Cholinergic input to mouse visual cortex signals a movement state and acutely enhances layer 5 responsiveness

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ABSTRACT

Acetylcholine is released in visual cortex by axonal projections from the basal forebrain. The signals conveyed by these projections and their computational significance are still unclear. Using two-photon calcium imaging in behaving mice, we show that basal forebrain cholinergic axons in the mouse visual cortex provide a binary locomotion state signal. In these axons, we found no evidence of responses to visual stimuli or visuomotor prediction errors. While optogenetic activation of cholinergic axons in visual cortex in isolation did not drive local neuronal activity, when paired with visuomotor stimuli, it resulted in layer-specific increases of neuronal activity. Responses in layer 5 excitatory neurons to both top-down and bottom-up inputs were increased in amplitude and decreased in latency, whereas those in layer 2/3 neurons remained unchanged. Using opto- and chemogenetic manipulations of cholinergic activity, we found acetylcholine to underlie the locomotion-associated decorrelation of activity between excitatory neurons in both layer 2/3 and layer 5. Our results suggest that acetylcholine augments the responsiveness of layer 5 neurons to inputs from outside of the local network, enabling faster switching between internal representations during locomotion.

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INTRODUCTION

Acetylcholine is one of the key neuromodulators involved in cortical function and plasticity. A rich body of work has shown that acetylcholine gates experience-dependent changes in the responses of cortical neurons to sensory inputs. This includes receptive field plasticity in auditory cortex (Bakin et al., 2012; Froemke et al., 2007; Kilgard and Merzenich, 1998; Metherate and Weinberger, 1990), somatosensory

cortex (Ego-Stengel et al., 2001; Rasmusson and Dykes, 1988; Sachdev et al., 1998), and olfactory cortex (Wilson et al., 2004), or ocular dominance plasticity in visual cortex (Bear and Singer, 1986; Greuel et al., 1988; Gu and Singer, 1993; Kirkwood et al., 1999). In all cases, the central tenet is that pairing of acetylcholine release or its application concurrent with a sensory stimulus results in plasticity that increases the responsiveness of neurons to subsequent presentations of the same stimulus. While the evidence that acetylcholine gates plasticity is overwhelming and unequivocal, it remains less well understood what drives the release of acetylcholine in cortex during behavior and what its functional consequences are on cortical computations acutely.

Cholinergic projections to cortex arise from a population of neurons in the basal forebrain (Li et al., 2018). Within this population of neurons, there is a relatively broad functional heterogeneity (Laszlovszky et al., 2020; Robert et al., 2021), with responses observed during movement – be it locomotion or licking (Harrison et al., 2016), to reinforcers (Hangya et al., 2015), to auditory stimuli (Guo et al., 2019; Robert et al., 2021), and weakly to visual stimuli (Robert et al., 2021). However, inferring acetylcholine release in specific cortical targets based on cholinergic activity within the basal forebrain is complicated by the fact that different regions (Kim et al., 2016; Pinto et al., 2013) and cholinergic cell types (Laszlovszky et al., 2020) in the basal forebrain project to different areas of cortex. This projection specificity likely underlies the heterogeneity in cholinergic activity across dorsal cortex (Collins et al., 2022; Lohani et al., 2022). Measurements of cholinergic activity in cortex have found that movement in the form of a lever press is associated with cholinergic activity in mouse motor cortex, somatosensory cortex, and prefrontal cortex (Ren et al., 2022). Whisking is associated with cholinergic axonal activity in barrel cortex (Eggermann et al., 2014), while sound (Zhu et al., 2023) and movement (Nelson and Mooney, 2016; Zhu et al., 2023) are associated with cholinergic activity in auditory cortex, and locomotion (Larsen et al., 2018; Lohani et al., 2022), facial movement (Lohani et al., 2022), and pupil dilation (Larsen et al., 2018; Reimer et al., 2016) are associated with cholinergic activity in visual cortex. Overall, cholinergic activity in cortex is closely related to movement, but whether movement is the primary determinant of cholinergic activity and how movement related activity compares to sensory driven activity is less clear.

