Anesthesia Induces Shifts in Spatial Frequency Preference in the Primary Visual Cortex

Jiahao Wu[1,2,3], Taisuke Yoneda[1,2], Kallum Robinson[4,5], Naotsugu Tsuchiya[4,6,7,8,9], Yumiko Yoshimura[1,2] †

[1.] Physiological Sciences Program, Graduate Institute for Advanced Studies, SOKENDAI, Okazaki, Japan.

[2.] Division of Visual Information Processing, National Institute for Physiological Sciences, Okazaki, Japan.

[3.] Institute of Neuroscience, Key Laboratory of Brain Cognition and Brain-Inspired Intelligence Technology, Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences, Shanghai, China.

[4.] School of Psychological Sciences, Faculty of Medicine, Nursing and Health Science, Monash University, Victoria, Australia.

[5.] Berlin School of Mind and Brain, Faculty of Philosophy, Humboldt-Universität zu Berlin, Berlin, Germany

[6.] Turner Institute for Brain and Mental Health, Faculty of Medicine, Nursing and Health Science, Monash University, Victoria, Australia.

[7.] Center for Information and Neural Networks (CiNet), National Institute of Information and Communications Technology (NICT), Osaka, Japan.

[8.] Advanced Telecommunications Research Computational Neuroscience Laboratories, Kyoto, Japan.

[9.] Theoretical Sciences Visiting Program (TSVP), Okinawa Institute of Science and Technology Graduate University, Onna, 904-0495, Japan.

† correspondence to yumikoy@nips.ac.jp

Abstract

General anesthesia markedly reduces overall brain responsiveness to external stimuli. Animal studies reveal that in individual neurons of the primary visual cortex (V1), anesthesia modulates the gain of neural responses while leaving orientation and direction tuning unaltered. However, the impact of anesthesia on spatial frequency (SF) tuning is largely unexplored despite it being a fundamental characteristic of visual perception. Furthermore, compared to excitatory neurons, the effects of anesthesia on the visual responsiveness of specific inhibitory neuron subtypes are poorly understood. Here, we directly compared visual responses from the same V1 neurons in mice under both anesthetized and awake states. We found that isoflurane anesthesia significantly shifts the preferred SFs of excitatory neurons to lower values. This downward shift was even more pronounced in inhibitory neurons. These downshifts selectively impaired the neural coding of high-SF information while leaving low-SF coding unaffected. We observed distinct patterns of anesthesia-induced downward shifts of preferred SFs between two major cortical inhibitory neuron subtypes: somatostatin-expressing (SOM) and parvalbumin-expressing (PV) neurons. Furthermore, anesthesia-induced changes in response gain and tuning sharpness were evident in SOM neurons but not in PV neurons. These results highlight the diverse effects of anesthesia on sensory responses, varying significantly based on both the visual feature processed and the specific neuron subtype involved. The consistent decrease in preferred SFs during anesthesia not only suggests a potential mechanism for anesthesia-induced alterations in sensory experience but also emphasizes the critical role of heightened SF preference during wakefulness for fine object perception.

Introduction

General anesthesia is indispensable for the safe and humane execution of surgical, diagnostic, and therapeutic procedures. It produces an acute state of unconsciousness and unresponsiveness to external stimuli, representing one of the most extreme shifts in behavioral states. Extensive research has explored anesthesia's effects and mechanisms, from identifying target molecules and neural pathways to understanding neuronal population dynamics and its relation with fluctuations in the level of consciousness (Alkire et al., 2008; Franks, 2008; Schwartz et al., 2010; Brown et al., 2011; Koch et al., 2016; Mashour, 2024). Notable studies in rodents have demonstrated that multiple types of general anesthesia induce synchronous spontaneous activity across local and inter-regional neuronal populations, as revealed by electrophysiological and optical imaging techniques (Steriade et al., 1993; Greenberg et al., 2008; Afrashteh et al., 2021; Nivinsky Margalit et al., 2022; Horváth et al., 2024).

A critical step in understanding the considerable reduction of sensory perception under anesthesia involves characterizing precisely which sensory encodings are affected. It has been reported in humans and mice that while higher sensory cortical areas exhibit a significant reduction in their sensory responses under anesthesia, primary sensory cortices are less profoundly affected, even at similar anesthetic levels (Krom et al., 2020; Nivinsky Margalit et al., 2022).

The rodent primary visual cortex (V1), with its well-characterized receptive field properties, is thus a suitable area for cell-level changes in primary cortical sensory responses induced by anesthesia. Previous studies report that orientation selectivity in V1 layer 2/3 is not affected under either urethane or isoflurane anesthesia (Niell and Stryker, 2010a; Goltstein et al., 2015; Durand et al., 2016). In contrast, size tuning and spatial selectivity in V1 neurons are reported to decrease under isoflurane anesthesia (Adesnik et al., 2012; Haider et al., 2013). These results suggest that the effects of anesthesia are visual parameter-specific. Yet, how anesthesia modulates spatial frequency (SF) tuning, a fundamental component of vision, has received little attention. Since SF preference is known to vary with receptive field and laminar location (Ji et al., 2015; Zhang et al.,

2015; Salinas et al., 2017), isolating the effects of anesthesia necessitates tracking the same neurons across both awake and anesthetized conditions.

Previous studies in rodent V1 indicate that under isoflurane anesthesia, the response magnitude of excitatory neurons remains largely unchanged, while that of inhibitory neurons decreases (Adesnik et al., 2012; Kimura et al., 2014). Additionally, visual responses are prolonged during isoflurane- or urethane-anesthesia, possibly due to decreased inhibition (Haider et al., 2013; Aasebø et al., 2017). These findings suggest that inhibitory neurons are generally more sensitive to anesthesia than excitatory neurons. Cortical inhibitory neurons comprise multiple subtypes, each expressing different channel molecules sensitive to anesthesia (Franks, 2008; Speigel and Hemmings, 2022). Furthermore, each subtype possesses distinct connectivity and functions (Kawaguchi and Kubota, 1997; Markram et al., 2004; Defelipe et al., 2013; Pfeffer et al., 2013). Consequently, the effect of anesthesia on sensory responses may differ across individual interneuron subtypes (Adesnik et al., 2012). However, these subtype-specific effects of anesthesia have not yet been systematically studied.

In this study, we directly compared visual responses recorded from the same V1 neurons in mice across anesthetized and awake states using two-photon Ca^{2+} imaging. To evaluate neuron type-specific effects of anesthesia, we utilized transgenic mice to label pan-inhibitory neurons, somatostatin-expressing (SOM) and parvalbumin-expressing (PV) interneurons. We found that anesthesia induced a shift in preferred SF to lower frequencies, irrespective of neuron subtype, and that the degree and pattern of SF shift were distinct for each subtype.

Results

Anesthesia Decreased Preferred Spatial Frequencies

To investigate how anesthesia affects visual response properties, we compared visual responses recorded from the same neurons in layer 2/3 of mouse V1 under both isoflurane-anesthetized (0.7–0.9%) and awake conditions. To this end, we used two-photon Ca^{2+} imaging. GCaMP7b was

expressed pan-neuronally using AAV vectors. Glutamatergic excitatory neurons were distinguished from GABAergic interneurons (pan-inhibitory neurons) which were labeled with red fluorescence in Vgat-Cre;Ai14 mice (N = 9 mice; Fig. 1A). We recorded visual responses to full-field drifting sinusoidal grating stimuli, varying across five SFs and eight directions (Fig. 1B). For quantification, we selected neurons responsive to visual stimuli in both states ($\Delta F/F > 25\%$, ANOVA $p < 0.01$; 610 excitatory cells, 111 inhibitory cells).

