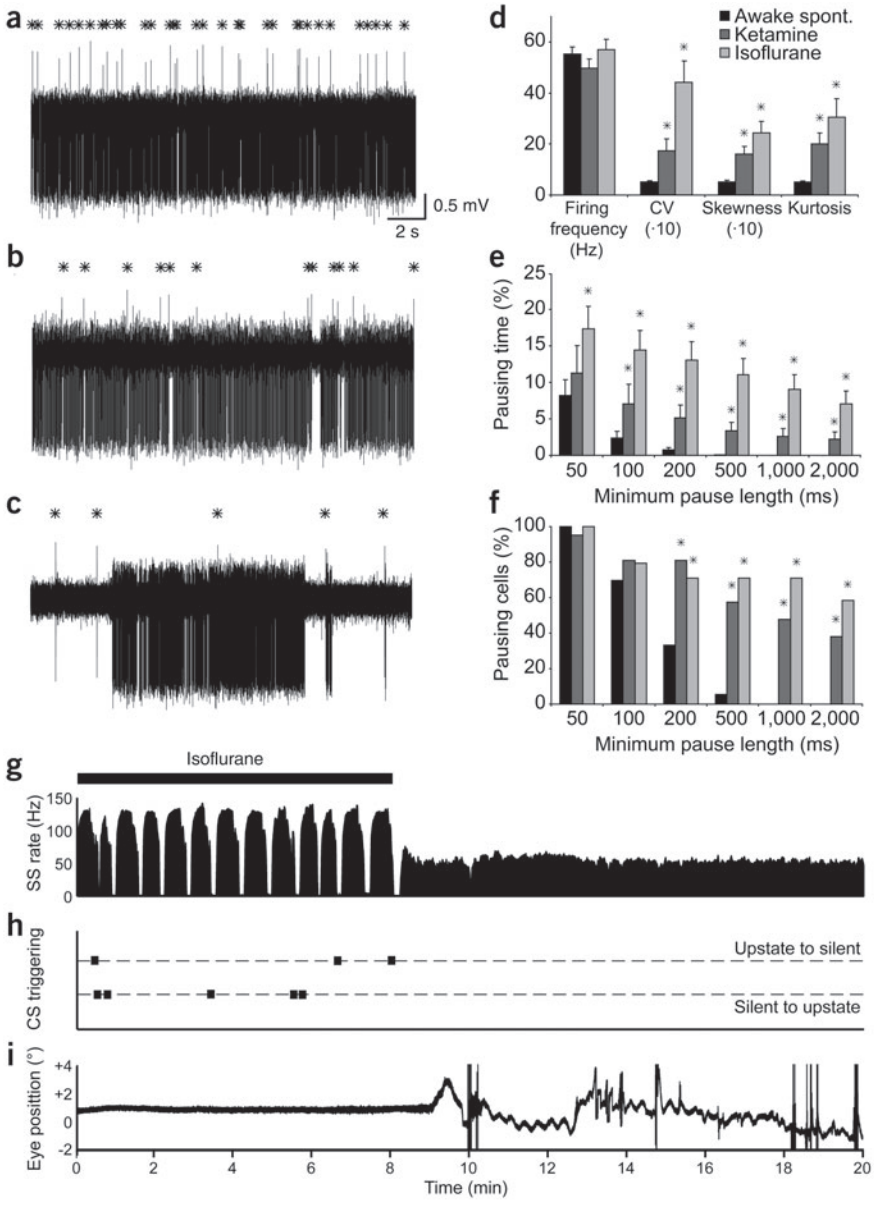

Figure 1 Simple spike pauses and signs of toggling are dramatically increased by anesthetics. (**a**) Typical extracellular recording of spontaneous Purkinje cell activity in an awake mouse showing few or no signs of pauses or toggling. Asterisks indicate complex spikes. (**b**) Purkinje cell activity recorded extracellularly in a mouse under ketamine/xylazine anesthesia showing pauses in simple spike firing. (**c**) Extracellular recording of a Purkinje cell under isoflurane anesthesia showing a highly irregular firing pattern consisting of pauses and bursts. (**d**) Although simple firing frequency is not affected, measures for the shape of the ISSI distributions (CV, skewness and kurtosis) are significantly higher in ketamine/xylazine ($n = 21$) and isoflurane ($n = 24$) anesthetized mice compared to awake ($n = 36$) mice (asterisks, $P < 0.05$). (**e**, **f**) Histograms comparing the percentage of total recording time that individual cells were pausing (**e**) and the percentage of cells showing one or more pauses (**f**) for different minimum pause lengths. Purkinje cells in anesthetized animals display significantly more pausing than those in awake mice (asterisks, $P < 0.05$). (**g**–**i**) Extracellular Purkinje cell recording of a mouse starting under isoflurane anesthesia, which ceased after 8 min. Pauses in simple spike firing frequency (**g**) and signs of toggling (**h**) that can be seen during anesthesia are no longer present after cessation of the application of isoflurane. Recordings of eye position during the experiment (**i**) show that eye movements start approximately 1 min after cessation of isoflurane, confirming that the mouse recovered from the anesthetics. For raw trace and climbing fiber pause of this cell, see **Supplementary Figure 3**.

Under both forms of anesthesia, Purkinje cells showed multiple stable states, of which the silent downstate and firing upstate were predominant. The transitions between states were more prominent under isoflurane than under ketamine/xylazine; moreover, under both forms of anesthesia, the complex spikes affected simple spike firing by shifting the membrane potential from down- to upstate or vice versa, known as the toggle phenomenon (for numerical data, see **Supplementary Fig. 1** and **Supplementary Data** online).