Likely the primary functional effect of increased levels of acetylcholine in cortex is increased sensory responsiveness. Local application of acetylcholine has been shown to increase sensory responses in somatosensory cortex (Chatfield and Dempsey, 1941), auditory cortex (Forster and McCARTER, 1946), as well as visual cortex (Sato et al., 1987) in anesthetized cats. A similar gain in visually evoked responses in rodent visual cortex can be induced by iontophoretic application of acetylcholine in cortex (Sillito and

Kemp, 1983), by systemic acetylcholine release following basal forebrain stimulation (Goard and Dan, 2009; Pafundo et al., 2016), or by optogenetic stimulation of basal forebrain cholinergic axons in visual cortex (Pinto et al., 2013). Interestingly, this increase of sensory responses caused by acetylcholine is not homogeneous across cortical layers. Neurons responsive to iontophoretic application of acetylcholine are preferentially found in deep cortical layers (Krnjević and Phillis, 1963). The effects of acetylcholine are primarily one of facilitation of neuronal responses in deep cortical layers and one of suppression in superficial layers (Sillito and Kemp, 1983; Soma et al., 2013). However, depending on how locally acetylcholine is applied, it can also result in selective hyperpolarization of layer 5 neurons (Gulledge et al., 2007). Triggering systemic increase of acetylcholine by nucleus basalis stimulation results in firing rate increases in layers 4, 5, and 6 of visual cortex, but decreased firing rates in layer 2/3 (Goard and Dan, 2009). While in the experiments that use systemic modulation of cholinergic activity it is unclear whether the effect is cortical or reflects a combination of changes in subcortical and cortical processing, a direct layer-specific effect on cortical processing would be consistent with differential expression of acetylcholine receptors across cortical layers (Obermayer et al., 2017). Based on these findings it is often assumed that the local release of acetylcholine in cortex differentially influences deep and superficial cortical layers.

A second functional effect of acetylcholine is a decorrelation of cortical activity. Both basal forebrain cholinergic stimulation and local cholinergic axon stimulation in visual cortex desynchronizes activity in visual cortex (Pinto et al., 2013). This effect is thought to be mediated by the influence of acetylcholine on somatostatin positive interneurons and their interaction with the excitatory neurons. This is based on the finding that optogenetic inhibition of somatostatin interneurons blocks acetylcholine induced decorrelation (Chen et al., 2015), and that optogenetically induced acetylcholine release selectively enhances the input from excitatory neurons onto somatostatin interneurons (Urban-Ciecko et al., 2018). A similar decrease in correlation has also been observed during locomotion (Aydın et al., 2018; Erisken et al., 2014), and the effect was stronger in deeper layers of cortex (Dadarlat and Stryker, 2017). It is possible that the decrease of correlations observed during locomotion is driven by locomotion related increases in acetylcholine.

More generally, following the discovery of the modulation of sensory evoked responses by locomotion in visual cortex (Niell and Stryker, 2010), it has been speculated that acetylcholine is involved in locomotion related changes in neuronal activity. Based on studies in rodents, a circuit was delineated that projects from mesencephalic locomotor region to the basal forebrain, which when active, can bring about a gain

