

Delayed integration of somatostatin interneurons into visual circuits

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In primary visual cortex (V1), neuronal receptive fields are generally thought to be fully established prior to eye-opening, with subsequent experience-dependent refinement controlled by GABAergic inhibition and regulated by homeostatic mechanisms. However, GABAergic interneurons (INs) are diverse and relatively little is known about the early postnatal roles of dendrite-targeting interneurons. Surprisingly, we find that somatostatin-expressing interneurons (SST-INs) in mouse V1 are not visually responsive at eye opening, instead developing visual sensitivity during the third postnatal week. Over the same period, SST-INs exhibit a rapid increase in excitatory innervation without compensatory synaptic scaling. Simultaneous imaging and optogenetic manipulation in juvenile animals reveals that SST-INs largely exert a multiplicative modulation of nearby excitatory neuron responses at all ages, but this effect increases over time. Our results identify a uniquely delayed developmental window for maturation of this inhibitory circuit and its contribution to visual gain normalization.

Prior to eye opening, spontaneous waves of activity arising from the retina promote the early formation of cortical circuits underlying basic visual receptive field features in primary visual cortex^{1–5}. Individual excitatory pyramidal neurons (PNs) in V1 exhibit mature receptive field architecture and visual feature selectivity at eye opening^{6,7}. Soma-targeting, parvalbumin-expressing GABAergic interneurons (PV-INs) likewise exhibit mature receptive fields and robust visual responses at eye opening^{8,9}, suggesting that INs may be fully integrated into early juvenile visual cortical circuits. However, GABAergic INs are highly diverse¹⁰ and little is known about the impact of other IN populations on cortical circuit function during postnatal development.

Recent work has highlighted the unique roles of somatostatin-expressing (SST), dendrite-targeting INs in cortical circuit function. SST-INs regulate synaptic integration in PNs^{11–14}, influence calcium-dependent dendritic plasticity^{15–17}, and contribute to gain modulation¹⁸. In V1, SST-INs exhibit strong visual responses that are broadly tuned for stimulus size, orientation, and spatial frequency^{19–23}.

These INs are unique in exhibiting strong responses to relatively large visual stimuli^{19,24}, potentially leading to surround suppression of local PNs²⁵ and long-range coordination of visually evoked activity²⁶. Moreover, SST-IN activity is robustly modulated by changes in behavioral state, such as arousal and locomotion^{19,22,24,27}, and may contribute to state-dependent visual perceptual performance via inhibition of nearby PN dendrites.

Cortical SST-INs originate in the medial ganglionic eminence and migrate to their final destinations by postnatal day 5 (P5) in mice, but their connectivity and function are not yet fully mature^{28,29}. Recent work suggests global changes in the activity of SST-INs across cortical areas during the first postnatal week, leading to a shift in synchronization of cortical activity^{8,30,31}. The intrinsic electrical properties of SST-INs in V1 and other cortical areas mature well after eye opening at the end of the second postnatal week, leading to increased excitability by P28–29^{32,33}. In infragranular somatosensory but not visual cortical circuits, SST-INs transiently receive thalamic input and regulate the

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maturation of PV-INs³⁴, a circuit motif associated with early postnatal SST-IN responses to whisker stimuli^{8,31}. SST-INs may also exert a critical influence on the late postnatal maturation of cortical circuits, including the refinement of binocular receptive fields in V1³⁵. Early disruption of SST-IN function is associated with dysregulated neural activity in the adult cortex^{36–38}, further indicating a potentially critical developmental role for these cells. However, the early postnatal function of SST-INs remains poorly understood.

Here, we examined the postnatal integration of SST-INs into V1 circuits using 2-photon imaging in juvenile mice. In contrast to the majority of V1 neurons, we find that SST-INs exhibit little visual sensitivity at eye opening and gradually develop responses to visual stimuli during the third postnatal week. This emergence of visual responses is independent of visual experience and arises in coordination with a rapid increase in excitatory synaptic innervation of SST-INs without compensatory changes in synaptic scaling. SST-INs begin to exhibit size tuning and state-dependent modulation of activity during this period, whereas nearby PNs and vasoactive intestinal peptide-expressing interneurons (VIP-INs) exhibit visual responses and state-dependence from the earliest age studied. Targeted optogenetic manipulation of juvenile SST-INs in combination with simultaneous 2-photon imaging reveals that SST-INs have robust functional connectivity with local PNs at eye opening, but exert progressively more influence on PN visual gain over time. Together, our results highlight a uniquely delayed integration of SST-INs into visual cortical circuits, along with the developmental emergence of this GABAergic cortical circuit mechanism for visual gain normalization.

Results

Emergence of visual sensitivity in SST-INs

In adult mice, both SST-INs and PNs in layer 2/3 of V1 exhibit reliable, robust visual responses. Although extensive previous work has highlighted the early onset of well-tuned visual responses in PNs^{6,7}, relatively little is known about the development of visual sensitivity in SST-INs. We therefore used in vivo 2-photon imaging to directly test the developmental trajectories of visual sensitivity in both cell types following eye opening at P14. We imaged cellular activity in juvenile SST-INs;Ai148^{F/0} and Thy1-GCaMP6s mice constitutively expressing the calcium indicator GCaMP6 in SST-INs or PNs, respectively. We measured the activity of either SST-INs or PNs in head-fixed, awake behaving mice that ranged in age from P15, the first day after eye opening, to P29 (see “Methods”) (Fig. S1a, c). We observed no changes in the density of GCaMP-expressing SST-INs or the number of cells identified per imaging field of view in vivo for SST-INs or PNs, suggesting that GCaMP6 expression remained stable across ages (Fig. S1b, d–f). All numbers and statistical results are shown in Supplementary Data 1.

Only ~20% of SST-INs in awake behaving mice were responsive to visual stimulation at P15 (Fig. 1). In contrast, by P21, over 80% of SST-INs were sensitive to visual stimuli, a proportion that was maintained at later ages (Fig. 1c, d). The lack of early postnatal SST-IN visual responses was not due to changes in locomotion across ages, as we found a similar trajectory of visual responsiveness even when analysis was restricted to periods of quiescence without locomotion (Fig. S2a). In contrast, most VIP-INs already exhibited robust visual responses at P15–17 (Fig. S2j). In good agreement with previous studies^{6,7}, we found a stable proportion of visually responsive PNs from P15 through P29 (Fig. 1e, f). The relatively low proportion of visually responsive PNs is consistent with the stimulation protocol, in which we used drifting gratings of a single orientation (see “Methods”). Together, these data suggest distinct developmental timelines for visual sensitivity in PNs, whose receptive fields are well established prior to eye opening^{6,7}, and SST-INs, whose visual sensitivity increases rapidly starting at the end of the second postnatal week.

Given the substantial increase in visual sensitivity in developing SST-INs, we examined whether excitatory innervation of these interneurons exhibited plasticity over the same period. We performed targeted whole-cell patch clamp recordings in acute slices from SST-INs;Ai9^{F/0} mice that constitutively expressed the red fluorescent indicator tdTomato selectively in SST-INs. We recorded miniature excitatory postsynaptic currents (mEPSCs) in SST-INs in layer 2/3 of V1 of mice ranging from P15 to P23. Although mEPSC frequency can be affected by temperature and other variables, we maintained consistent recording conditions for all groups to reduce contributions from these effects. We found that the frequency of mEPSCs increased between P15 and P18 (Fig. 2a–c), whereas mEPSC amplitudes (Fig. 2d) and 10%–90% rise times (Fig. 2e) remained unchanged. Together, these data suggest a rapid increase in excitatory synaptic innervation of SST-INs following eye opening, leading to the emergence of visual responses before P21.