In the excitatory neurons, anesthesia decreased preferred SFs by 0.15 octaves ($p = 7\times10^{-45}$, linear mixed-effects model; Fig. 2A). The differences in preferred SFs were statistically significant as revealed by posthoc tests (median: anesthetized 0.059 c/d, awake 0.074 c/d; $p = 3\times10^{-25}$, Wilcoxon signed-rank test; $p = 6\times10^{-3}$, Kolmogorov–Smirnov test, n = 610 cells; Fig. 2A). However, preferred orientation did not considerably change between anesthesia and wakefulness (Fig. S1), indicating that anesthesia specifically modified SF preference. To rule out order effects, visual responses were also recorded in five of nine mice after full recovery from anesthesia (>4h in the home cage; Fig. S2). Preferred SFs did not differ between the initial awake session and the recovered condition (second awake session) ($p = 0.87$, Wilcoxon signed-rank test; $p = 0.97$, Kolmogorov–Smirnov test, n = 314; Fig. S2B). Thus, the anesthesia-induced changes in SF tuning were not due to the recording order or representational drift (Clopath et al., 2017; Rule et al., 2019).

Next, we examined whether similar anesthetic effects occurred in inhibitory neurons. On average, preferred SFs in inhibitory neurons decreased by 0.42 octaves under anesthesia compared to wakefulness ($p = 2\times10^{-21}$, linear mixed-effects model, Fig. 2B). This decrease was statistically significant (median: anesthetized 0.079 c/d, awake 0.13 c/d; $p = 4\times10^{-8}$, Wilcoxon signed-rank test; $p = 1\times10^{-5}$, Kolmogorov–Smirnov test, n = 111; Fig. 2B). A significant downward shift in preferred SF during anesthesia compared to the awake state was observed in excitatory neurons (34%) and in inhibitory neurons (45%) (bootstrap analysis, Fig. 2E, Fig. S3A–E). The proportion for inhibitory neurons was significantly greater than that for excitatory neurons ($p = 0.01$, Chi-

square test, Fig. 2E, S3F). These results suggest that anesthesia modulates the SF preference of inhibitory neurons more strongly than excitatory neurons.

Anesthetic Effects on Inhibitory Neuron Subtypes

We further investigated whether anesthesia-induced modulation varied across inhibitory neuron subtypes (Kawaguchi and Kubota, 1997; Markram et al., 2004; Defelipe et al., 2013). We focused on somatostatin-expressing (SOM) and parvalbumin-expressing (PV) neurons, the two major classes in mouse V1 that form monosynaptic inhibitory synapses onto excitatory neurons (Kawaguchi and Kubota, 1997; Markram et al., 2004; Defelipe et al., 2013; Pfeffer et al., 2013). SOM neurons were labeled using SOM-Cre;Ai14 mice (N = 5), and PV neurons with PV-Cre;Ai14 mice (N = 6).

The SOM neurons displayed significantly lower preferred SFs under anesthesia compared to the awake condition (median: anesthetized 0.046 c/d, awake 0.068 c/d; $p = 3\times10^{-9}$, Wilcoxon signed-rank test, $p = 4\times10^{-5}$, Kolmogorov–Smirnov test, n = 60 cells; Fig. 2C, Fig. S4A). On average, preferred SFs decreased by 0.49 octaves after anesthesia ($p = 8\times10^{-14}$, linear mixed-effects model). The PV neurons also showed a significant decrease in preferred SFs under anesthesia (median: anesthetized 0.13 c/d, awake 0.22 c/d; $p = 2\times10^{-6}$, Wilcoxon signed-rank test, $p = 3\times10^{-7}$, Kolmogorov–Smirnov test, n = 33 cells; Fig. 2D, Fig. S4B). The preferred SFs in the PV neurons were considerably higher than those in the SOM neurons, regardless of brain state (Fig. 2C–D, Fig. S4C). However, the proportion of neurons exhibiting SF downshifts during anesthesia was not considerably different between the SOM and the PV neurons (Fig. 2E). Taken together, these findings indicate that anesthesia commonly modulates SF preference across different neuron subtypes.

Anesthetic Effects on the Neuronal Population Coding

To investigate the effect of anesthesia on population coding of visual stimuli, we performed a

neural decoding analysis using a Bayesian decoder. A decoder was trained on visual responses from both excitatory and inhibitory neuron populations in the awake state and subsequently used to decode visual stimuli from neuronal activity during both awake and anesthetized conditions. We found that while decoding errors for low-SF stimuli (≤ 0.04 c/d) were comparable between the two states, errors for high-SF stimuli (> 0.04 c/d) were significantly higher under anesthesia (Fig. 3A). This impairment was specific to spatial frequency, as decoding accuracy for orientation remained similar between the two conditions (Fig. 3B and D). Notably, the errors under anesthesia were biased, with an increased probability of decoding a higher SF than the actual stimulus presented (Fig. 3B–C). As a control, all stimuli were decoded with high fidelity from responses in the awake state (Fig. 3B–D). Taken together, these results strongly suggest that anesthesia selectively impairs the neural coding of high-SF information.

Characterization of Preferred SF-Shift Patterns

The anesthesia-induced downward shift of the optimal spatial frequency (SF) can be attributed to two potential factors: a reduced response to high SFs and/or an enhanced response to low SFs. To investigate this possibility, we calculated the average population responses to all tested SFs for neurons that exhibited significant downward SF shifts under anesthesia compared to the awake state. In excitatory neurons, the downward shift primarily resulted from an increased response to low SF stimuli under anesthesia, rather than a decreased response to high SFs (Fig. 4A). Conversely, in pan-inhibitory neurons, the downward shift was attributed to a specific reduction in responses to high SFs (Fig. 4B). Analysis of inhibitory neuron subtypes revealed that the population response to high SFs in the SOM neurons selectively decreased in the anesthetized condition (Fig. 4C). In contrast, the response tuning of the PV neurons shifted comprehensively toward lower frequencies during anesthesia (Fig. 4D). Therefore, while the excitatory, SOM, and PV neurons all exhibited a preference for lower SFs during anesthesia, the patterns underlying these shifts appear to be distinct.

Modification of Tuning Sharpness and Response Magnitude

To assess how anesthesia affects the sharpness of SF selectivity, we calculated the full width at half maximum (FWHM) of the SF tuning curve in the optimal direction for individual neurons. The excitatory neurons showed a significantly broader SF tuning width in the anesthetized condition (median: 1.7 octaves) compared to wakefulness (1.6 octaves), indicating that SF selectivity became broader during anesthesia ($p = 3\times10^{-6}$, Wilcoxon signed-rank test; Fig. 5A). However, we found no significant differences in orientation or direction selectivity of the excitatory neurons between anesthesia and awake states ($p = 0.43$ for orientation selectivity, $p = 0.50$ for direction selectivity; Fig. S5A and E), consistent with previous studies (Niell and Stryker, 2010b; Kimura et al., 2014; Durand et al., 2016). These results suggest that anesthesia specifically modifies SF tuning more than orientation and direction tuning in excitatory neurons.

The pan-inhibitory neurons displayed no significant differences in the sharpness of SF and direction selectivity between the awake and anesthetized conditions, but a significant difference in orientation selectivity ($p = 0.458$ for SF tuning, $p = 1\times10^{-4}$ for orientation selectivity, $p = 0.54$ for direction selectivity, Wilcoxon signed-rank test; Fig. 5B, Fig. S5B and F). Interestingly, and in contrast to excitatory neurons, the SOM neurons exhibited significantly sharper SF tuning (i.e., a smaller FWHM) under anesthesia than during awake state (median SF bandwidth: 1.6 octaves for anesthetized, 2.2 octaves for awake; $p = 4\times10^{-6}$, Wilcoxon signed-rank test, Fig. 5C). The SOM neurons also exhibited sharper selectivity for orientations and directions during anesthesia ($p = 0.002$ for orientation selectivity, $p = 0.009$ for direction selectivity; Fig. S5C and G). On the other hand, the PV neurons showed significantly broader SF tuning during anesthesia than during wakefulness (median SF bandwidth: 2.0 octaves for anesthetized1.7 octaves for awake; $p = 0.02$, Wilcoxon signed-rank test, Fig. 5D). The PV neurons showed no significant differences in the sharpness for orientation and direction between the anesthetized and awake conditions ($p = 0.99$ for orientation selectivity; $p = 0.37$ for direction selectivity, Fig. S5D, H). These results

demonstrate that the SOM neurons, but not the PV neurons, exhibited sharper visual tuning during anesthesia compared to wakefulness.