Subsequently, we validated to what extent extracellular recordings can be used to study bistability and toggling, because stable intracellular recordings in awake behaving animals present technical complications. We therefore first made simultaneous intracellular and extracellular recordings from single Purkinje cells *in vivo* under ketamine/xylazine anesthesia using double electrode recordings. Each extracellularly recorded simple spike consistently corresponded to an intracellularly recorded action potential (**Supplementary Fig. 2** and **Supplementary Methods** and **Data** online). Moreover, the recordings demonstrated that the state of the membrane potential of a Purkinje cell can be deduced from the duration of the intervals between simple spikes that have been recorded extracellularly.

To find out whether anesthetics can influence the level of bistability and/or toggling, we compared the extracellular Purkinje cell activities in awake mice with those in mice anesthetized with either isoflurane or ketamine/xylazine (**Fig. 1a–c** and **Supplementary Data**). We analyzed inter–simple spike interval (ISSI) distributions and found that the skewness, kurtosis and the coefficient of variation (CV) of the simple spike activities were all significantly higher in Purkinje cells recorded in anesthetized mice, whereas their mean firing frequency was not affected (**Fig. 1d**). In addition, anesthetized mice showed a marked increase in ISSI distributions that were bi- or multimodal. For a minimum pause length criteria of 200–2,000 ms, the percentages of both pausing times and pausing cells were significantly higher in anesthetized mice than in awake mice, in which these percentages were negligible (**Fig. 1e**, **f**). Moreover, the toggling phenomenon found under anesthesia was virtually absent in the awake animal (**Supplementary Data**). Finally, continuous recordings from single Purkinje cells during and after the application of isoflurane showed that the abundant pauses and signs of complex spike toggling that were apparent during the application of isoflurane disappeared within one minute after cessation (**Fig. 1g–i** and **Supplementary Fig. 3** online).