91 of responses in visual cortex to visual stimuli (Lee et al., 2014). It has been argued that nicotinic activation
92 of vasoactive intestinal peptide (VIP) expressing interneurons in visual cortex is necessary for this
93 locomotion induced gain of visual responses (Fu et al., 2014). While VIP interneurons are clearly
94 influenced by acetylcholine (Gasselin et al., 2021), cholinergic receptors are expressed in all cortical cell
95 types (Colangelo et al., 2019), and there are likely direct functional effects of acetylcholine on many other
96 cell types (Alitto and Dan, 2012; Chen et al., 2015; Gulledge and Stuart, 2005; Hay et al., 2016; Hedrick
97 and Waters, 2015; Unal et al., 2012; Xiang, 1998; Zolles et al., 2009). This has called into question the
98 interpretation that acetylcholine is involved in locomotion related changes in neuronal activity primarily
99 through nicotinic activation of VIP neurons (Pakan et al., 2016). The specific subtypes of muscarinic and
100 nicotinic receptors expressed across these different cell types further complicate causal attribution of
101 effects. Nevertheless, all these lines of evidence come together to suggest that basal forebrain cholinergic
102 axons in visual cortex increase their activity on locomotion and release acetylcholine to bring about a gain
103 of visually evoked responses. However, a direct measurement of the activity of cholinergic projection from
104 basal forebrain to visual cortex during locomotion has not been made. Throughout the manuscript, we
105 will refer to basal forebrain cholinergic axons simply as cholinergic axons. This distinction notably excludes
106 potential axons that arise from local VIP-ChAT$^+$ neurons (Granger et al., 2020), that are labelled when
107 using a cross of ChAT-Cre mice with a reporter line.

108 In this work, we sought to understand the signals conveyed by the basal forebrain cholinergic system to
109 visual cortex, the consequent changes to activity of layer 2/3 and layer 5 excitatory neurons in visual
110 cortex upon release of acetylcholine, and the potential implications this could have on the computations
111 performed by visual cortex. We used two-photon calcium imaging of cholinergic axons in visual cortex in
112 behaving head-fixed mice and found that cholinergic activity in visual cortex was best approximated as a
113 locomotion state signal. The effects of optogenetic activation of cholinergic axons locally in visual cortex
114 were layer-specific: the responsiveness was increased in neurons in layer 5 but not in layer 2/3.
115 Furthermore, we provide evidence that acetylcholine release underlies the locomotion related
116 decorrelation of activity in visual cortex.

117 **RESULTS**

118 To investigate the function of cholinergic input to visual cortex, we first characterized the calcium activity
119 in these axons. We used an AAV vector to express an axon-targeted GCaMP6s (Broussard et al., 2018) in
120 basal forebrain cholinergic neurons using ChAT-IRES-Cre mice (Rossi et al., 2011) and recorded calcium

activity of their axons in visual cortex using two-photon microscopy (**Figures 1A and 1B**). For imaging experiments, mice were head-fixed on a spherical treadmill surrounded by a toroidal screen (**Figure 1C**). We used a set of different visuomotor conditions known to activate neurons in visual cortex to probe for activation of cholinergic axons. First, mice were exposed to a closed loop condition during which locomotion velocity was coupled to movement in a virtual tunnel. We then measured activity in an open loop condition during which locomotion and movement in the virtual tunnel were uncoupled, in darkness, and during presentation of full field drifting gratings. Throughout all experimental conditions mice were free to locomote on the spherical treadmill.

We found that the activity in many cholinergic axons strongly increased during locomotion (**Figures 1D and 1E**). Activity increased with locomotion onset (**Figure 1F**) with an average lag across all responsive axons of 477 ms relative to locomotion onset (**Figure S1A**). Thus, consistent with results reported for auditory cortex (Nelson and Mooney, 2016), we found that locomotion preceded the increase in cholinergic activity in visual cortex. Locomotion onset also results in an increase in activity of neurons in primary visual cortex (Ayaz et al., 2013; Keller et al., 2012; Saleem et al., 2013). It has been speculated that cholinergic input is a driver of locomotion related activity in visual cortex (Lee et al., 2014). We thus compared the observed response latency between cholinergic input and activity of layer 2/3 and layer 5 excitatory neurons. The response latency of cholinergic axons was longer than the latency we observed in responses of layer 2/3 excitatory neurons (**Figure S1B**), but shorter than that of layer 5 excitatory neurons (**Figures S1C and S1D**). This would suggest that the initial increase in neuronal activity in layer 2/3 of the visual cortex was not driven by cholinergic input from basal forebrain.

