

## Choice of cranial window type for *in vivo* imaging affects dendritic spine turnover in the cortex

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**Determining the degree of synapse formation and elimination is essential for understanding the structural basis of brain plasticity and pathology. We show that *in vivo* imaging of dendritic spine dynamics through an open-skull glass window, but not a thinned-skull window, is associated with high spine turnover and substantial glial activation during the first month after surgery. These findings help to explain existing discrepancies in the degree of dendritic spine plasticity observed in the mature cortex.**

Recent studies using two-photon microscopy have shown markedly different turnover rates for dendritic spines in normal and sensory-deprived mouse cortex<sup>1–6</sup>. In the superficial layer of several cortical regions in adult mice, dendritic spines imaged through a thinned-skull window are remarkably stable with 1–2% turnover over 3 d, ~5% over 1 month and ~20–26% over 19 months<sup>1,5,6</sup>. In contrast, in the same cortical regions, when the skull is removed and replaced with a glass window (open-skull), >20% of adult dendritic spines turn over in 1–4 d and 30–50% turn over in 1–4 weeks<sup>2–4</sup>. These results lead to contradictory views on the structural plasticity of synapses in the mature brain and have different implications for information storage and maintenance in neuronal circuits<sup>7,8</sup>.

To determine the possible factors contributing to the differences in spine turnover seen in previous reports, we measured dendritic spine dynamics through either open-skull or thinned-skull windows in the barrel cortex of adult transgenic mice expressing yellow fluorescent protein (YFP) in layer V pyramidal neurons. The surgical procedures, two-photon imaging and data quantification were done as previously described<sup>1–6</sup> (**Supplementary Methods** online). Prior studies with open-skull windows usually started imaging ~1–2 weeks after surgery, presumably because of the increased opacity of the preparation soon after craniotomy<sup>2–4</sup>. We found that 1–2 weeks (12.3 ± 0.8 d) after craniotomy, open-skull preparations in ~30% (19/59) of the mice became transparent, and allowed imaging of spines with a signal-to-noise ratio that was comparable to that of thinned-skull preparations. When these mice (4.1 ± 0.9 months of age) were imaged over the subsequent days to weeks, we found that spine turnover in the barrel cortex was 9.9 ± 2.4% over 1–2 d, 19.8 ± 4.1% over 7 d, 24.7 ± 4.0% over 2 weeks and 34.3 ± 4.1% over 1 month (**Fig. 1a,b**; an average of 7 animals and 1,200 spines were analyzed for each time interval;

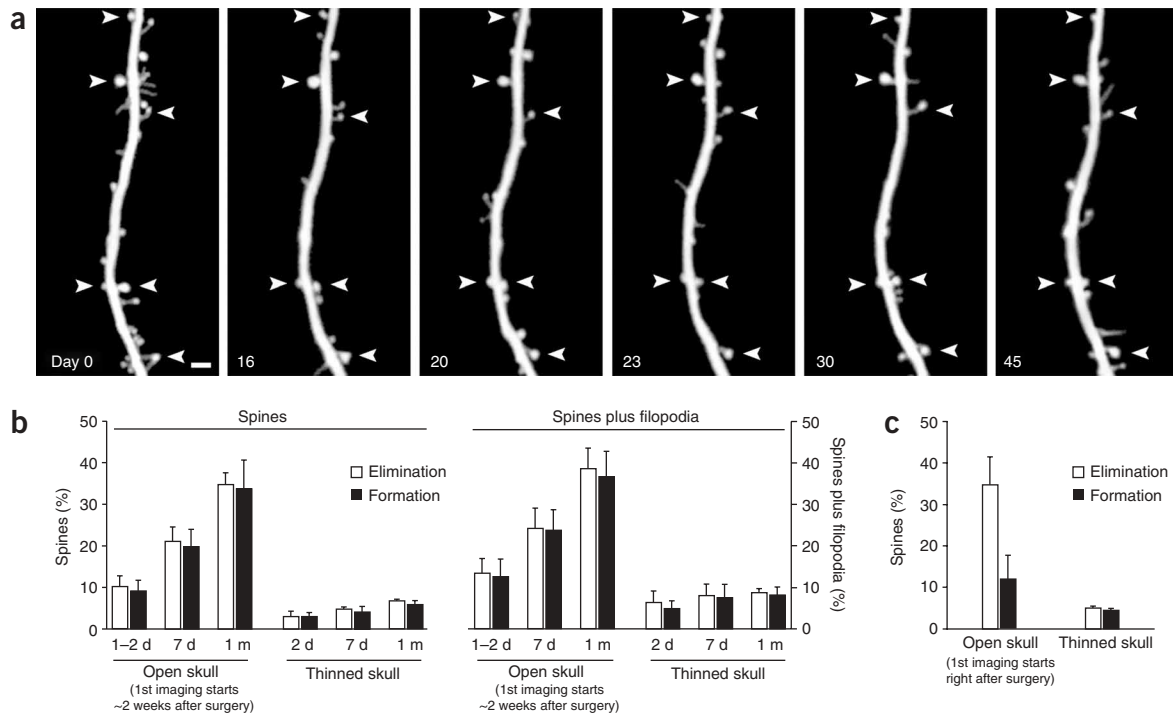
**Supplementary Fig. 1** online). Furthermore, spine turnover over 7 d was 21.5 ± 3.5% in mice at 2–3 months of age and 17.1 ± 3.7% at 7 months of age, suggesting that there was only a slight decrease in spine dynamics in mature adults. In addition, we found a small percentage of transient filopodia in the open-skull preparations 2 weeks after craniotomy (4.2 ± 2.6% of dendritic protrusions, 1,620 protrusions analyzed from 11 animals). The high turnover rates of total dendritic protrusions (spines and filopodia, **Fig. 1b**) that we observed over a period of days or weeks are comparable to those reported previously with open-skull imaging in mice at similar ages<sup>2–4</sup>, but are in sharp contrast to the low turnover rates of adult spines (~5% over a month) reported with thinned-skull windows<sup>1,5,6</sup>.