Distinct developmental trajectories of stimulus selectivity in PNs and SST-INs

Previous work found that mature SST-INs and PNs in V1 exhibit robust, state-dependent feature selectivity for visual stimulus size^{19,24,25}. Indeed, SST-INs are highly sensitive to arousal and locomotion and are thought to potentially mediate surround suppression in nearby PNs and INs due to their responsiveness to large visual stimuli^{22,24,25}. However, little is known about the development of feature selectivity or state-dependent modulation of evoked activity in SST-INs. We found that neither SST-INs nor PNs exhibited overall population modulation of spontaneous activity levels by locomotion at P15–17, although individual cells in each population were positively or negatively modulated (Fig. S2). By P18–21, SST-INs broadly exhibited positive modulation by locomotion (Fig. S2b, c, e). These changes were accompanied by altered spontaneous SST-IN activity (Fig. S2i). In contrast, the PN population did not exhibit a change in state-dependent modulation of spontaneous activity across the P15–P29 age range (Fig. S2d, f) and retained a broad distribution of modulation consistent with previous observations in adult animals^{19,24,39,40}. In contrast, VIP-INs in V1 already showed robust state-dependent modulation at P15–17 (Fig. S2k).

At P15–17, SST-INs exhibited little visual sensitivity or modulation of visual responses by locomotion (Fig. 3a, b). Even within the subpopulation of visually responsive SST-INs, visual response amplitudes at P15–17 were modest (Figs. 3 and S3). In the subset of visually responsive SST-INs, visual responses to stimuli of all sizes emerged by P18–20 and continued to increase in amplitude through P27–29 (Fig. 3b–d). The visually responsive subset of SST-INs showed responses to a broad range of stimulus diameters by P21–23 (Figs. 3b and S3a–c), suggesting that progressive excitatory innervation drives changes in visual responses over this period. SST-IN visual responsiveness did not vary with depth within layer 2/3 (Fig. S3f, g). In contrast, PNs exhibited robust visual responses that were selective for smaller stimuli by P15–17 and did not change significantly in amplitude between P15 and P29 (Figs. 3b–d and S3b, d), further supporting a model where PN receptive fields and feature selectivity emerge prior to eye opening. Surround suppression in PNs did not change between P15 and P29 (Fig. S3e). The developmental trajectory of visual responses was similar across cells within each population, regardless of whether stimuli were well matched to the cell’s receptive field position (Fig. S3c, d), suggesting that these results did not arise from visual stimulus misalignment. SST-INs did not exhibit locomotion-mediated modulation of visual response magnitude at P15–17 but developed robust modulation by P18–20 (Fig. 3e). We did not observe an effect of dark rearing on the visual responses of SST-INs (Fig. S3h–k). Together, these data indicate that SST-INs develop robust, selective visual responses and sensitivity to large stimuli well after PNs exhibit stable visual responses, sharp size tuning, and consistent surround suppression.

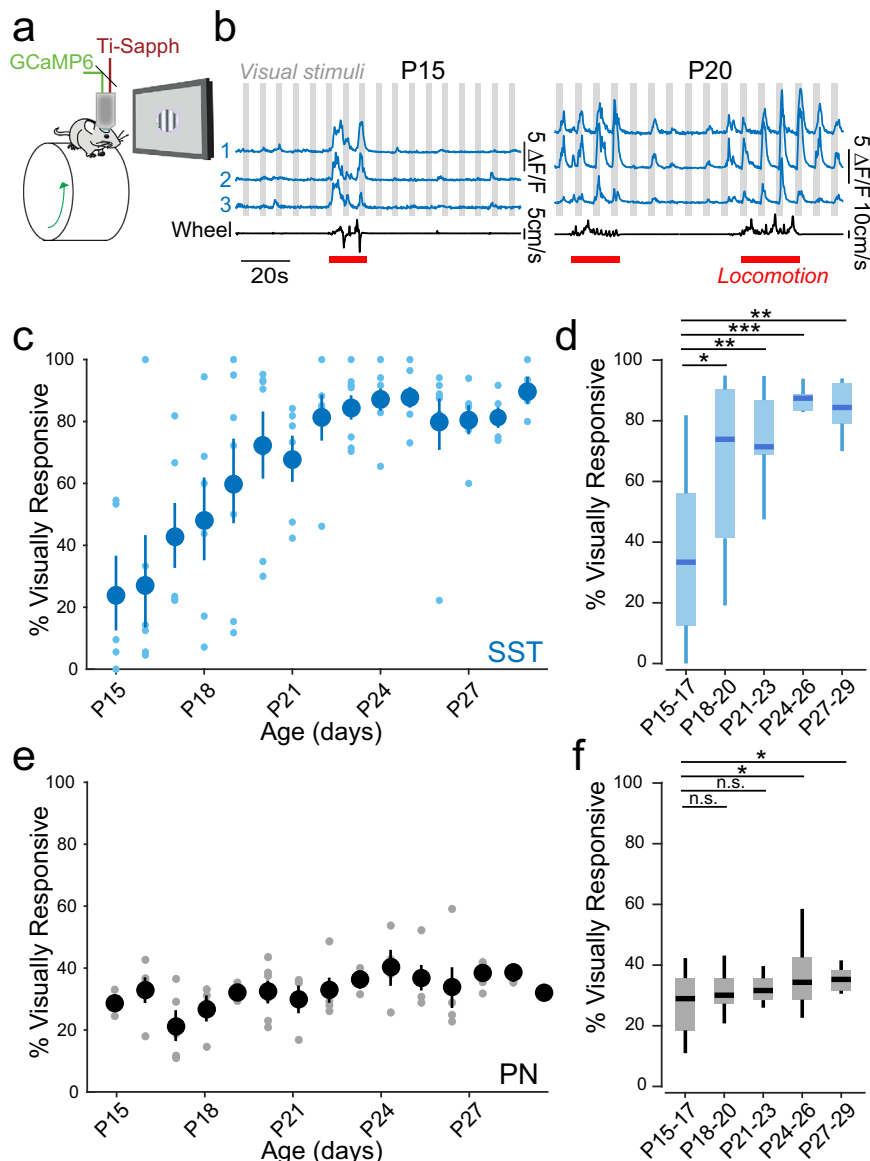


Fig. 1 | Visual sensitivity in SST-INs emerges following eye opening. **a** Schematic of the in vivo 2-photon imaging configuration. **b** Left: Ca²⁺ traces of three example P15 SST-INs (blue) recorded during the presentation of visual stimuli (gray) and wheel speed tracking (black) to identify locomotion bouts (red). Right: Ca²⁺ traces of three example P20 SST-INs. **c** Proportion of SST-INs that were visually responsive at each age. Large dark circles represent mean values, and small light circles represent individual animals. Vertical lines show SEM. **d** Boxplots of the values in (c), aggregated into 3-day age groups (P15–17: *n* = 217 cells, 7 mice; P18–20: *n* = 184

cells, 8 mice; P21–23: *n* = 211 cells, 9 mice; P24–26: *n* = 230 cells, 10 mice; P27–29: *n* = 139 cells, 6 mice). The central mark indicates the median, box boundaries indicate the 25th and 75th percentiles, and the whiskers indicate the range of the data. **e**, **f** Same as in (c, d) but for PNs (P15–17: *n* = 3301 cells, 6 mice; P18–20: *n* = 2791 cells, 7 mice; P21–23: *n* = 2425 cells, 6 mice; P24–26: *n* = 3638 cells, 6 mice; P27–29: *n* = 2099 cells, 6 mice). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, all statistics calculated with a 0/1 inflated beta mixed-effects regression model with age as a fixed effect and mouse as a random effect.