Regarding response magnitude, the responses to preferred visual stimuli during anesthesia slightly increased in the excitatory neurons compared to wakefulness ($p = 1\times10^{-5}$, Wilcoxon signed-rank test; Fig. 5E) and decreased in the pan-inhibitory neurons (Fig. 5F) ($p = 0.01$, Wilcoxon signed-rank test). In the SOM neurons, visual response magnitude significantly decreased during anesthesia ($p = 1.5\times10^{-5}$, Wilcoxon signed-rank test, Fig. 5G), consistent with previous electrophysiological recordings (Adesnik et al., 2012). Conversely, the PV neurons showed no significant difference in response strength between the awake and the anesthetized conditions ($p = 0.70$; Wilcoxon signed-rank test, Fig. 5H), which also aligns with a previous electrophysiological analysis (Adesnik et al., 2012). Taken together, these findings suggest that visual responses in the SOM neurons are more sensitive to anesthesia.

The Effect of Arousal Levels on Visual Responses

We finally examined whether preferred SFs fluctuate during wakefulness due to transient modulation in arousal levels. We used pupil size as a proxy for arousal levels(Vinck et al., 2015; Mineault et al., 2016). Within awake sessions, trials were categorized as "low arousal" if the average pupil size during visual stimulation was in the lower 25% of all trials, and "high arousal" if in the upper 25% (Fig. 6A). Visual responses were analyzed in excitatory neurons obtained from Vgat-Cre;Ai14 mice in which pupil size can be well analyzed (N = 6 mice, 216 cells).

The magnitude of visual responses in excitatory neurons was significantly smaller during low-arousal than during high-arousal trials, consistent with previous research ($p = 3\times10^{-7}$, Wilcoxon signed-rank test with Bonferroni correction; Fig. 6B)(Mineault et al., 2016). Furthermore, the response magnitude under anesthesia was significantly larger than that during low-arousal trials but was comparable to that during high-arousal trials. We then compared the preferred SFs of the excitatory neurons in the high and low arousal states with those measured under the anesthetized

condition. The preferred SFs were significantly higher in both low and high arousal states than during anesthesia (Fig. 6C-D). The preferred SFs did not differ significantly between the high and low arousal states, a finding consistent with previous research(Mineault et al., 2016). Therefore, the downward shift in preferred SFs appears to be a specific effect of anesthesia.

SF tuning sharpness was significantly lower (i.e., a larger FWHM) in the anesthetized state than in the low-arousal state ($p = 2\times10^{-5}$, Wilcoxon signed-rank test with Bonferroni correction, Fig. 6E). However, SF tuning sharpness was similar between the anesthetized and high-arousal states ($p > 0.99$), consistent with the corresponding changes in response magnitude.

Discussion

By directly comparing visual responses in the same layer 2/3 neurons of V1 between the awake and anesthetized states, we demonstrate a systematic downward shift in preferred SF under isoflurane anesthesia. While this downward shift was observed in both excitatory and inhibitory neurons, the effect was more pronounced in inhibitory neurons, affecting both the SOM and PV interneuron subtypes. In line with these cellular-level observations, population decoding analyses revealed that the fidelity of representing high-SF stimuli was compromised under anesthesia, whereas the representation of low-SF stimuli remained robust.

In excitatory neurons, the anesthesia-induced downshift in optimal SF was accompanied by a broadening of SF tuning. Conversely, and in agreement with previous reports, anesthesia did not alter the preference or tuning sharpness for orientation and direction in excitatory neurons (Niell and Stryker, 2010; Durand et al., 2016). Moreover, among inhibitory neuron subtypes tested, anesthesia-induced changes in response gain and tuning sharpness were specific to the SOM neurons, and largely absent in the PV neurons. Notably, optimal SFs were significantly higher in both low and high arousal awake states compared to the anesthetized condition. Taken together, these results indicate that anesthesia universally drives SF preference toward lower frequencies across neuronal subtypes, but modulates response gain and tuning sharpness in a highly subtype-

specific manner.

Anesthetic Modulation of SF Preference

Distinct anesthetic agents may induce various modulations in visual responses. Previous electrophysiological work in rat V1 has documented a decrease in visual responses to high SFs under isoflurane anesthesia compared to the awake state (Aasebø et al., 2017). Our data are consistent with this, demonstrating that isoflurane-induced downshifts in preferred SFs occur at the level of individual, identified neuron types in layer 2/3 of mouse V1. However, a previous study has shown no significant change in the preferred SFs of mouse V1 neurons between urethane-anesthetized and awake states (Durand et al., 2016). This suggests that the anesthesia-induced modulation of receptive field properties may depend on the choice of anesthetics. Another potential explanation for the different effects of anesthesia in the previous and current studies is that the earlier study did not restrict its analysis of preferred SFs to layer 2/3.

Ketamine, a dissociative anesthetic known for inducing hallucinations, exhibits distinct effects on cortical spontaneous activity patterns compared to isoflurane (Michelson et al., 2018; Aggarwal et al., 2024). Furthermore, its primary molecular target is the NMDA receptor, which, despite some overlap, differs considerably from isoflurane's primary target of $GABA_A$ receptors (Franks and Lieb, 1994). Therefore, comparing awake and ketamine-anesthetized states may reveal different changes in SF selectivity than those observed between awake and isoflurane-anesthetized states.

Potential Circuit Mechanisms for State-Dependent Changes in SF Preference

Clarifying the mechanisms that regulate spatial frequency (SF) preference across brain states remains challenging, as anesthesia's effects are widespread and not limited to a specific neuronal population or specific circuits in V1. For instance, anesthetic-sensitive channels are expressed differentially across neuron subtypes (Speigel and Hemmings, 2022). Similarly, brain-state-

dependent neuromodulators exert cell- or synapse-type-specific influences (Luhmann, 2023). These specific sensitivities to both anesthetics and neuromodulators likely represent a primary mechanism for modulating neural activity. Consequently, these individual neuronal effects likely coalesce at the circuit level to produce the observed modulation of SF preference.

Acknowledging the challenges inherent in elucidating SF modulation in V1, the mechanisms potentially involved encompass peripheral inputs, local neural circuits, and top-down inputs.

The contribution of peripheral inputs seems unlikely, as visual responses in the lateral geniculate nucleus are similar between the anesthetized and awake states (Durand et al., 2016).

During wakefulness, excitatory neurons showed reduced responses to low SFs, shifting their preference toward high SFs. Although the SOM neuron responses were enhanced during wakefulness, this is unlikely to explain the suppression of low SF responses in excitatory neurons. This is because the enhanced SOM neuron activity was specific to high SFs, while their responses to low SFs remained unchanged.

Top-down inputs from higher cortical areas are known to be considerably stronger during wakefulness compared to anesthesia (Alkire et al., 2008; Suzuki and Larkum, 2020). Feedback neurons in the posteromedial area (PM), a higher visual area, preferentially respond to high SF stimuli and provide distinct excitatory inputs to V1 neurons that also prefer high SFs (Andermann et al., 2011; Marshel et al., 2011; Huh et al., 2018). During wakefulness, these feature-selective feedback inputs are likely significantly enhanced compared to anesthesia, potentially modulating the preferred SFs in V1.

In addition, top-down inputs from the cingulate cortex preferentially activate vasoactive intestinal peptide-expressing (VIP) interneurons in V1 (Zhang et al., 2014). Optogenetic activation of VIP neurons leads to an increase in the preferred SFs in excitatory neurons (Ayzenshtat et al., 2016). Since VIP neurons directly inhibit SOM neurons, SOM neuron activity can serve as an inverse proxy for VIP activity. Our observation of elevated SOM neuron responses during wakefulness thus suggests low VIP neuron activity. Therefore, VIP neurons are unlikely to mediate the

wakefulness-dependent increase in preferred SF.

To elucidate the mechanisms of anesthesia-induced changes in SF preference, further investigation is warranted. The evidence of anesthesia- or wakefulness-induced SF modulation could contribute to developing neuronal network models for SF representation in individual neurons(Zhu et al., 2009; Carandini and Heeger, 2012; Baruzzi et al., 2025), potentially leading to the elucidation of circuit mechanisms that cannot be revealed solely through physiological experiments.