Consistent with previous studies of spine dynamics using thinned-skull windows<sup>1,5,6</sup>, we found that the spine turnover measured through a thinned skull was 2.5 ± 0.5% over 2 d, 4.4 ± 0.4% over 7 d, 4.9 ± 0.1% over 2 weeks and 6.4 ± 0.5% over 1 month in the barrel cortex of mice at ~6 months of age (**Fig. 1b**, an average of 4 animals and 570 spines were analyzed for each time interval; **Supplementary Fig. 1**). We found that ~3.1 ± 2.1% of dendritic protrusions were long and thin filopodia in the thinned-skull preparations (11 adult mice), as has also been reported previously<sup>1,5,6</sup>. Previous thinned-skull imaging studies distinguished dendritic spines from filopodia<sup>1,5,6</sup>, whereas open-skull imaging studies did not<sup>2–4</sup>. However, because only a small percentage of filopodia exist in open-skull or thinned-skull preparations, we found that there was an approximately fivefold difference in the turnover rate of either spines or total protrusions measured with the two imaging methods, regardless of whether or not filopodia were included in the analysis (**Fig. 1b**). Because the parameters, such as transgenic mice, cortical regions, imaging settings and data quantification, were the same or nearly identical in all of our experiments, our results strongly suggest that the use of either open- or thinned-skull windows for imaging leads to the different spine dynamics seen in previous reports<sup>1–6</sup>.

To better understand how the choice of cranial window type affects spine dynamics, we examined spine formation and elimination in adult barrel cortex during various time periods after surgery. Within the 2 d after the open-skull surgery (before the open-skull window becomes opaque), we found that spine elimination and formation were 7.2 ± 1.3% and 5.1 ± 1.4%, respectively, significantly higher than those under the thinned-skull window (both ~2.5%) ( $P < 0.0002$ ). Over the first 1–2 weeks (12.3 ± 0.8 d) after craniotomy, the percentage of spine elimination (34.6 ± 3.3%) was significantly higher than that of spine formation (12.0 ± 1.3%,  $P < 0.0002$ , 1154 spines, 9 mice at ~5 months of age; **Fig. 1c**). In contrast, the rates of spine elimination and formation over 2 weeks under thinned-skull windows were comparable (elimination, 5.2 ± 0.3% and formation, 4.6 ± 0.4%;  $P > 0.2$ , 635 spines, 4 animals; **Fig. 1c**). Thus, the open-skull window, but not the thinned-skull window, is associated with a ~22% net loss of adult spines in the first 2 weeks after surgery. Consistent with this