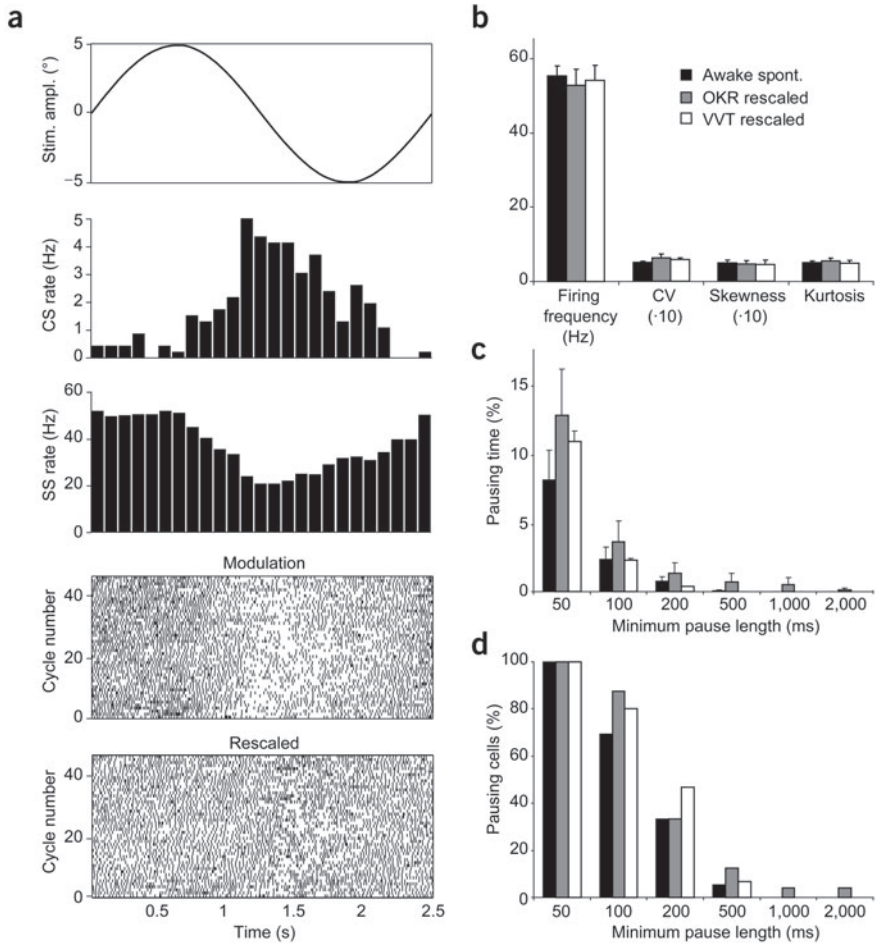

Figure 2 Motor performance or motor learning does not significantly affect bistability in Purkinje cells. (**a**) Sinusoidal optokinetic stimulation (top) modulated complex and simple spike firing as can be seen in both peristimulus time histograms (top: CS; bottom: SS). Raster plots show the effect of the rescaling on the SS modulation (top: before; bottom: after). (**b**) Rescaled ISSI distributions of recordings reveal that the shapes are not affected by optokinetic stimulation (OKR; peak velocity of 8° per s at 0.05–0.4 Hz; motor performance task) or visuovestibular training (VVT; combined optokinetic and vestibular stimulation both with an amplitude of 5° at 0.4 Hz; motor learning task) relative to spontaneously active conditions. (**c**,**d**) Both the percentages of pausing time (**c**) and pausing cells (**d**) do not change when mice are subjected to these protocols.

Possibly, however, the potential roles of bistability and/or toggling might come into play during sensorimotor behavior or motor learning under physiological circumstances. We therefore investigated activities of floccular Purkinje cells in awake mice responding to optokinetic stimulation[6] or visuovestibular training[7]. When we mathematically removed the direct effects of the sinusoidal stimulations used, we found no differences in ISSI distribution parameters (that is, percentages of pausing times and cells) among the awake mice responding to natural stimulation and those that were spontaneously active (**Fig. 2** and **Supplementary Data**).

Our experiments show that bistability can only occur infrequently in awake animals and that it can barely be influenced by natural sensory stimulation, motor performance or motor learning, whereas it is prominently present under isoflurane or ketamine/xylazine anesthesia (summarized in **Supplementary Fig. 4** online). These data agree with extracellular recordings in other labs that investigated simple spike and complex spike behavior in awake behaving and learning animals, including monkeys, cats and rabbits[8–10]. Moreover, inspection of these data obtained by other labs in other awake, behaving animals showed virtually no sign of toggling, which is so prominently present under anesthesia. Thus, although technical difficulties obstruct whole-cell recordings of Purkinje cells in awake animals, it is parsimonious to assume that Purkinje cells in awake behaving animals in general operate almost continuously in the upstate and that this state of their membrane potential is not significantly modified by sensory

stimulation, motor performance or motor learning. The current data raise the possibility that the bistability found in neurons in other brain regions[1] is also related to anesthesia, and they indicate that one should, in general, be careful in assigning important physiological roles to the phenomenon of bistability.