Differential Effects of Anesthesia on Visual Responses of SOM and PV Neurons

SOM and PV neurons are functionally distinct in their firing patterns, synaptic targets, and afferent input sources (Kawaguchi, 1997; Markram et al., 2004; Defelipe et al., 2013; Pfeffer et al., 2013). SOM neurons primarily mediate feedback or lateral inhibition and are implicated in the formation of size tuning (Adesnik et al., 2012; Adesnik, 2017) In contrast, PV neurons predominantly drive feedforward inhibition and contribute to response gain regulation and the generation of high-frequency oscillations (Sohal et al., 2009; Chen et al., 2017; Znamenskiy et al., 2024). In this study, we revealed that the preferred SF range of the SOM neurons was consistent with that of excitatory neurons and lower than that of the PV neurons, regardless of brain state (anesthetized or awake). Furthermore, we identified distinct patterns of anesthesia-induced SF shift between the SOM and PV neurons. Consistent with previous electrophysiological investigations (Adesnik et al., 2012), isoflurane anesthesia, relative to the awake state, diminished visual responses in the SOM neurons but not in the PV neurons. Additionally, we observed changes in the tuning sharpness for SF, orientation, and direction in the SOM neurons following anesthesia, whereas the PV neurons displayed almost no such alterations. These results demonstrate that the visual responsiveness of SOM neurons is profoundly modulated by the anesthetized state, which may help elucidate the distinct functional roles of these interneuron subtypes.

Functional Significance

Our finding that isoflurane anesthesia alters SF selectivity strongly suggests that the associated decline in sensory perception stems from a degradation of spatial resolution. This interpretation is consistent with prior work showing that V1 neuron size tuning, a key correlate of spatial resolution, is also impaired under anesthesia (Haider et al., 2013). Taken together, these findings suggest that anesthesia impairs spatial resolution at the level of single neurons in V1.

Furthermore, it remains an open question whether this reduction is specific to isoflurane or is a more general feature of unconsciousness. To clarify this distinction, future experiments are warranted to examine the effects of other anesthetics, such as ketamine, and non-anesthetic states of unconsciousness like sleep (Franks, 2008a; Schwartz et al., 2010; Michelson et al., 2018; Aggarwal et al., 2024). Ultimately, the consistent downward shift of preferred SFs under anesthesia not only reveals a potential mechanism for altered sensory perception but also illuminates that high-SF tuning in the awake V1 is fundamental to perceiving fine visual detail.

Methods

Animals

All experimental procedures were performed in accordance with the guidelines of the Experimental Animal Committee of the National Institute for Physiological Sciences. Mice of either sex, aged 2–5 months (C57BL/6 background), were used. To visualize GABAergic, SOM-positive, and PV-positive interneurons, Vgat-IRES-Cre (B6J.129S6(FVB)-Slc32a1$^{tm2(cre)Lowl}$/MwarJ, The Jackson Laboratory, stock #16962), SOM-IRES-Cre (B6N.Cg-Sst$^{tm2.1(cre)Zjh}$/J; The Jackson Laboratory, stock #013044) and PV-IRES-Cre (B6;129P2-Pvalb$^{tm1(cre)Arbr}$/J, The Jackson Laboratory, stock #008069) mice were respectively crossed with Ai14 tdTomato reporter mice (B6;129S6-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J, The Jackson

Laboratory, stock #007908). The mice were housed under a standard visual environment (12/12 h light/dark cycles) with ad libitum access to food and water.

Virus injection

For surgical procedures, mice were anesthetized with an intraperitoneal (ip) injection of a cocktail containing medetomidine (0.75 mg/kg; Domitor, Zenoaq), midazolam (4.0 mg/kg; Midazolam Sandoz, Sandoz), and butorphanol (5.0 mg/kg; Vetorphale, Meiji Seika). The animals were then head-fixed in a stereotaxic frame (SR-5M-HT, Narishige). A small craniotomy (< 0.4 mm diameter) was performed over the left visual cortex (2.7 mm lateral and 1.0 mm anterior to lambda). A beveled glass micropipette attached to a nanoinjector (Nanoject II, Drummond) was used to inject 400 nL of AAV1-Syn-jGCaMP7b-WPRE (Addgene) into layer 2/3 (400–500 μm from the dural surface). The viral solution was diluted with saline to a final titer of approximately $2–3\times10^{12}$ genome copies/mL. Following the injection, the scalp was sutured. The mice were kept on a heating blanket until fully recovered from anesthesia.

Craniotomy

A cranial window was implanted 10–12 days after virus injection. Mice were anesthetized with an ip injection of a cocktail containing medetomidine (0.75 mg/kg), midazolam (4.0 mg/kg), and butorphanol (5.0 mg/kg). Throughout the surgery, body temperature was maintained with a heating blanket, and ofloxacin ophthalmic ointment was applied to the eyes to prevent corneal desiccation. To reduce inflammation and edema, mice were administered carprofen (6.0 mg/kg, ip), enrofloxacin (6.5 mg/kg, sc), and glycerol (0.15 ml/kg, ip).

The mice were mounted on a stereotaxic frame (SR-5M-HT, Narishige) with ear bars and a bite bar. The scalp overlying the parietal bones was resected, and the exposed skull was cleaned. A 3.5-mm-diameter craniotomy and durotomy were performed over the GCaMP7b expression site. A double-coverslip window was constructed by adhering a 3-mm-diameter coverslip (#3 thickness, Matsunami) to a 5-mm-diameter coverslip (#1 thickness, Matsunami) using a UV-curable optical adhesive (NOA61, Norland). This assembly was placed in the craniotomy. The

edge of the window was then sealed to the surrounding skull first with cyanoacrylate adhesive (Aron Alpha A, Sankyo) and then reinforced with dental resin cement (Super-Bond C&B, Sun Medical). Finally, a custom-made head plate was attached to the skull using the same dental cement. The head plate was positioned to be as parallel as possible with the coverslip surface. Mice were monitored on a heating pad until they had fully recovered from anesthesia.

Wide-Field Calcium Imaging

For wide-field Ca^{2+} imaging, visual stimuli were generated using PsychoPy (Peirce, 2009) and presented on a 23-inch LCD monitor (FS2333, EIZO). The monitor was placed 20 cm from the mouse's right eye, at a 45° angle to the right of the anteroposterior axis to be parallel to the right eye. Retinotopy mapping utilized a sweeping bar composed of a flickering black-and-white checkerboard pattern (Kalatsky et al., 2003). Each 15° square of the checkerboard inverted its contrast at 2 Hz. The bar was swept across the screen four times in each of the four cardinal directions (horizontal and vertical) at a speed of 10°/s. All stimuli were spherically corrected to account for the geometry of the planar monitor (Marshel et al., 2011).

Wide-field Ca^{2+} signals were recorded from head-fixed, awake mice. The cortical surface of the left hemisphere, including V1, was imaged at a 4-Hz frame rate using a Leica M165 FC stereomicroscope equipped with a cooled CCD camera (ORCA-ER, Hamamatsu Photonics) controlled by AQUACOSMOS software. Excitation light was filtered through a 450–490 nm bandpass filter, and emission signals were collected through a 500–550 nm bandpass filter. Analysis of the retinotopic mapping data was conducted using the 'Visual Area Segmentation' repository (https://snlc.github.io/ISI/; Marshel et al., 2011). The boundaries of V1 were identified from the resulting visual field sign map, and 500 × 500 μm regions of interest (ROIs) were defined within V1 for subsequent two-photon imaging.

***In vivo* two-photon calcium imaging**

GCaMP and tdTomato fluorescence signals were acquired using a two-photon microscope (A1MP, Nikon) equipped with a Ti:Sapphire laser (Mai Tai DeepSee, Spectra-Physics) and a 25× water-

immersion objective (NA 1.10, MRD77220, Nikon). A custom-made light shield surrounded the objective to prevent light contamination from the stimulus monitor. Images (512 × 512 pixels; pixel size = 0.99 μm) were acquired at 15 frames/s using a resonant scanner. Both GCaMP7b and tdTomato were excited at 950 nm, and their respective emission signals were collected through bandpass filters (500/50 nm for GCaMP7b and 663/75 nm for tdTomato). Imaging was performed at depths ranging from 180–300 μm below the pial surface, corresponding to layer 2/3.