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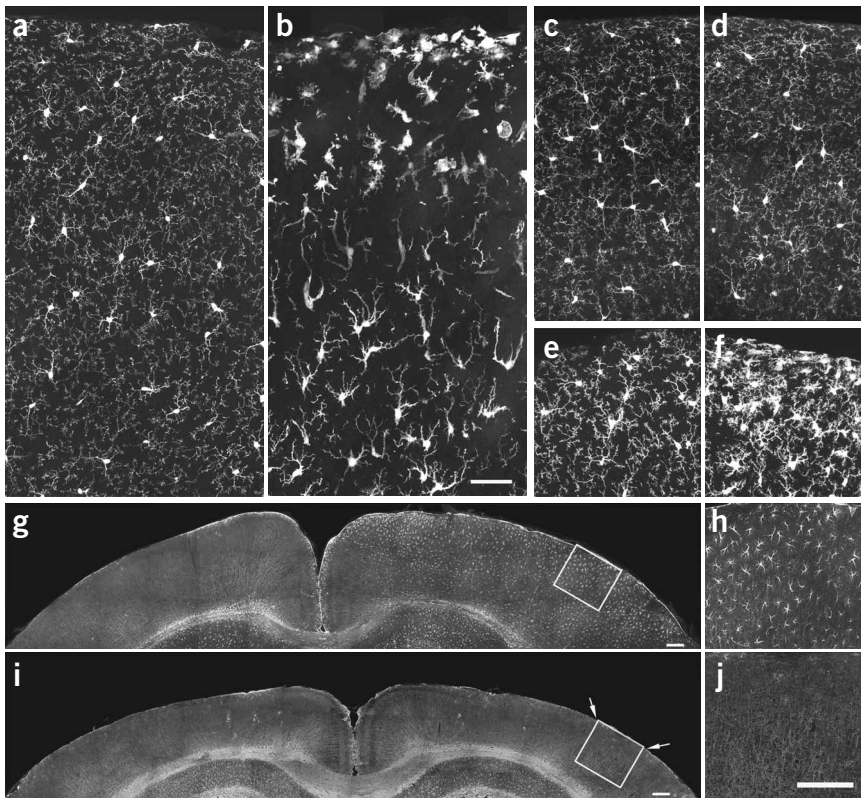
**Figure 1** High spine turnover was associated with the open-skull window. **(a)** Repeated imaging through an open-skull window revealed a high turnover of spines over 45 d in the barrel cortex. The first image (day 0) was acquired immediately after the implantation of a glass window. Arrows indicate spines that were present in all of the images. **(b)** Percentage of spines eliminated or formed as a function of viewing intervals. The turnover of spines or total protrusions over days to weeks was significantly higher under the open-skull window than under the thinned-skull window. Spine imaging through open-skull windows started ~2 weeks after craniotomy, whereas imaging through thinned-skull started immediately after surgery. The dynamics of both spines and total protrusions (spines and filopodia) were plotted for comparison with previous studies under thinned- and open-skull windows. **(c)** Percentage of spines eliminated or formed over 2 weeks under open-skull and thinned-skull conditions. Here spine imaging through both open- and thinned-skull windows was done immediately after surgery and then 10–14 d later. Within the first 2 weeks after open-skull surgery, spine elimination was significantly higher than spine formation, resulting in a substantial loss of spines in adult mice (**Supplementary Fig. 2**). Scale bar, 2  $\mu$ m **(a)**.

finding, we found that spine density decreased from  $0.37 \pm 0.02$  spines per  $\mu$ m to  $0.25 \pm 0.03$  spines per  $\mu$ m within 2 weeks of open-skull surgery (9 animals,  $160 \pm 21$  spines per animal; **Supplementary Fig. 2** online). These results help to explain why spine densities that were reported previously using the open-skull window (1–2 weeks after surgery)<sup>2–4</sup> are not only highly variable, but also lower than those reported using a thinned-skull window<sup>1,5,6</sup>. Lastly, we found that at 3 weeks after craniotomy, spine turnover over 1 week ( $18.2 \pm 2.3\%$ , 494 spines, 4 animals) was still significantly higher under open-skull windows than that under thinned-skull windows. Two months after open-skull surgery, spine turnover over 2 d ( $4.8 \pm 0.1\%$ , 700 spines, 3 animals) was close to that observed under thinned-skull conditions, suggesting that the effects of craniotomy on spine dynamics subside by 2 months, but not 3 weeks, after surgery. Together, these results indicate that the use of an open-skull window, but not a thinned-skull window, is associated with a substantial net loss of spines in the first 2 weeks after surgery and, subsequently, a high spine turnover that lasts for at least an additional 3–4 weeks.

In addition to the time-dependent changes in spine dynamics in the first 1–2 months after craniotomy, we also found that the open-skull window was associated with the extensive activation of microglia and astrocytes. Within 1–2 d after open-skull surgery, microglia in the superficial layers had either assumed amoeboid shapes or extended most of their processes toward the pial surface (**Fig. 2a–d**, **Supplementary Fig. 3** online, 6 animals). We also observed the activation of astrocytes in the cortical areas surrounding the open-skull window, as