Note: Supplementary information is available on the Nature Neuroscience website.

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Loewenstein *et al.* reply:

We reported[1] that the membrane potential of Purkinje cells *in vivo* can exist in multiple stable states that correspond to firing and silent periods. Toggling between these states can be triggered by current injection or synaptic input, but the maintenance of the states is an intrinsic membrane property of these neurons[1–4]. Our experiments were done in rats and guinea pigs using ketamine-xylazine or barbiturate anesthesia, but similar behavior occurs in awake monkeys[5–7], frogs[8] and cats[9–11]. Schonewille *et al.* investigated Purkinje cell bistability in awake mice and concluded that membrane potential bistability is rare or absent in awake animals. However, in our view, their data is insufficient to support this conclusion.

Because intracellular recordings in awake animals are technically challenging, Schonewille *et al.* used extracellular recordings of spikes as an indirect measure of membrane potential. However, the extent to which the membrane potential of a Purkinje cell can be inferred from the firing pattern of simple spikes is questionable. For example, they report that in intracellular recordings from isoflurane-anesthetized mice, 57 ± 16% of transitions from down to up state were preceded by a complex spike, compared to only 9.3 ± 3.0% when the cell's state was determined by the timing of extracellularly recorded simple spikes under the same anesthesia conditions. Because the timing of complex spikes is determined unequivocally in both cases, the parsimonious conclusion is that although the timing of simple spikes recorded extracellularly matches the timing of simple spikes recorded intracellularly, the timing of transitions between states is not detected correctly from this analysis. Their most compelling indirect evidence against bistability is the statistically significant difference in the coefficient of variation, percentage of pausing time, and percentage of cells with a pause larger than a specific value between awake and anesthetized animals. However, a statistically significant difference in these same variables is also evident between ketamine-xylazine and isoflurane anesthetized animals, where in both cases Purkinje cells exhibit bistability (as confirmed in intracellular recordings). Therefore, these criteria do not seem sufficient to rule out bistability.

Additionally, Schonewille *et al.* analyzed the histogram of the logarithm of the inter–simple spike interval and found that only 5–10% of the cells recorded in the awake condition are bimodal. However, the same analysis applied to the firing pattern of Purkinje cells recorded extracellularly in anesthetized conditions gives a large percentage of unimodal cells, whereas the membrane potential of these neurons in the same conditions is mostly bimodal. This discrepancy between the firing pattern and the corresponding changes in membrane potential undermines the validity of the test used by Schonewille *et al.* Moreover, strong parallel fiber input could trigger occasional simple spikes from the down state, which renders all the above criteria irrelevant for determining membrane potential state.

Finally, there seem to be significant species differences between the dynamics of Purkinje cells in rats and guinea pigs compared to mice. For example, we reported that 24 of 24 Purkinje cells in ketamine-xylazine anesthetized rats were bimodal, whereas Schonewille *et al.* report that only 6 of 10 Purkinje cells are bimodal in mice using the same anesthesia. Furthermore, the percentage of time that the membrane potential was in the down state in mice was only 3 ± 2%, much less frequently than in rats (52 ± 4%). Thus, the generality of the Schonewille *et al.* results for other species is questionable.

Our results highlight a key biophysical feature of Purkinje cells—the ability to adopt two intrinsic states that can be sustained without persistent synaptic input—that provides a new framework for interpreting Purkinje cell activity patterns *in vivo*. Although it is plausible that the amount of time that Purkinje cells spend in the down state in awake animals is reduced compared to that in anesthetized animals or that occasional simple spikes are generated in the down state, the fundamental issue is that the biophysical mechanisms underlying bistability are likely to be present in Purkinje cells in awake animals, and could thus influence network function in the cerebellum. Further experiments in awake behaving animals, preferably using intracellular recording, are required to understand the role of membrane potential bistability in different species and under different behavioral conditions.

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