For all imaging sessions, mice were head-fixed under the microscope. Imaging was first performed in awake mice, followed by a session under light isoflurane anesthesia (0.6–0.8% in air). Each imaging session lasted approximately 50 min. During anesthetized recordings, body temperature was maintained at ~37°C with a heating pad. In a subset of experiments, an additional awake session was conducted following a recovery period of at least 4 hours in the home cage. To compensate for slow axial drift between sessions, the imaging depth was re-adjusted using a custom ImageJ plugin by aligning the vasculature in the field of view to a reference stack recorded before the initial awake session. Data from sessions with axial motion artifacts exceeding 4 μm were excluded from further analysis.

Visual stimulation

Visual stimuli were generated with PsychoPy (Peirce, 2009)and presented on a gamma-corrected 23-inch LCD monitor (FS2333, EIZO; 60 Hz refresh rate) positioned 20 cm from the mouse's right eye. The display covered approximately 100° in azimuth and 70° in elevation of the visual field. The precise extent of this coverage was confirmed at the start of each experiment by mapping neuronal responses to a flickering checkerboard stimulus.

The stimuli consisted of full-screen, sine-wave drifting gratings (100% contrast; 100 cd/m² mean luminance) presented to the right eye. These gratings utilized six spatial frequencies (SFs) (0.02–0.64 cycles/degree, in one-octave steps) and eight directions of motion (0–315°, in 45° steps), at a temporal frequency of 2 Hz. The stimulus set also included a blank condition (a gray screen at mean luminance) and a 2 Hz full-field flicker. A total of 50 unique stimuli were presented in a pseudo-random order, with 10 repetitions per imaging session. Each trial consisted of a 2-s stimulus presentation, followed by a 4-s inter-trial interval showing a gray screen.

Estimation of visual tuning preferences

Raw two-photon imaging data were motion-corrected, and regions of interest (ROIs) corresponding to individual neurons were identified using Suite2p (Pachitariu et al., 2017). For mice expressing both GCaMP and tdTomato, the structurally-expressed tdTomato fluorescence (red channel) was used for motion correction, while ROIs were detected from the functionally-driven GCaMP signal in the green channel. Putative non-neuronal ROIs were manually curated and excluded in the Suite2p GUI.

To correct for neuropil contamination, the neuropil signal for each ROI was defined as the average fluorescence within a circular annulus (inner edge 3 μm from the ROI boundary; width 5 μm). The fluorescence time series of neurons (F_{soma}) was then corrected by subtracting the scaled neuropil signal ($F_{neuropil}$) according to the formula:

$$F_{corrected} = F_{soma} - \alpha\left(F_{neuropil} - \text{median}\left(F_{neuropil}\right)\right)$$

for each neuron was estimated using robust linear regression between its cell fluorescence and the corresponding neuropil signal. If α exceeded 1, α was set to 0.93 (median value across all cells). The relative change in fluorescence (ΔF/F) was calculated for each trial as (Fcorrected−F0)/F0, where F0 is the mean fluorescence during the 1.3-s period immediately preceding stimulus onset. The response amplitude for each stimulus condition was computed as the average ΔF/F across 10 repetitions. A neuron was classified as visually responsive if it met two criteria: (1) its activity during periods of visual stimulation was significantly different from the blank condition (one-way

ANOVA, $p<0.01$), and (2) its trial-averaged response to the preferred stimulus exceeded a threshold of 0.25 ΔF/F.

The direction and SF tuning properties for individual neurons were characterized by fitting their trial-averaged responses to the product of a double Gaussian in direction space and a Gaussian in SF space. The response R is described as:

$$R(\theta, SF) = C + R_{max} \left(\exp\left(-\frac{w^2(\theta - \theta_{pref})}{2\sigma_{Dir}^2}\right) + Q\exp\left(-\frac{w^2(\theta + \pi - \theta_{pref})}{2\sigma_{Dir}^2}\right)\right) \exp\left(-\frac{(SF - SF_{pref})^2}{2\sigma_{SF}^2}\right)$$

$w(\theta)$ wrap angles onto the interval between 0 and π:

$$w(\theta) = \min\{|\theta|, |\theta + 2\pi|, |\theta - 2\pi|\}$$

where $R(\theta, SF)$ is the trial-averaged ΔF/F for the stimulus with direction θ and spatial frequency SF. This model assumed that the peaks of the two Gaussians were 180° apart. θ_{pref} is the preferred direction, σ_{Dir} is the width of the direction tuning curve, Q is the relative magnitude of the response in the null direction, SF_{pref} is the preferred SF, σ_{SF} is the width of the SF tuning curve, R_{max} is the response to the preferred SF and direction, and C is a constant amplitude offset. The values of the fit parameters were determined by minimizing the squared error using the lsqcurvefit function in MATLAB (R2020b, MathWorks). Fits with a low coefficient of determination ($R^2<0.3$) were excluded from the SF analysis.

To assess the reliability of the estimated tuning parameters, a bootstrapping procedure was performed. For each neuron, we generated 1000 surrogate datasets by randomly resampling (with replacement) the 10 trials for each grating stimulus. The tuning curve was re-fit to each surrogate dataset, yielding a distribution of tuning parameters, from which 95% confidence intervals (CIs) were derived. A neuron was considered to exhibit a significant difference in a tuning parameter (e.g., preferred SF) between the anesthetized and awake conditions if the respective 95% CIs did not overlap.

The sharpness of the SF tuning was assessed by the full width at half maximum (FWHM) of the tuning curve at the optimal direction. The orientation selectivity index (OSI), computed as follows:

$$OSI = \sqrt{\left(\sum R_i(\sin(2\theta_i))\right)^2 + \left(\sum R_i \cos(2\theta_i)\right)^2} / \sum R_i$$

where R_i is the response to the ith direction with the optimal SF.

The direction selectivity index (DSI) was calculated as follows:

$$DSI = (R_{pref} - R_{null})/(R_{pref} + R_{null})$$

where R_{pref} is the response in the preferred direction, and R_{null} is the response in the opposite direction.

Bayesian decoding

To examine how anesthesia influenced the representation of visual information in the neural population activity, we implemented a Bayesian decoding analysis based on the framework of Jazayeri and Movshon (Jazayeri and Movshon, 2006). This method estimates the probability that a given population response, $r = \{r_1, r_2, ..., r_N\}$, was generated by a stimulus, s, from the set of all possible stimuli, S.

We assumed that the neural response, r_i, from each neuron, i, on a given trial was drawn from an independent Poisson distribution with a mean response defined by its tuning curve, $f_i(s)$. For each neuron i, we calculated the tuning function $f_i(s)$ as the mean response across trials to stimulus s. Assuming a uniform prior probability for all stimuli, the log-likelihood of a stimulus s given a population response r can be computed as a weighted sum of the responses from all N neurons:

$$L(s_j \mid \mathbf{r}) = \sum_{i=1}^{N} r_i \log f_i(s_j)$$

The posterior probability of the stimulus, $P(s_j \mid \mathbf{r})$, was calculated by normalizing the likelihood across all possible stimuli:

$$P(s_j \mid \mathbf{r}) = \frac{\exp\big(L(s_j \mid \mathbf{r})\big)}{\sum_k \exp\left(L(s_k \mid \mathbf{r})\right)}$$

For each trial, the posterior probability distribution over the stimulus set was computed. The decoded stimulus was defined as the one with the highest posterior probability (the maximum a posteriori, or MAP, estimate). Decoding error was quantified as the absolute difference between the decoded stimulus orientation and the true stimulus orientation. We used level-one-out cross-validation to decode the awake state response with the awake state tuning curve. Trials that had been decoded were excluded from calculating the tuning curve.