indicated by increased expression of glial fibrillary acidic protein (GFAP) (**Supplementary Fig. 4** online). Furthermore, 10 d after surgery, the densities of microglia in cortical layer I/II of the open-skull side of the brain were  $62 \pm 25\%$  higher than those on the contralateral side (**Fig. 2e,f**, **Supplementary Table 1** online, 3 animals). Extensive GFAP expression in astrocytes was observed throughout the entire cortical hemisphere containing the open-skull window 10–20 d after surgery (**Fig. 2g,h**, **Supplementary Fig. 5** online, 4 animals for each time point). Thirty days after the craniotomy, the density of microglia was comparable between the open-skull and contralateral sides, whereas GFAP expression in astrocytes was mainly restricted to the superficial layer located under the open-skull window (**Supplementary Fig. 5**, 2 mice), suggesting that glial activation subsided at this stage. Notably, when the thinned-skull surgery was properly performed, with a skull thickness of ~20  $\mu$ m after thinning (**Supplementary Methods** online), no obvious signs of activation of microglia and astrocytes were observed 2 or 10 d after surgery (**Fig. 2c,d,i,j**, **Supplementary Fig. 3–4**, 4 animals for each time point). Because glia are important in regulating synaptic strength and structure<sup>9–11</sup>, these results raise the possibility that extensive activation of glia, which can last for at least 4 weeks, may be linked to the substantial changes in dendritic spine turnover that are observed after open-skull surgery.

In summary, we show that imaging through an open-skull, but not a thinned-skull, cranial window is associated with high spine turnover and extensive glial activation that lasts for at least 4 weeks after surgery. Previous studies with the thinned-skull window showed that spines in



**Figure 2** Extensive glial activation is observed after open-skull surgery but not thinned-skull surgery. (**a,b**) Two days after open-skull surgery, GFP-labeled microglia on the contralateral control side of the barrel cortex appeared normal, with many ramified branches projected from somata (**a**). Microglia under the open-skull window appeared abnormal, having no or few processes or extending most of their branches toward the pial surface (**b**). (**c,d**) Two days after thinned-skull surgery, GFP-labeled microglia appeared normal both on the contralateral control side (**c**) and under the thinned-skull window (**d**). (**e,f**) Ten days after craniotomy, microglia still assumed reactive phenotypes with lower densities on the contralateral control side (**e**) compared with those under the open-skull window (**f**; **Supplementary Table 1**). (**g,h**) Ten days after craniotomy, immunostaining showed little GFAP expression in astrocytes on the contralateral control side (**g**, left hemisphere) but extensive GFAP expression in the entire hemisphere of the cortex subjected to open-skull surgery (**g**, right hemisphere). A higher-magnification view of GFAP staining (box in **g**) is shown in **h**. (**i,j**) Ten days after thinned-skull surgery, little or no GFAP expression in astrocytes was found under the thinned-skull window (between arrows, **i**) or on the contralateral control side (left hemisphere). The boxed region in **i** is shown in **j**. Scale bars, 50  $\mu\text{m}$  (**a-f**); 200  $\mu\text{m}$  (**g-j**).

adult mice are highly stable in different cortical regions and that sensory deprivation for weeks prevents spine loss in adolescent mice, but has no significant effect on adult spine turnover<sup>1,5,6</sup>. On the other hand, studies using an open-skull window suggest that there is a very high and variable spine turnover in different sensory cortices and that sensory deprivation for days further increases spine turnover in adulthood<sup>2-4</sup>. Our findings suggest that the choice of cranial window type for *in vivo* imaging is the major factor contributing to previous discrepancies observed in spine dynamics under both normal and sensory deprivation conditions (**Supplementary Notes** online). Further work, however, is needed to fully resolve and provide mechanistic insights into the differences in spine turnover that are measured under different cranial windows. We believe that an open-skull preparation is valuable because certain experiments cannot be done without using it<sup>12,13</sup>. Nevertheless, it is important to characterize the degree and duration of the structural and functional consequences associated with the open-skull window to best use such a preparation<sup>14,15</sup>. It is also important to note that under the thinned-skull window, various factors such as skull thinning, phototoxicity, anesthesia and inclusion of filopodia as spines may all result in artifactual changes of spine dynamics and contribute to measured spine turnover. The effects of these factors on spine dynamics remain to be determined, but seem to be rather limited under current experimental protocols, as only ~2–3% of spines change over 2 d under the thinned-skull window. Lastly, although the vast majority of spines are remarkably stable over months under thinned-skull windows, the progressive increase in spine turnover with time suggests that a certain degree of spine dynamics exists in the adult cortex<sup>1,5,6</sup>. Future studies are required to investigate the mechanisms underlying both the stability and plasticity of synaptic connections under normal and pathological conditions.

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

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#### AUTHOR CONTRIBUTIONS

H.-T.X., F.P. and G.Y. contributed equally to this work. All of the authors contributed to the experimental design. G.Y., F.P. and H.-T.X. did the *in vivo* imaging experiments and the data analysis. H.-T.X. did the immunostaining and imaging of glial cells. W.-B.G. initiated the project, contributed to the initial experiments and wrote the manuscript. H.-T.X., F.P. and G.Y. discussed the results, made the figures and commented on the manuscript.

#### COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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