Functional impact of SST-INs on visual selectivity of PNs

To determine the influence of SST-INs on the local cortical circuit during postnatal development, we performed simultaneous 2-photon imaging and targeted optogenetic manipulations in juvenile mice. Using SST^{Cre};Thy1-GCaMP6 mice expressing a Cre-dependent ChrimsonR⁴¹ construct (Fig. S4a, see “Methods”), we first selectively stimulated SST-INs while imaging the activity of local PNs at each age point to confirm functional connectivity between SST-INs and PNs. Activation of SST-INs during visual stimulation robustly suppressed the visually evoked activity of local PNs from P15 onwards (Fig. S4b–f), consistent with the early establishment of functional synaptic connectivity between SST-INs and PNs²⁸ despite minimal excitatory innervation of SST-INs at early ages (Fig. 2).

We next examined the impact of SST-IN activity on the visual responses of local PNs by using optogenetic suppression of SST-IN activity via activation of a Cre-dependent ArchT construct⁴² expressed in SST^{Cre};Thy1-GCaMP6 mice (Figs. 4a, b, S1g–k and S4e–g). We found that in juvenile PNs that exhibited tuning for stimulus size, the impact of optogenetic suppression of juvenile SST-INs was diverse, with individual PNs showing either enhanced or reduced visual responses (Figs. 4c and S4h–j). In both cells whose visual responses were enhanced and reduced by suppressing SST-INs, the impact on visual tuning was strongest for stimuli ~20°, largely multiplicative at both early and late age points (Fig. 4d, e), and did not exhibit selective modulation of responses to large stimuli (Fig. S4o). Indeed, the impact of SST-IN suppression in reduced cells became more selective for

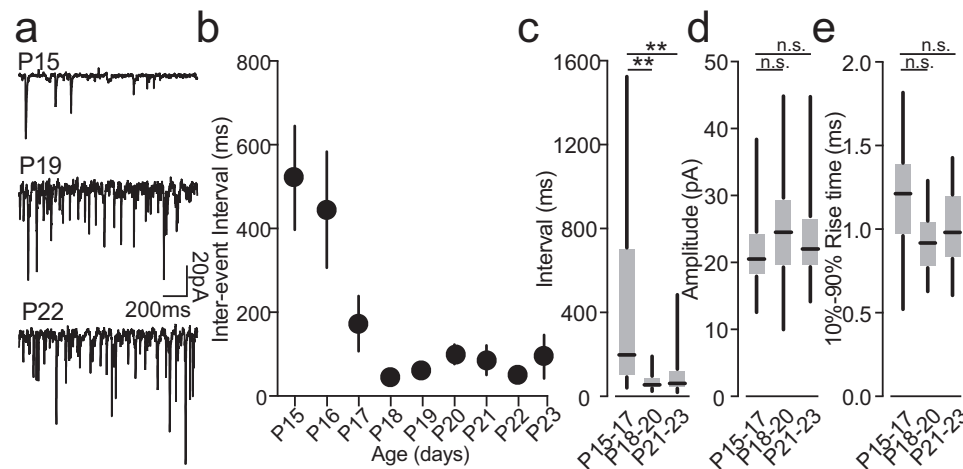


Fig. 2 | Rapid increase in excitatory synaptic input to SST-INs. a Example traces of miniature EPSCs (mEPSCs) recorded ex vivo in SST-INs at P15 (upper), P19 (middle), and P22 (lower). **b** Inter-event intervals at each age P15–P23. Circles represent mean values, and vertical lines show SEM. **c** Boxplots of the interval values in (b), aggregated into 3-day age groups. The central mark indicates the

median, box boundaries indicate the 25th and 75th percentiles, and whiskers indicate the range of the data. **d** Boxplots of mEPSC amplitude across ages. **e** Boxplots of the 10%–90% rise time of mEPSCs across ages. For all panels: P15–17: $n = 30$ cells. P18–20: $n = 31$ cells. P21–23: $n = 20$ cells. ** $p < 0.01$, all statistics calculated with one-way ANOVA with Tukey's multiple comparisons test.

smaller stimuli across ages (Figs. 4f and S4j). The overall impact of SST-IN suppression shifted towards enhancement of PN visual responses between P15–17 and P24–26 (Fig. S4k). Optogenetic suppression of SST-INs in adult mice revealed predominantly enhanced responses in PNs (Fig. S4l–n), suggesting that the shift from heterogeneous to consistently enhancing impacts of SST-IN suppression on PN visual responses represents a developmental shift in circuit interactions that extends beyond P30. In contrast, the population impact of SST-IN suppression on PN spontaneous activity did not change across ages (Fig. S4p). Together, these data suggest that SST-INs largely exert a multiplicative gain modulation effect on PN visual responses at early and late ages, but their net effect on the PN population is refined across the postnatal period as SST-INs develop robust visually evoked activity.

Discussion

Our results reveal a key developmental window for the integration of dendrite-targeting SST-INs into V1 circuits. We find that, unlike PNs, PV-INs, and VIP-INs, in which visual receptive fields are established prior to eye opening, layer 2/3 V1 SST-INs have little response to visual stimuli at eye opening. Over the third postnatal week, SST-INs develop visual responses in coordination with a substantial increase in mEPSCs, suggesting enhanced excitatory synaptic innervation. Over this same period, SST-IN responses exhibit increases in visual feature selectivity and modulation by changes in behavioral state. SST-INs are functionally connected to nearby PNs even at P15, suggesting that their synaptic output is established prior to eye opening, even though synaptic input to these cells is delayed. Finally, we find that SST-INs begin to exert a multiplicative impact on PN visual responses over the same time period.

SST-INs are among the earliest GABAergic populations to be integrated into the cortex, arriving in the cortical plate at P0 and concluding their laminar sorting by P5²⁹. SST-INs in layers 4 and 5 of the somatosensory cortex undergo transient phases of connectivity early in postnatal life that are important for the maturation of thalamocortical and corticocortical circuits^{34,43–45}. In adult mice, layer 2/3 SST-INs receive extensive excitatory inputs, which mostly arise from local, horizontally projecting PNs rather than from feedforward inputs^{11,25} and project to layer 1 cells and the dendrites of layer 2/3 PNs^{16,46,47}. An ongoing process of interneuron maturation and circuit refinement begins prior to eye opening and extends through the critical period.

The electrical properties of SST-INs in V1 and other cortical areas mature after eye opening, with intrinsic excitability increasing until P28–29^{32,33}, in contrast to PNs, which decline in intrinsic excitability⁴⁸. Synaptic connections between local SST-INs and PNs in layer 2/3 of V1 emerge by P7, with connectivity between SST-INs and PNs increasing markedly between P8 and P11, followed by a decrease in unitary SST-PN currents around eye opening at P14^{28,49}. SST-INs exhibit an increase in input resistance, depolarization of the resting membrane potential, and increased excitability between P15 and P30, but no further change in SST-PN synaptic strength, whereas PV-INs exhibit a change in membrane capacitance, maturation of fast-spiking properties, and increased PV-PN synaptic strength over this period^{32,50}. Our findings that SST-INs exhibit increased impact on PN responses over the P15–P20 period may thus reflect increased excitatory synaptic input driving visually evoked SST-IN activity and the increased excitability of SST-INs, rather than changes in the properties of SST-PN synapses.