Interestingly, we found no evidence of a response of cholinergic axons to other stimuli that drive responses in visual cortex such as full field drifting gratings (**Figure 1G**), visual flow onsets in open loop (**Figure 1H**), or visuomotor mismatches (**Figure 1I**) (Attinger et al., 2017; Jordan and Keller, 2020; Keller et al., 2012; Niell and Stryker, 2008; Vasilevskaya et al., 2023; Widmer et al., 2022). Visuomotor mismatches are brief visual flow halts that break the coupling between locomotion and visual flow feedback in a closed loop condition. Consistent with the absence of response to visual stimuli – full field drifting gratings or visual flow in open loop, we found that the response to locomotion onset was independent of whether the locomotion occurred in darkness, in the presence of visual stimuli, or with visual flow feedback coupled to locomotion (**Figure 1J**). For each imaging site, we then quantified the percentage of cholinergic axons that exhibited significant responses to locomotion onset, full field drifting gratings, or visuomotor mismatch, and found that 52.3% ± 4.4% (mean ± SEM) of the axons responded significantly to locomotion

152 onset, while the fraction of responsive axons to gratings and mismatch was not different from chance
153 (**Figure 1K**).

154 To confirm that an increase in calcium activity in cholinergic axons corresponds to an increase in
155 extracellular acetylcholine, we measured acetylcholine levels using the genetically-encoded acetylcholine
156 sensor GRAB-Ach3.0 (Jing et al., 2020). The sensor was expressed in visual cortex, and we performed two-
157 photon imaging in layer 2/3 during the same visuomotor conditions as described above. We found that
158 consistent with the calcium activity of cholinergic axons, acetylcholine levels in visual cortex increased on
159 locomotion onsets (**Figure S2A**), while grating onsets (**Figure S2B**) and visuomotor mismatches (**Figure**
160 **S2C**) resulted in no measurable increase when corrected for hemodynamic artifacts (**Figures S2A-S2F**).
161 Thus, consistent with previous reports (Larsen et al., 2018), we find that locomotion is associated with a
162 basal forebrain driven increase in acetylcholine levels in visual cortex.

163 Further characterizing the relationship between locomotion velocity and cholinergic activity, we found
164 that cholinergic axon activity was not linearly related to locomotion velocity, but instead exhibited a
165 locomotion state dependence. Over the course of a locomotion bout, we found that velocity decreased
166 systematically, such that towards the end of a locomotion bout, velocity was lower than at the beginning
167 (**Figure 2A**). For cholinergic activity, this decrease was absent. We found no evidence of a difference in
168 the average calcium activity of cholinergic axons after locomotion onset to that before locomotion offset
169 (**Figure 2B**). Indeed, the average cholinergic activity after locomotion onset and before locomotion offset
170 was highly correlated across locomotion bouts, while a similar analysis on locomotion velocity revealed
171 that the time-course of velocity is more variable and less correlated bout-by-bout (**Figure 2C**). The
172 similarity of locomotion onset and offset responses was not only true for the population average activity,
173 but the activity after locomotion onset, before locomotion offset, and the average activity during the
174 locomotion bout were all well correlated on an axon-by-axon level (**Figures 2D and S2G**). To quantify the
175 relationship between locomotion velocity and calcium activity in cholinergic axons more generally, we
176 computed the average calcium activity as a function of locomotion velocity (see Methods). This
177 relationship was more step-like than linear, and we found no evidence of an increase in calcium activity
178 between low and high locomotion velocities (**Figure 2E**). The same held true for extracellular acetylcholine
179 levels as measured by GRAB-ACh3.0 (**Figure S2H**). If calcium activity in cholinergic axons reflected a step-
180 like change during locomotion, we should find that calcium activity correlates better with a binarized
181 version of locomotion velocity than with locomotion velocity itself. This is indeed what we found (**Figure**
182 **2F**), independent of the value of the threshold used for binarization (**Figure S3**). Thus, calcium activity in

183 cholinergic axons in visual cortex is better described as a binary locomotion state signal, than a linear
184 locomotion velocity signal.