To further assess how anesthesia influenced the representation of visual information, we also examined the full posterior probability distribution. Unlike the single MAP estimate, the full distribution provides more information about the representational landscape, revealing changes such as bias or systematic shifts. Before MAP estimation, these probabilities were normalized to sum to 1 over all possible stimuli. After computing per-trial posteriors, they were aligned to the true stimulus by centering them on that trial's stimulus value. Then, the aligned posterior probabilities were averaged across trials within each state, and the resulting distributions were compared between the awake and anesthetized conditions.

Arousal modulation

The animal's arousal state was monitored by tracking pupil diameter, following established methods (Vinck et al., 2015; Mineault et al., 2016). To avoid interfering with visual stimulation of the right eye, the contralateral (left) eye was illuminated with an infrared (IR) LED, and videos of the eye were recorded at 15 Hz using a monochrome camera (MQ003MG-CM, XIMEA) equipped with a macro zoom lens (MLM-3XMP, COMPUTAR) and a long-pass filter (>830 nm; LP830, AZURE). Video acquisition was synchronized with frame acquisition triggers from the two-photon microscope using custom Python software.

Pupil size and position were extracted from the recorded videos offline using Facemap (https://github.com/MouseLand/facemap). For each session, a region of interest (ROI)

encompassing the pupil was manually drawn. To account for ambient light fluctuations, the minimum pixel value within the ROI was subtracted from all pixels on a frame-by-frame basis. A brightness threshold was then manually set to isolate dark pupil pixels. The pupil's area and center were calculated by fitting a 2D Gaussian to the covariance of these thresholded pixels.

The resulting pupil area trace was filtered to remove blink-related artifacts. Specifically, a median filter (2-s window) was applied, and any raw data point that deviated from the median by more than 0.5 standard deviations was replaced with the median-filtered value. The cleaned trace was then normalized to the maximum pupil area observed within that session.

For subsequent analysis, trials were categorized into low and high arousal states based on the session-wide pupil area distribution. 'Low arousal' corresponded to pupil areas below the 25th percentile, while 'high arousal' corresponded to areas above the 75th percentile. The arousal level for a given trial was defined as the average normalized pupil area during that trial's stimulus presentation. Trials were subsequently categorized as 'low arousal' or 'high arousal' if this average value fell into the corresponding percentile range.

Finally, to investigate the effect of arousal on sensory processing, SF and direction tuning curves were fitted separately to neuronal responses during low and high arousal trials. For SF tuning analysis, a stricter goodness-of-fit threshold ($R^2>0.4$) was used, to account for the relatively low response amplitudes observed in low arousal trials.

Statistical analysis

All quantitative and statistical analyses were performed using custom scripts written in MATLAB (R2020b, MathWorks). Data are presented as mean ± standard deviation (SD). In box plots, the central mark indicates the median, the bottom and top edges of the box indicate the 25th and 75th percentiles, and the whiskers extend to the most extreme data points not considered outliers. Outliers were defined as data points falling more than 1.5 times the interquartile range (IQR) beyond the box edges.

Statistical tests were chosen based on the data structure. The Wilcoxon signed-rank test was used for paired comparisons, while the Wilcoxon rank-sum test or the two-sample Kolmogorov-Smirnov test was used for unpaired group comparisons. For multiple comparisons, a Bonferroni correction was applied to adjust the significance threshold. Correlations were assessed using Pearson's correlation coefficient. The chi-squared (χ^2) test was used for comparing proportions between two groups. A p-value of < 0.05 was considered statistically significant.

To account for the non-independence of data from the same animal, linear relationships between preferred SFs in different brain states were tested using linear mixed-effects models (Statistics and Machine Learning Toolbox, MATLAB). In these models, individual animal identity was included as a random effect.

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Figures and legends

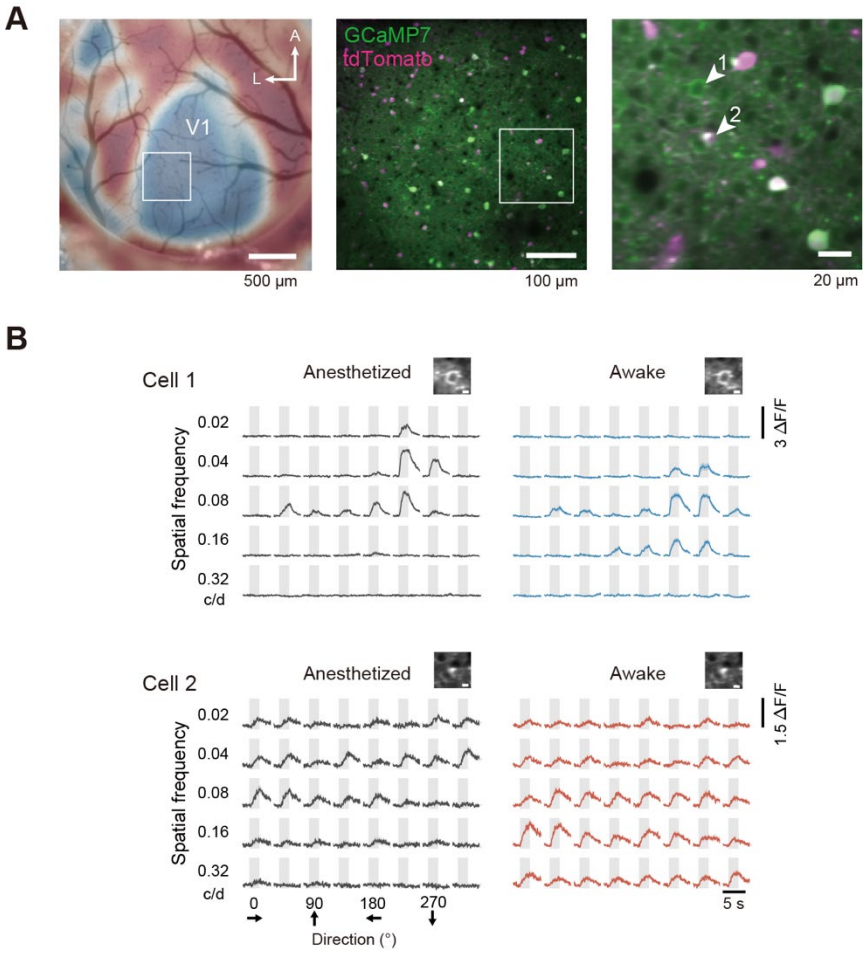

Figure 1. Two-photon Ca^{2+} imaging of the same neurons in anesthetized and awake states.

(A) The primary visual cortex (V1) was first mapped by wide-field Ca^{2+} imaging (left), followed by two-photon cellular-level imaging (middle). A higher-magnification image showing the distribution of GCaMP7b-expressing neurons, including tdTomato-labeled inhibitory neurons, is shown on the right.

(B) Example visual responses of an excitatory (cell 1) and an inhibitory neuron (cell 2) in the anesthetized (left) and the awake (right) conditions (mean ± SEM). Responses (ΔF/F) were evoked by visual stimuli with various spatial frequencies (1-octave steps, shown on the left) and directions (45°-steps, shown at the bottom). Each gray box indicates the stimulus presentation period (2 s). Top, mean image of the recorded neurons in each condition.