Previous work has found that layer 2/3 PNs in V1 exhibit an increase in mEPSC rates but a decrease in mEPSC amplitudes during the third postnatal week⁵¹. In contrast, we found that the frequency of mEPSC events in SST-INs rapidly increased after P15 without a change in their amplitude or rise time. These results indicate a likely increase in excitatory synaptic innervation and suggest that developmental synaptic scaling may not occur similarly in all populations. However, the developmental trajectory of the synaptic and functional properties of GABAergic interneurons may vary across cortical areas⁵². Indeed, SST-INs contribute to early cortical activity patterns across multiple areas^{30,53}, and previous findings in somatosensory (S1) cortex suggest that SST-INs in barrel cortex exhibit robust responses to whisker stimulation before P14^{8,31}. We found that activating SST-INs had a similar impact on PNs across the P15–P29 age range, supporting the idea that functional connectivity between these two cell types is established prior to eye opening and remains largely stable despite changes in excitatory inputs and visual responses of SST-INs. However, our imaging data were restricted to layer 2/3, leaving open the possibility that SST-INs in other cortical layers may exhibit distinct developmental trajectories^{31,34}.

The activity of both excitatory and inhibitory neurons in cortical circuits is modulated by changes in behavioral state, such as arousal and locomotion^{23,24,40,54–58}. Previous imaging and electrophysiological studies found that mature PNs exhibit a broad distribution of responses to locomotion with a modest bias towards positive

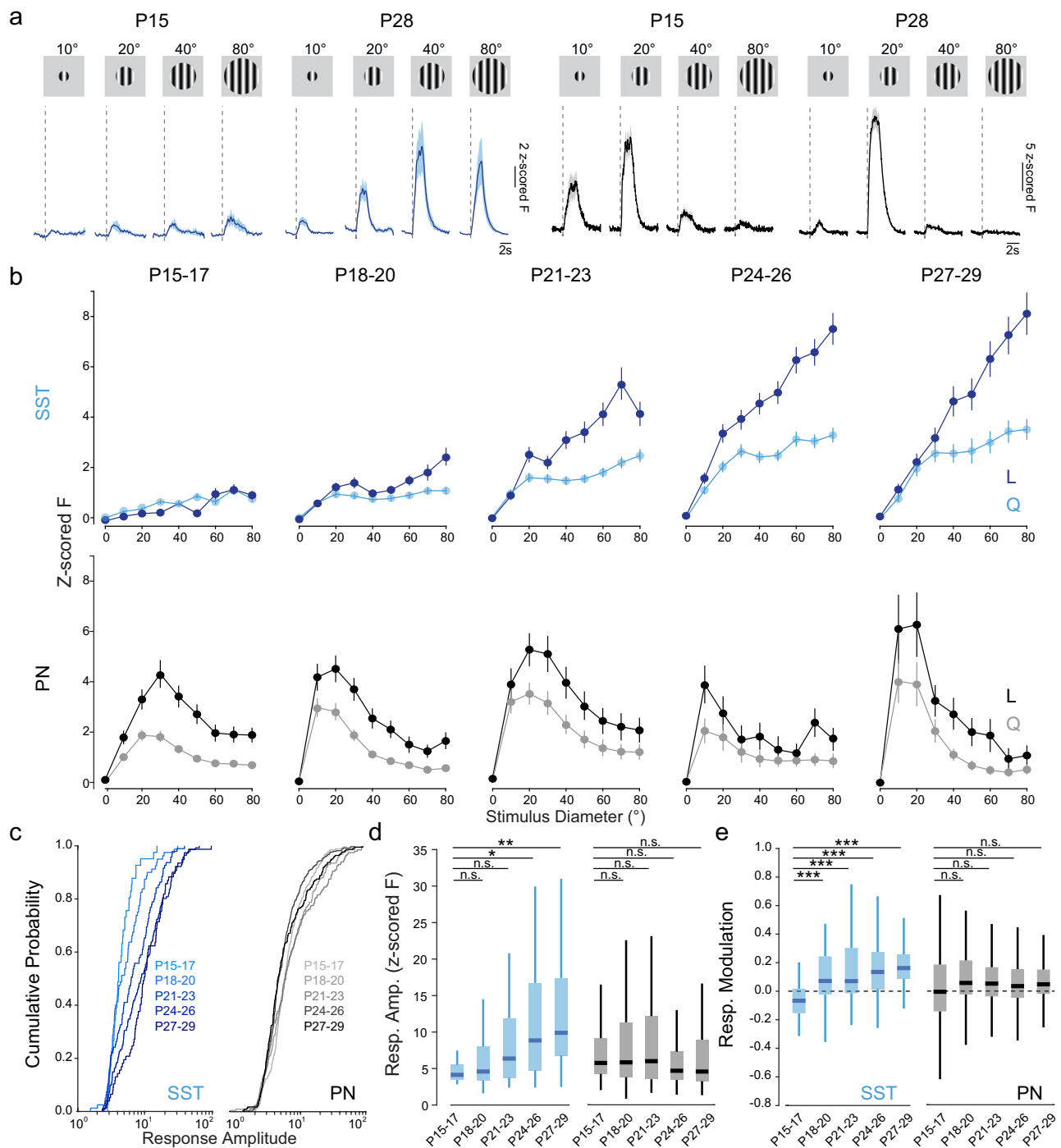


Fig. 3 | Developmental trajectory of SST-IN visual response amplitude and selectivity. **a** Responses of example SST-INs (blue) and PNs (black) to drifting grating stimuli of varying sizes at P15 and P28. Vertical dashed lines indicate visual stimulus onset. Shaded areas indicate mean $\pm$ SEM. **b** Population average visual responses of the subset of visually responsive SST-INs (upper, blue) and PNs (lower, black) to stimuli of varying size at each age. Responses are Z-scored to the 1-s baseline period before the stimulus onset for periods of quiescence (Q, light colors) and locomotion (L, dark colors). **c** Cumulative probability distribution of response amplitude at the preferred stimulus size for visually responsive SST-INs in each age group from (b) (left; P15–17: $n = 41$ cells, 5 mice; P18–20: $n = 80$ cells, 7 mice; P21–23: $n = 95$ cells, 9

mice; P24–26: $n = 141$ cells, 10 mice; P27–29: $n = 77$ cells, 6 mice) and PNs (right; P15–17: $n = 268$ cells, 6 mice; P18–20: $n = 217$ cells, 7 mice; P21–23: $n = 201$ cells, 6 mice; P24–26: $n = 397$ cells, 6 mice; P27–29: $n = 225$ cells, 6 mice). **d** Boxplots of response amplitudes at the preferred stimulus size for each age group from (c) for SST-INs (blue) and PNs (black). The central mark indicates the median, box boundaries indicate the 25th and 75th percentiles, and whiskers indicate the range of the data. **e** Boxplots of locomotion-mediated gain modulation of visual response amplitudes in SST-INs (blue) and PNs (black) across age groups from (c). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, all statistics calculated with a linear mixed-effects regression model with age as a fixed effect and mouse as a random effect.

modulation of spontaneous activity, whereas the spontaneous activity of mature SST-INs is largely positively modulated^{19,22,24,39,40}. We found that at the beginning of the third postnatal week, the spontaneous activity of both PNs and SST-INs exhibited diverse responses to

locomotion onset. SST-INs gradually developed positive state-dependent modulation over the following week. Locomotion also induces visual response modulation in diverse populations of mature V1 neurons, enhancing encoding of visual information during periods