185 A number of behavioral variables, like pupil dilation, facial movements, as well as the overall level of
186 arousal are all correlated with locomotion (Lohani et al., 2022; Reimer et al., 2014; Vinck et al., 2015). It
187 has been shown that cholinergic activity in visual cortex is also well correlated with pupil dilation and facial
188 movements (Larsen et al., 2018; Lohani et al., 2022; Reimer et al., 2014). Disambiguating the effects of
189 locomotion, pupil dilation, and intensity of facial movements on cholinergic activity is complicated by the
190 fact that these three variables are strongly correlated during behavior. It has been shown that under
191 certain conditions, facial movements are a better predictor of cholinergic activity than locomotion across
192 large parts of dorsal cortex (Lohani et al., 2022). To quantify this relationship in visual cortex, we compared
193 the correlation between cholinergic activity, locomotion velocity, pupil diameter, and intensity of facial
194 movements in different visuomotor conditions. This analysis was motivated by our observation that
195 average light levels have a stronger influence on pupil size than locomotion state. We found that the
196 correlation of locomotion velocity and cholinergic activity was independent of visuomotor condition
197 (**Figure 3A**). This was not the case for the correlation between pupil diameter and cholinergic activity,
198 which was systematically lower in darkness than in conditions with visual stimuli, and lower during closed
199 loop visuomotor coupling than during open loop or grating conditions (**Figure 3B**). This pattern was also
200 reflected in the correlation between locomotion and pupil diameter (**Figure 3C**). The reduced correlation
201 between locomotion velocity and pupil diameter in darkness was not a consequence of a decrease in
202 variability due to ceiling-effects in pupil diameter in darkness (**Figure S4A**). Finally, we also computed the
203 correlation between cholinergic activity and facial movements. The correlation of cholinergic activity with
204 locomotion was higher than that with facial movements under all conditions that we tested (**Figure S4B**).
205 Thus, of these three behavioral variables, locomotion, pupil diameter, and facial movements, locomotion
206 exhibited the most robust correlation with cholinergic activity in visual cortex (**Figure S4C**).

207 Next, we turned to the question of what the influence of increased cholinergic activity in visual cortex is
208 on the activity of excitatory neurons in layer 2/3 and layer 5. We know that locomotion influences
209 neuronal activity in visual cortex in a variety of ways. One of these effects is that during locomotion, visual
210 responses in visual cortex are increased (Niell and Stryker, 2010). This effect was apparent in both layer
211 2/3 (**Figure 4A**) and in layer 5 excitatory neurons (**Figure 4B**). To test whether this increase in visual
212 responsiveness during locomotion was driven by acetylcholine, we expressed the excitatory opsin
213 ChrimsonR (Klapoetke et al., 2014) in basal forebrain cholinergic neurons using an AAV2/1-hSyn-DIO-