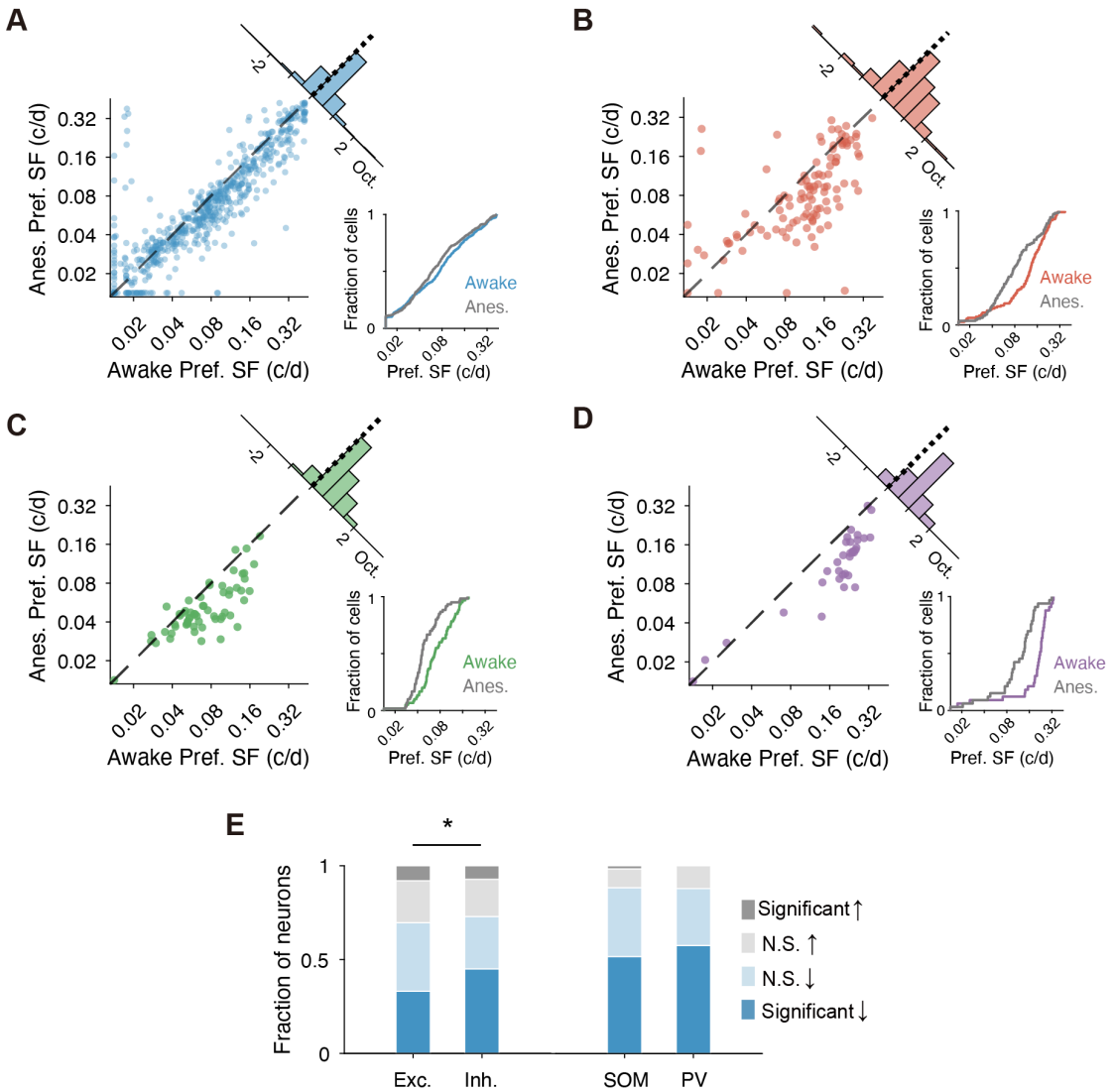

Figure 2. Anesthetize-induced downward shift of the preferred spatial frequency.

(A and B) Preferred SF of individual neurons in the anesthetized condition (y-axis) is plotted against that in the awake condition (x-axis) for tdTomato-negative (presumed excitatory neurons, n = 610 cells, N = 9 mice, A) and tdTomato-positive (inhibitory neurons, n = 111 cells, B) neurons. The corner histogram shows the distribution of the pairwise difference in preferred SF between the two conditions. Bottom right: cumulative distributions of preferred SFs in each condition.

(C–D) Same plots for SOM cell (n = 60 cells, N = 5 mice, C) and PV cell (n = 33 cells, N = 6 mice, D).

(E) Fraction of neurons exhibiting a significantly decreased (blue), non-significantly (N.S.) changed (light blue or light gray), or significantly increased (gray) preferred SF under anesthesia across the four neuron groups. The asterisk denotes a significant difference in these distributions across the groups ($P < 0.05$, Chi-square test).

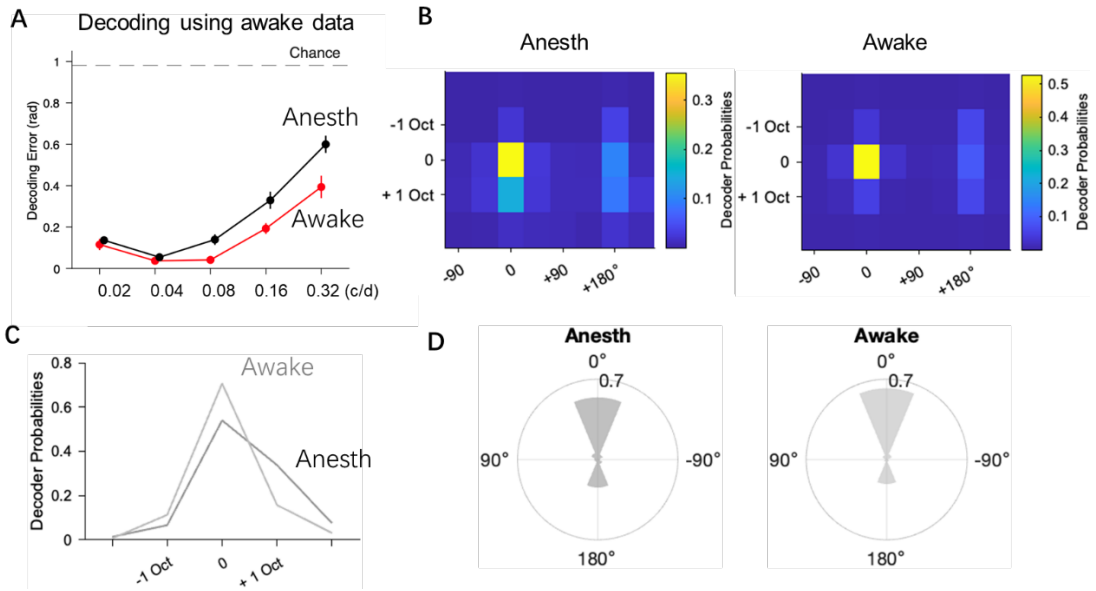

Figure 3. Effects of anesthesia on neuronal population coding.

(A–D) A decoder was trained using visual responses from the awake condition.

(A) Decoding error for visual stimuli as a function of SF from visual responses in the awake (red) and anesthetized conditions (black). The error represents the difference between the decoded and actual orientations. The dotted line indicates the chance level.

(B) Distributions of decoder probabilities for the visual responses in the anesthetic (left) and awake (right, control) conditions. The value of 0 on the SF and orientation axes indicates that the decoded stimulus matches the true stimulus.

(C–D) Comparison of decoder probability profiles for the awake and anesthetized conditions. The profiles were extracted from (B) along the SF (C) and orientation axes (D), respectively. Under anesthesia, the probability of decoding at higher SFs was increased compared to the awake state, while the orientation did not change.

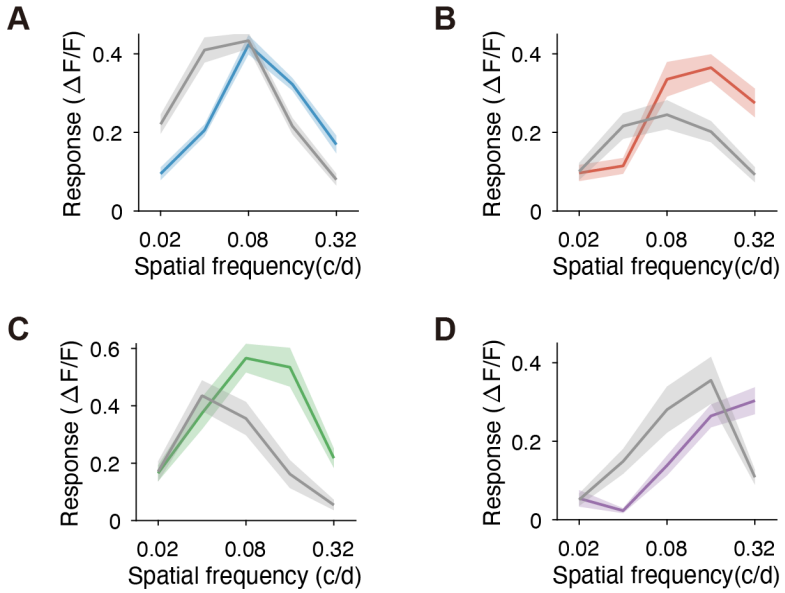

Figure 4. Characterization of the preferred SF shift patterns

Population-averaged SF tuning curves for neurons that showed a significant anesthesia-induced shift in preferred SF. (A) Excitatory neurons. (B) Pan-inhibitory neurons. (C) SOM neurons. (D) PV neurons. Colored lines represent the awake condition, and gray lines represent the anesthetized condition. The error band represents the SEM.