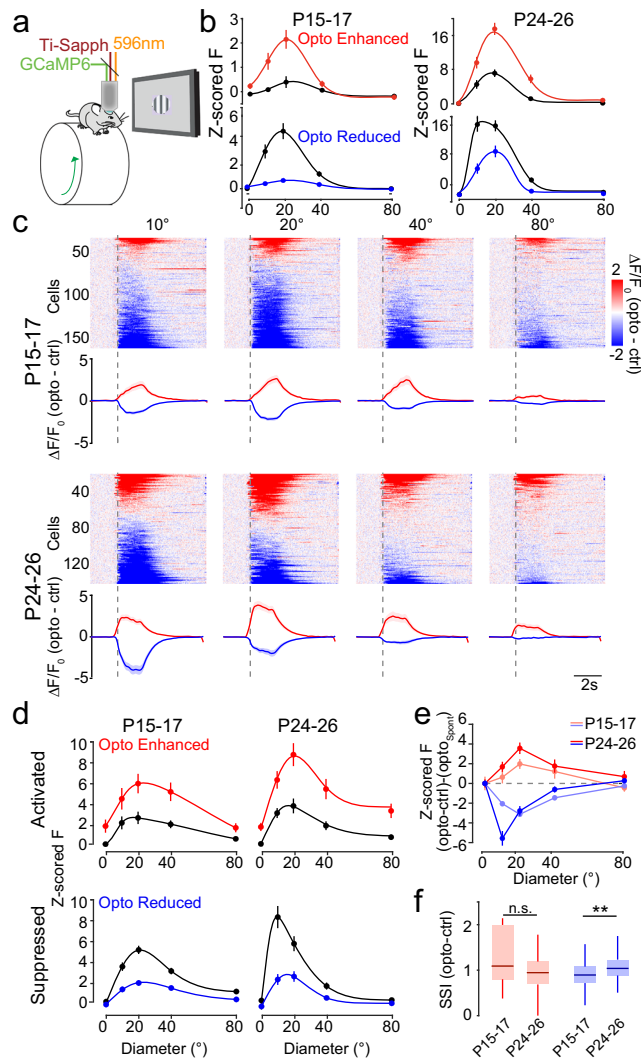


Fig. 4 | Emergence of SST-IN influence on visual selectivity in PNs. **a** Schematic of experimental configuration for simultaneous in vivo optogenetics and 2-photon imaging. **b** Example size tuning curves of four individual PNs in P15–17 and P24–26 animals expressing Cre-dependent ArchT in SST-INs and GCaMP6 in PNs. Individual PNs showed selective visual responses to drifting grating stimuli of varying sizes during baseline conditions (black) and either enhanced (red) or reduced (blue) visual responses during optogenetic suppression of SST-INs. Error bars indicate mean $\pm$ S.E.M. **c** Subset of PNs exhibiting significant modulation of visually evoked responses by SST-IN suppression at varying stimulus sizes across ages. Each colored line represents a single PN's activity, with either enhancement (red) or reduction (blue) of visual responses by the optogenetic stimulus. The difference in z-scored response between optogenetic and control trials for PNs showing enhanced (red) and reduced (blue) visual responses is plotted below each heat map. P15–17 (upper row; $n = 151$ cells, 6 mice), P24–26 (lower row; $n = 137$ cells, 4 mice). **d** Population average visual responses of PNs that were enhanced (upper row) and reduced (lower row) by SST-IN suppression across ages. Size tuning curves for enhanced (red) and reduced (blue) PNs are plotted against their control responses (black). **e** Change in visual response amplitude at each stimulus size in enhanced (red) and reduced (blue) PNs at P15–17 (light colors) and P24–26 (dark colors) from **(d)**, calculated as $(\text{Opto-ctrl}) - (\text{Opto}_{\text{spont}})$, where $\text{Opto}_{\text{spont}}$ is the effect of optogenetic manipulation on spontaneous PN activity. **f** Surround suppression index of the effect of optogenetic suppression of SST-INs for enhanced (red) and reduced (blue) PNs at each age. The central mark indicates the median, box boundaries indicate the 25th and 75th percentiles, and whiskers indicate the range of the data. Enhanced PNs: P15–17 $n = 28$ cells, 6 mice; P24–26 $n = 55$ cells, 4 mice. Reduced PNs: P15 $n = 123$ cells, 6 mice; P24–26 $n = 82$ cells, 4 mice. $^{**}p < 0.01$, two-sided Mann–Whitney U test.

of arousal^{23,24,27,40,54–57}. Locomotion-induced visual modulation was initially absent in the SST-IN population at P15–17 and developed gradually through P27–29, suggesting distinct developmental trajectories for visual responses and state modulation. In contrast, PNs exhibited a more modest increase in locomotion-modulated visual responses throughout this postnatal period. Previous work has implicated SST-INs in the mature cortex as a part of a VIP-SST-PN disinhibitory circuit, where state-dependent cholinergic activation of VIP-INs may suppress SST activity and enhance PN activity^{19,24,58–60}. However, we found that VIP-INs exhibited robust locomotion modulation at eye opening, indicating that the lack of juvenile SST-IN modulation is not directly inherited from VIP-INs. However, little is known about the maturation of VIP-SST synaptic connections. SST-INs are also directly and indirectly sensitive to cholinergic signals associated with locomotion and arousal^{61,62}, suggesting that the emergence of state modulation in these cells may be linked to changes in neuromodulatory regulation. Together, these findings suggest a potential developmental window for state-dependent regulation of this circuit.

We found that the proportion of SST-INs exhibiting visually evoked responses was low at P15 and increased throughout the third postnatal week. Although SST-INs exhibited increased state-dependent modulation across this period, the emergence of visual responsiveness at later ages was not due to the effects of locomotion. Mature SST-INs in V1 exhibit some selectivity for visual stimulus orientation and direction^{20,21,23} and are more responsive to large stimulus sizes than PNs or other INs^{19,24,25}. In juvenile animals at P15–17, most SST-INs had no visual response, and the minority of visually responsive SST-INs exhibited weak, untuned visual responses. In comparison, parvalbumin-expressing (PV) interneurons exhibit strong, selective visual responses by P17 and less tuning thereafter^{9,63}, suggesting distinct developmental trajectories in different GABAergic populations. Likewise, we found that VIP-INs also exhibited strong visual responses at eye opening. These results also highlight potential differences in cortical development across species, as GABAergic interneurons in ferret V1 exhibit robust untuned responses to stimuli prior to eye opening and rapidly develop tuning following the onset of visual experience⁶⁴. Between P15 and P24, SST-INs developed stronger visual responses and adult-like selectivity for stimulus size, indicating mature receptive field architecture. The development of SST-IN visual responses over the third postnatal week was not dependent on visual experience, as SST-INs in dark-reared animals also developed visual responses. In good agreement with previous reports^{6,7}, we found that PNs at P15–17 already exhibited robust responses that were selective for small stimuli.

Previous work has suggested that adult V1 SST-INs exhibit tuning for relatively large visual stimuli (~ 20 – 30° in diameter) and may contribute to visual surround suppression in nearby PNs^{19,25}. Indeed, SST-INs in layer 2/3 receive extensive horizontal excitatory input²⁵ as well as strong inhibition from VIP-INs⁶⁵, and the VIP-SST circuit is thought to mediate contextual suppression in PNs^{26,66,67}. However, we observed robust selectivity for small stimulus size in PNs at an age when SST-INs largely do not exhibit any visually evoked activity, suggesting that surround suppression is not regulated by SST-INs in this early postnatal period. Indeed, surround suppression of PN visual responses remained stable across the juvenile age range despite the emergence of visually evoked SST-IN responses. Consistent with our finding that SST-IN size tuning does not fully mature until the end of this period, we found no effect of SST-INs on PN surround suppression at P15–17. However, it is possible that another population of GABAergic interneurons that are visually active at P15 may contribute to surround suppression at this developmental stage. Given the evidence for early, tuned visual responses in PV-INs, developmental changes in SST-PV interactions and their influence on PN visual response amplitude and tuning are an intriguing target for future study.

Optogenetic suppression of SST-IN activity during visual stimulation revealed a heterogeneous⁶⁸ and age-dependent impact on PN visual responses, with juvenile PNs exhibiting both enhanced and suppressed responses to visual stimuli. In contrast, suppression of SST-INs exerted a more homogeneously enhancing impact on adult PN responses, suggesting developmental changes in the local circuit targeted by SST-INs. In the mature cortex, layer 2/3 SST-INs robustly innervate local PV-INs^{65,69}, which exhibit small receptive fields and narrow tuning for small stimulus sizes and inhibit local PNs. SST-INs exerted an age-dependent multiplicative modulation of PN responses, consistent with the developmental emergence of a key inhibitory circuit mechanism for normalization^{70–72} where recurrent excitatory input to SST-INs promotes gain modulation in the local circuit¹⁸. SST-INs innervate PNs early in postnatal life²⁸ and we found that optogenetic activation of SST-INs suppressed PNs equally from P15 onwards, suggesting that the increasing SST-IN impact on PN visual responses is not due to the initial formation of SST-PN synapses. We found that suppressing SST-IN activity at P15–17 led to a modest gain modulation of PN visual responses, and the amplitude of this effect grows between P15 and P26. The enhanced effect of SST-INs on PN visual responses over time could arise from increasing visually evoked SST-IN activity or broadening of SST-IN axonal arborization.

Overall, our results highlight a window for the maturation of the functional properties of SST-INs in the postnatal cortex. We find a unique developmental trajectory for SST-INs in mouse primary visual cortex that is distinct from that of nearby excitatory PNs and VIP-INs, suggesting that the window between eye opening and the beginning of the classical critical period is a key time for the refinement of cortical operations that rely on these interneurons. The maturation of functional properties in dendrite-targeting interneurons remains relatively poorly understood. However, the potential involvement of SST-INs in mechanisms of neurodevelopmental disorders, including schizophrenia and autism^{36,37,73,74}, suggests that these cells may be critical mediators of cortical maturation and particularly vulnerable targets of postnatal dysregulation.

Methods

Animals

All animal handling and maintenance is approved by the Institutional Animal Care and Use Committee of the Yale University School of Medicine (Protocol # 11317). Juvenile male and female Sst-IRES-Cre^{+/+} (Jax stock no. 018973) crossed with Ai148^{F/F} (Ai148(TIT2L-GC6f-ICLT2A2)-D, Jax stock no. 030328) (SST^{Cre+}; Ai148^{F/F}), juvenile male and female VIP-IRES-Cre^{+/+} (Jax stock no. 031628) crossed with Ai148^{F/F}, Thy1-GCaMP6s (Jax stock no. 024275), and Sst-IRES-Cre^{+/+} crossed with Thy1-GCaMP6s (SST^{Cre+};Thy1-GCaMP6) mice were kept on a 12 h light/dark cycle, provided with food and water ad libitum, and were returned to their parents and littermates following headpost implants. All mice used in the study were confirmed to have opened their eyes at P14. Juvenile mice were separated from their parents and housed by sex once they reached weaning age (P21). A subset of animals were imaged as adults (P90–120). Imaging experiments were performed during the light phase of the cycle.

Neonatal local injections

Local expression of the channelrhodopsin ChrimsonR or archaeorhodopsin ArchT in V1 was achieved by intracranial injection. P0–P1 litters of Sst^{Cre+};Thy1-GCaMP6s mice were removed from their home cage and placed on a heating pad. Pups were kept on ice for 8 min to induce anesthesia via hypothermia and then maintained on a metal plate surrounded by ice for the duration of the injection. Under a dissecting microscope, viral injections were made via beveled glass micropipette into the primary visual cortex (V1) at a depth of ~350 μ m (QSI, Stoelting Co.). Pups were injected unilaterally with 1 μ l of AAV9-hSyn-DIO-ChrimsonR-mRuby2-ST (2×10^{12} gc ml⁻¹; Addgene #105448)

or 1 μ l of AAV9-CAG-Flex-ArchT (2×10^{12} gc ml⁻¹; Addgene #209779). Pups were then placed back on the heating pad with their littermates. Once the entire litter was injected, pups were gently rubbed with home cage bedding and nesting material and returned to their home cage.

Headpost and cranial window implantation procedure

Implant surgeries were performed on juvenile mice (P15–P29), anesthetized with 1–2% isoflurane mixed with pure oxygen. Mice were required to weigh at least 6.0 grams at P15 to be considered for headpost implantation. During surgery, the scalp was first cleaned with Betadine solution. An incision was then made at the midline, and the scalp was resected to each side to leave an open area of the skull. After cleaning the skull and scoring it lightly with a surgical blade, a custom titanium headpost was secured with C&B-Metabond (Butler Schein) with the left V1 centered. Skull screws were not used, given the relative thinness of the mouse skull at these ages. A 3 mm² craniotomy was made over the left V1. A glass window made of a 3 mm² rectangular inner cover slip adhered with an ultraviolet-curing adhesive (Norland Products) to a 5 mm round outer cover slip (both #1, Warner Instruments) was inserted into the craniotomy and secured to the skull with Cyanoacrylate glue (Loctite). A circular ring was attached to the titanium headpost with glue, and additional Metabond was applied to cover any exposed skull. An analgesic (5 mg/kg Carprofen) and anti-inflammatory steroid (2 mg/mL Dexamethasone) was given immediately after surgery and on the two following days to aid recovery. Mice were given a course of antibiotics (Sulfatrim, Butler Schein) to prevent infection and returned to their littermates to provide maximum comfort for recovery.

Histology

Following experiments, GCaMP-expressing animals were given a lethal dose of sodium pentobarbital and perfused intracardially with 0.9% saline, followed by cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer. Brains were removed and fixed in 4% PFA/PBS solution for 24 h and subsequently stored in PBS. Tissue was sectioned at 50 μ m using a vibrating blade microtome.

Widefield and confocal images were taken with a Zeiss LSM 900. To minimize counting bias, we compared sections of equivalent bregma positions, defined according to the Mouse Brain atlas (Franklin and Paxinos⁷⁵). Cell counting was performed manually using a standardized 100 $\times$ 100 μ m grid overlay to determine the average cell density in layers 2/3 of V1 across three consecutive sections.

Fluorescent in situ hybridization histochemistry

For RNAscope® experiments, samples were processed according to the ACDBio Hplex12 v2 Fluorescent Kit protocol for fixed paraffin-embedded tissue. Briefly, tissue from SST^{Cre+};Thy1-GCaMP6 animals injected with AAV9-CAG-ArchT was fixed overnight in 4% paraformaldehyde, rinsed for 24 h, and pretreated with a series of dehydration steps, followed by paraffin embedding and sectioning at 5 μ m. Sections were then mounted on slides, imaged for GCaMP expression, baked at 60 °C, and cleared in xylene and ethanol prior to treatment with antigen retrieval and protease reagents. Finally, sections were incubated with probes for Somatostatin (ACDBio # 404631) and ArchT (ACDBio # 1289351) at 40 °C. Sections were then rinsed, counterstained with DAPI, and mounted using Prolong Gold antifade mounting medium (Molecular Probes #P369300) for further imaging.

In vivo calcium imaging and optogenetic stimulation

All imaging was performed during the second half of the light cycle in awake, behaving mice that were head-fixed so that they could freely run on a cylindrical wheel. A magnetic angle sensor (Digikey) attached to the wheel continuously monitored wheel motion. Mice were allowed to recover for at least an hour after window implantation before being fixed to the wheel, and would comfortably run on the

wheel within 3 consecutive days of imaging. The face (including the pupil and whiskers) was imaged with a miniature CMOS camera (Blackfly s-USB3, Flir) with a frame rate of 10 Hz.