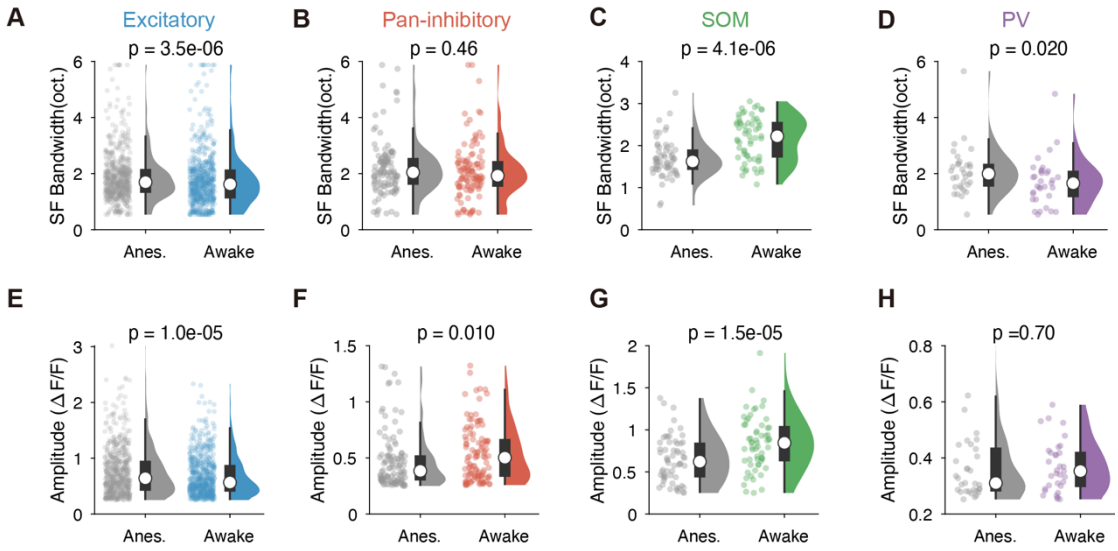

Figure 5. Comparison of SF selectivity and response magnitude between anesthetized and awake conditions.

(A–D) Box and raincloud plots comparing the bandwidth of SF tuning curve for individual neurons in the anesthetized and awake conditions. (A) The excitatory neurons. (B) The pan-inhibitory neurons. (C) The SOM neurons. (D) The PV neurons. The p-value of the Wilcoxon signed-rank test is indicated in the plot.

(E–H) Similar to A–D, but for response amplitude.

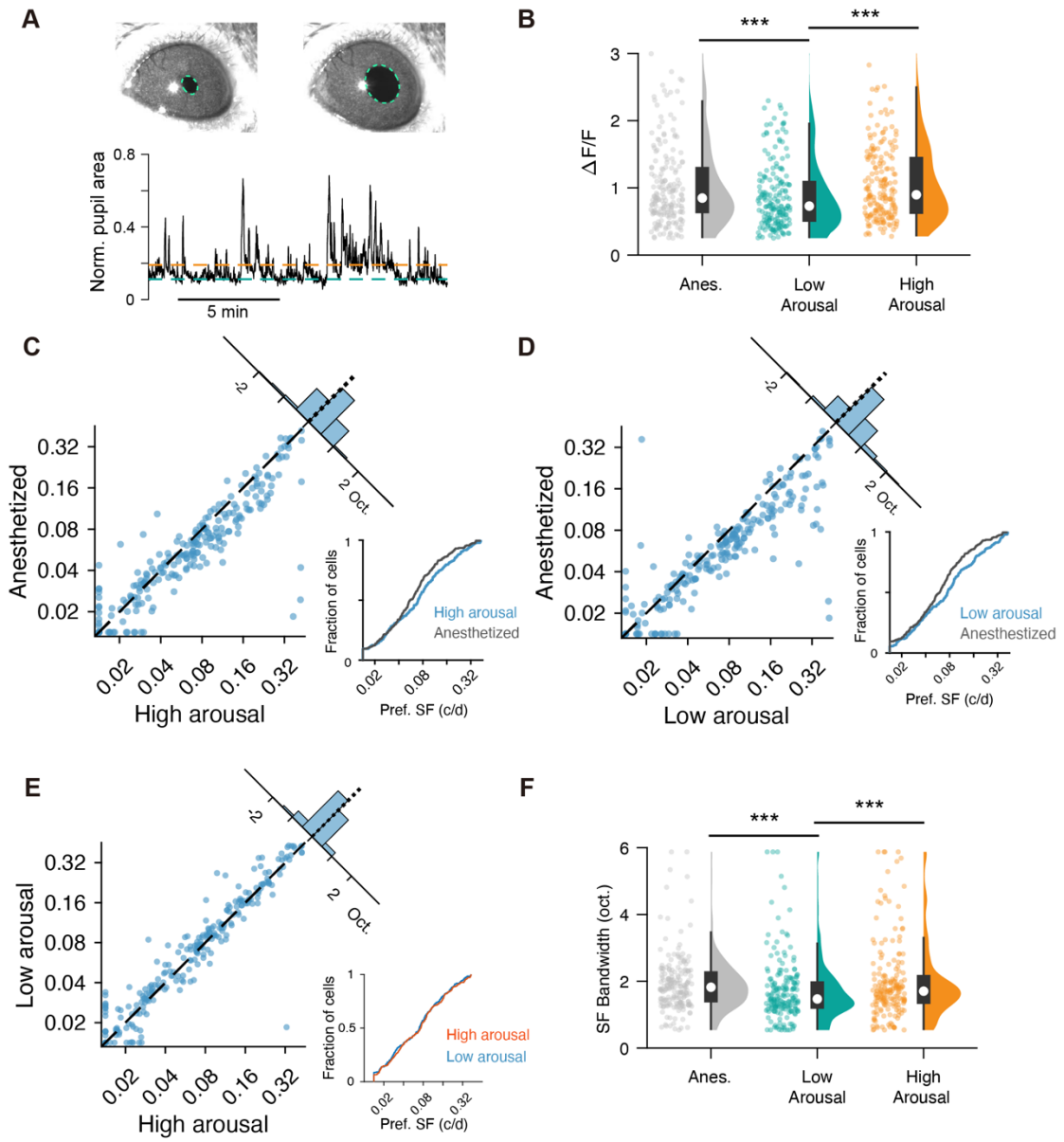

Figure 6. Comparison of visual responses in excitatory neurons between the anesthetic condition, and low-arousal and high-arousal states during wakefulness.

(A) Estimation of arousal level from pupil area. Top: Example raw pupil trace. Bottom: Normalized pupil area trace. Dotted lines represent the 75th and 25th percentiles, used as thresholds for high- and low-arousal trials, respectively.

(B) Box and raincloud plots comparing the visual response amplitude across the anesthetized, low-arousal, and high-arousal conditions (n = 216 cells, N = 6 mice). Asterisks denote a significant difference across the groups ($P < 0.001$, Wilcoxon signed-rank test).

(C) Preferred SF of individual neurons in the anesthetized condition (y-axis) is plotted against that in the high arousal condition (x-axis). The corner histogram shows the distribution of the pairwise difference in preferred SF between the two conditions. Bottom right: cumulative

distributions of preferred SFs in each condition.

(D–E) Similar to (C), but for comparison between the anesthetized and low-arousal conditions (D) and between the low-arousal and high-arousal conditions (E).

(F) Similar to (B), but for SF tuning bandwidth.