Imaging was performed using a resonant scanner-based two-photon microscope (MOM, Sutter Instruments) coupled to a Ti:Sapphire laser (MaiTai DeepSee, Spectra Physics) tuned to 920 nm for GCaMP6. Emitted light was collected using a 25×1.05 NA objective (Olympus). Mice were placed on the wheel and head-fixed under the microscope objective. To prevent light contamination from the display monitor, the microscope was enclosed in blackout material that extended to the headpost. Images were acquired using ScanImage 4.2 at 30 Hz, 512×512 pixels. Imaging of layer 2/3 was performed at 150–350 μm depth relative to the brain surface. Each mouse was imaged for as many consecutive days as possible. However, due to cranial growth, it was not possible to follow individual cells across the age range. Visual stimulation, wheel position, and Ca^{2+} imaging microscope resonant scanner frame ticks were digitized (5 kHz) and collected through a Power 1401 (CED) acquisition board using Spike 2 software.

Optogenetic stimulation was achieved by aligning a 594 nm laser to the same light path as the Ti:Sapphire laser of the two-photon microscope, allowing us to activate ChrimsonR or ArchT without affecting imaging quality. The main dichroic of the microscope was replaced with one with a 594 nm notch to allow dual IR and 594 nm excitation. Optogenetic stimulation was delivered through the two-photon microscope objective lens as a single pulse of light beginning 250 ms prior to the onset of the visual stimulus and concluding at the end of the 2 s visual stimulus period. This stimulation was delivered during alternating visual stimuli. To avoid optical noise from fluorophores at higher stimulation levels, an untagged AAV-CAG-ArchT construct was used for optogenetic suppression experiments.

Visual stimulation

Visual stimuli were generated using Psychtoolbox-3 in MATLAB and presented on a gamma-calibrated LCD monitor (17 inches) at a spatial resolution of 1280×960 , a real-time frame rate of 60 Hz, and a mean luminance of 30 cd/m^2 positioned 20 cm from the right eye. Stimuli had a temporal frequency of 2 Hz, spatial frequency of 0.04 cycles per degree, and orientation of 180° . To center stimuli on the receptive field, 100% contrast stimuli were randomly presented in nine 3×3 sub-regions to identify the location that evoked the largest population response in the field of view. The screen was centered, and the process was repeated until a center was identified. Stimuli in each session were randomized and presented in blocks with a fixed duration of 2 s and an interstimulus interval of 5 s, with a mean-luminance gray screen between stimuli. For size tuning, the visual angle was linearly spaced from 0° to 80° in diameter in steps of 10° , where each size was presented 45 times. For the optogenetics experiments, the sizes presented were limited to 0, 10, 20, 40, and 80° in diameter in order to accommodate a sufficient number of optogenetic and control trials for statistical comparison.

Dark rearing

Litters of $\text{SST}^{\text{Cre+}};\text{Ai148}^{\text{F/0}}$ mice were raised in an enclosed habitat in complete darkness from birth through P29. Dark-reared mice were implanted with a cranial window as described above on P29–30, and SST-IN activity was imaged between P30 and P32.

Ex vivo electrophysiology

Under isoflurane anesthesia, mice from each age group were decapitated and transcardially perfused with ice-cold choline-artificial cerebrospinal fluid (choline-ACSF) containing (in mM): 110 choline, 25 NaHCO_3 , 1.25 NaH_2PO_4 , 2.5 KCl, 7 MgCl_2 , 0.5 CaCl_2 , 20 glucose, 11.6 sodium ascorbate, 3.1 sodium pyruvate. Acute coronal slices (300 μm) were prepared from the left hemisphere and transferred to ACSF solution containing (in mM): 127 NaCl, 25 NaHCO_3 , 1.25 NaH_2PO_4 , 2.5 KCl, 1 MgCl_2 , 2 CaCl_2 , and 20 glucose bubbled with 95% O_2 and 5%

CO_2 . After an incubation period of 30 min at 32°C , the slices were maintained at room temperature until use.

Visualized whole-cell recordings were performed by targeting fluorescently labeled SST-INs in the primary visual cortex (V1). All recordings were performed at room temperature. Series resistance (R_s) values were $<20\text{ M}\Omega$ and uncompensated. For miniature excitatory postsynaptic current recordings (mEPSC), the ACSF contained $1\text{ }\mu\text{M}$ TTX to block sodium channels and $10\text{ }\mu\text{M}$ gabazine to block GABAergic currents. The internal solution contained (in mM): 126 cesium gluconate, 10 HEPES, 10 sodium phosphocreatine, 4 MgCl_2 , 4 Na_2ATP , 0.4 Na_2GTP , 1 EGTA (pH 7.3 with CsOH). Cells were voltage-clamped at -70 mV . For miniature postsynaptic current recordings, the ACSF contained $1\text{ }\mu\text{M}$ TTX to block sodium channels. For mEPSCs, the internal solution contained (in mM): 126 cesium gluconate, 10 HEPES, 10 sodium phosphocreatine, 4 MgCl_2 , 4 Na_2ATP , 0.4 Na_2GTP , 1 EGTA (pH 7.3 with CsOH).

Data analysis

Wheel position and changepoints. The wheel position was determined from the output of the linear angle detector. The circular wheel position variable was first transformed to the $[-\pi, \pi]$ interval. The phases were then circularly unwrapped to get running distance as a linear variable, and locomotion speed was computed as a differential of distance (cm/s). A change-point detection algorithm detected locomotion onset/offset times based on changes in the standard deviation of speed. Locomotion onset or offset times were estimated from periods when the moving standard deviations, as determined in a 0.5 s window, exceeded or fell below an empirical threshold of 0.1. Locomotion trials were required to have an average speed exceeding 0.25 cm/s and last longer than 1 s. Quiescence trials were required to last longer than 2 s and have an average speed of $<0.25\text{ cm}/\text{s}$.

Quantification of calcium signals. Analysis of imaging data was performed using ImageJ and custom routines in MATLAB (The Mathworks). Motion artifacts and drifts in the Ca^{2+} signal were corrected with the moco plug-in in ImageJ (Dubbs et al.⁷⁶), and regions of interest (ROIs) were selected as previously described (Chen et al.⁷⁷). All pixels in each ROI were averaged as a measure of fluorescence, and the neuropil signal was subtracted (Chen et al.⁷⁷; Lur et al.⁷⁸; Tang et al.⁷⁹). Excitatory pyramidal cell data were processed through Suite2p (Pachitariu et al.⁸⁰) due to the high prevalence of these cells in the fields of view imaged, but the output was similarly analyzed in the same custom routines in MATLAB. When the same cell was recorded across multiple sessions within a single age group, responses from that cell were averaged across sessions to prevent overrepresentation.

Spontaneous responses and modulation indices. To determine how behavioral state affected neural activity, we first calculated the change in fluorescence over time, $\Delta F/F_0$, as $(F(t)-F_0)/F_0$, where F_0 was the lowest 10% of values from the neuropil-subtracted trace for each session. For mean spontaneous responses, we calculated the average $\Delta F/F_0$ during locomotion (L) and quiescent (Q) periods that occurred in the absence of visual stimulation.

To quantify the amount of overall modulation by behavioral state, we selected locomotion trials across each session that lasted 5 s or longer and quiescent trials that lasted 20 s or longer. To determine whether Ca^{2+} activity was altered during behavioral state transitions, $\Delta F/F_0$ from [0,5]s after locomotion onset ($\text{Ca}_{\text{L-ON}}$) was compared with $\Delta F/F_0$ from [10,15]s after locomotion offset (Ca_{Q}) by computing a modulation index (MI), where $\text{MI} = (\text{Ca}_{\text{L-ON}} - \text{Ca}_{\text{Q}})/(\text{Ca}_{\text{L-ON}} + \text{Ca}_{\text{Q}})$. A minimum of 5 s of quiescence after this period [15,20]s was required to prevent anticipatory effects on Ca_{Q} . To ascertain the significance of this MI, we used a shuffling method in which the wheel trace was randomly circularly shifted relative to the fluorescence trace 1000 times. Cells were deemed significantly modulated if their MI was outside of the 95% confidence interval of the shuffled comparison.

Visual responses. Visual response amplitude was determined as the peak of the z-scored change in fluorescence during the 2 s visual stimulus (F) compared to the 1 s baseline before the stimulus (F_0), given by $(F - \mu_{F_0})/\sigma_{F_0}$. To reduce high-frequency noise when selecting peak amplitude, we applied MATLAB's zero-phase filtering (*filtfilt*) using a second-order infinite impulse response low-pass filter with a half-power frequency of 3 Hz. When separating the effects of state, the mouse was required to be running (or sitting) during the majority (>50%) of each of the 1 s baseline and the 2 s visual stimulation. The locomotion-mediated modulation of visual responses was defined as $\text{RespMod} = (z\text{Ca}_L - z\text{Ca}_Q)/(z\text{Ca}_L + z\text{Ca}_Q)$, where $z\text{Ca}$ are the z-scored visual responses during locomotion (L) or quiescence (Q) trials.

To evaluate visual responsiveness, we conducted a t -test comparing peak response amplitudes to the preferred stimulus size against responses to a blank stimulus (0°). The preferred stimulus size for each cell was determined as the size yielding the highest mean response amplitude across all tested sizes. We identified suppressed cells using bootstrap analysis with 1000 resamplings to compute the median response amplitudes from the z-scored fluorescence traces. Cells with a median bootstrap amplitude below zero were classified as suppressed and excluded from the positively visually responsive group.

Size tuning of all cell types, and particularly of SST-INs, prefers larger stimulus sizes when not well centered (Dipoppa et al.¹⁹). Tuned cells were identified using a t -test comparing the responses to their preferred stimulus size with those to the largest size. Cells with a statistically significant difference ($\alpha = 0.05$) in peak responses were classified as tuned, and those without a significant difference as untuned.

To compare surround suppression across cells with differing response amplitudes, visual response amplitudes were normalized to a 0–1 range (minimum subtracted and scaled by the response range) in lieu of baseline subtraction used in firing rate measures. The surround suppression index for each cell was then calculated as the difference between the normalized response at the preferred size and the normalized response at the largest size.

Optogenetic responses. We assessed whether a cell was significantly modulated by optogenetic stimulation by comparing peak response to the preferred stimulus size during optogenetic versus control trials using the Mann–Whitney U test ($\alpha = 0.05$). Modified cells were categorized as enhanced or reduced based on whether their response amplitude significantly increased or decreased, respectively, during optogenetic trials compared to control (Mann–Whitney U test, $\alpha = 0.05$). The amount of optogenetic modulation on visual responses was determined by calculating the difference in peak visual response amplitude between optogenetic and control trials and subtracting the effect of optogenetic manipulation on spontaneous PN activity (Opto-Control)–Opto_{spont}. To determine how the response varies over stimulus sizes, we calculated a suppression index for this optogenetic response change. To facilitate comparison across cells with varying response amplitudes, we normalized the data to the peak response of the control trials at the preferred stimulus size for each cell. The suppression indices were the difference between the response to the preferred stimulus size and the response to the largest size.

Quantification and statistical analyses

When possible, we used mixed-effect regression models for imaging data due to its nested structure with multiple cells recorded within each mouse. We treated the age group as the fixed effect, while individuals (mice) were the random effects. For the PN control data, which uniquely included multiple fields of view per mouse, fields of view were additionally modeled as nested random effects within mice to capture within-subject variability. Model complexity, including the addition of nested random effects, was evaluated based on Akaike and Bayesian Information Criteria. We additionally evaluated the fit of our models by

checking homoscedasticity in plots of residuals vs. fitted values, Q–Q plots to assess residual normality, and an analysis of deviance residuals to detect overdispersion and model fit anomalies.

For response variables that were continuous and normally distributed, we used a linear mixed-effect model, implemented with the *lmer* function from the *lme4* package in R (Version 4.2.2), R Core Team⁸¹. For continuous but bounded variables, such as the percent of visually responsive cells, we instead used a 0/1 inflated beta mixed-effect regression model. For these data, we fit a 0/1 inflated beta mixed-effect regression model using the *gamlss* package in R using the family “BEINF” (R Version 4.2.2), R Core Team⁸¹. After fitting the model, we transformed the estimates back to probabilities using the inverse link function to model coefficients to facilitate their interpretation.

To compare the number of SST-INs per field of view (counts), we used a Poisson mixed effects model, with age as the fixed effect and the individuals (mice) as the random effect. The PN data were overdispersed (the variance was significantly larger than the mean). To address overdispersion in our data, we evaluated both Poisson and negative binomial distributions for our mixed effects modeling. The models were fitted using the Poisson family from *glmer* and *glmer.nb* for the negative binomial model (*lme4* package) in R. Model selection was based on the likelihood ratio test (*lrtest* from *lmerTest* package in R, Version 4.2.2), R Core Team⁸¹. The negative binomial model was chosen due to a significant improvement in model fit ($p < 0.05$).

To assess differences among age groups, post-hoc analyses were conducted using the *emmeans* package in R, employing the Dunnett correction method for multiple comparisons. This method facilitated the comparison of each older age group to the youngest age group (P15–17) as a reference, effectively controlling for the family-wise error rate in the context of multiple testing.

For one subset of cells, the mixed-effect models did not converge. In this circumstance, we were comparing visually responsive and tuned PNs that were either positively or negatively modulated by the suppression of SST-INs across two age groups (P15–17 and P24–26). We instead employed the Mann–Whitney U test (after a Shapiro–Wilk test that showed the data did not follow a normal distribution).

For the linear mixed-effect model, the response variable was modeled as: response - age + (1|mouse),

which has the following mathematical form:

$$y_{ij} = \beta_0 + \beta_1 x_{ij} + b_{0j} + \epsilon_{ij} \quad (1)$$

where y_{ijk} is the i th observation for the j th mouse. x_{ij} is the age group for the observation i of the j th mouse. β_0 is the intercept, β_1 is the effect of age, b_{0j} is the random intercept for the j th mouse and captures the deviation of the j th mouse's baseline level from the overall intercept β_0 , and ϵ_{ijk} is the residual error for the i th observation within the j th mouse. The random effects have prior distributions $b_{0j} \sim N(0, \sigma_b^2)$, and the error term has the distribution $\epsilon_{ijk} \sim N(0, \sigma^2)$.

All of the details of the statistical tests used, including n 's and the definition of center and dispersion, are provided in Supplementary Data 1. Sex was not considered in any analysis. No tests were used to justify sample size, but sample sizes in the current study are comparable to several recent studies in behaving mice^{19,26,40,66}.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The full datasets generated and analyzed in this study are freely available from the corresponding author on request due to the large volume of data and will be provided for permanent access. Source data for each figure are provided as ‘Source Data’ files. Source data are provided with this paper.

Code availability

Analysis code is available at <https://github.com/cardin-higley-lab/Wang-Ferguson-2024>, <https://doi.org/10.5281/zenodo.13694907>.

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Author contributions

A.W., K.A.F., M.J.H. and J.A.C. designed the experiments. A.W., J.G., S.S., D.B. and V.F. collected the data. K.A.F., V.F. and D.B. analyzed the in vivo data, and J.G. analyzed the ex vivo data. A.W., K.A.F. and J.A.C. wrote the manuscript.

Competing interests

The authors declare no competing interests.

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