214 ChrimsonR-tdTomato vector in ChAT-IRES-Cre mice. We then performed two-photon calcium imaging in
215 visual cortex to measure the responses of layer 2/3 or layer 5 excitatory neurons to the optogenetic
216 activation of local cholinergic axons paired to the presentation of full field drifting grating stimuli while
217 the mouse was stationary (see Methods). We found that in layer 2/3, optogenetic activation of cholinergic
218 axons did not result in a detectable increase in grating onset responses (**Figure 4C**), while the responses
219 of layer 5 excitatory neurons to the same stimulus increased with concurrent optogenetic activation of
220 cholinergic axons (**Figure 4D**). Interestingly, this increase in grating onset responses in layer 5 neurons
221 driven by direct cholinergic activation was smaller than that observed during locomotion (**Figures 4B and**
222 **4D**). This could be either because the acetylcholine release triggered by the optogenetic stimulation of
223 cholinergic axons was weaker than that occurring during locomotion, or because the increase in responses
224 during locomotion is only partially explained by the co-release of acetylcholine. If the former were the
225 case, we would expect that responses occurring during locomotion are less influenced by optogenetic
226 activation of cholinergic axons. This was indeed the case for grating onset responses during locomotion
227 which were not modulated by optogenetic activation of cholinergic axons (**Figures S5A and S5B**).
228 Interestingly, visuomotor mismatch responses, which also occur only during locomotion, were
229 significantly increased in layer 5 neurons (**Figure 4F**), but not in layer 2/3 (**Figure 4E**). Similarly, we found
230 that optogenetic activation of cholinergic axons increased locomotion onset responses in layer 5 neurons
231 (**Figure 4H**), while layer 2/3 neurons exhibited no significant difference (**Figure 4G**). Thus, while in layer 5
232 neurons the effect of optogenetic activation appears to saturate for bottom-up grating responses during
233 locomotion, this was not the case for responses that rely on top-down input. Furthermore, the effects of
234 optogenetic activation of cholinergic axons on layer 5 neurons could not be explained by stimulation
235 artifacts. In mice that did not express ChrimsonR in cholinergic axons (no ChrimsonR controls), we found
236 no evidence of an optogenetic light stimulation effect (**Figures S5C-S5E**). Overall, consistent with previous
237 findings (Goard and Dan, 2009), we find that acetylcholine primarily increases both bottom-up and top-
238 down driven responses in layer 5 but not layer 2/3 neurons.

239 In addition to increased visual responses, a second effect of locomotion on neuronal activity in visual
240 cortex is that locomotion itself drives neuronal responses (Keller et al., 2012; Saleem et al., 2013). This
241 effect was observed in both layer 2/3 (**Figure 5A**) and layer 5 (**Figure 5B**) excitatory neurons. To test
242 whether artificial activation of cholinergic axons would result in increased neuronal activity, we again
243 performed two-photon calcium imaging in visual cortex neurons to measure responses of layer 2/3 or
244 layer 5 excitatory neurons upon optogenetic activation of local cholinergic axons. We found no evidence
245 of any increase in calcium activity in either layer 2/3 (**Figure 5C**) or layer 5 excitatory neurons (**Figure 5D**).

246 This absence of a stimulation response is consistent with previous reports that found that cholinergic axon
247 stimulation in visual cortex does not result in a response of layer 2/3 neurons (Chen et al., 2015) and that
248 electrical stimulation of basal forebrain primarily activates inhibitory neurons in superficial layers of visual
249 cortex (Alitto and Dan, 2012). This, combined with our finding that on average the layer 2/3 locomotion
250 onset related activity preceded the activity increase in cholinergic axons (**Figure S1**), would indicate that
251 the locomotion related increase in layer 2/3 and layer 5 activity observed in visual cortex is not driven by
252 acetylcholine.

253 A third effect locomotion has on neuronal activity in visual cortex is to decorrelate the activity of nearby
254 neurons (Dadarlat and Stryker, 2017; Erisken et al., 2014). Consistent with these studies, we found that
255 locomotion results in a reduction of pairwise correlations between activity of layer 2/3 excitatory neurons
256 (**Figures 6A and 6E**) as well as between layer 5 excitatory neurons (**Figures 6B and 6F**). We could induce
257 this decorrelation by optogenetically activating the cholinergic axons while mice were stationary. This
258 optogenetically induced decorrelation in layer 2/3 (**Figures 6C and 6E**) was stronger than that driven by
259 locomotion, while the two were similar in layer 5 (**Figures 6D and 6F**). This decorrelation was not due to
260 a visual response to the stimulation laser (**Figure S5F**). Interestingly, we found that in both layer 2/3 and
261 layer 5, the locomotion induced decorrelation was observed primarily for neurons with low pairwise
262 correlations. Pairs of neurons with high correlation of activity tended to further increase their correlation
263 during locomotion (**Figure S6**). Finally, we tested whether the correlation in visual cortex was similarly
264 influenced by chemogenetic manipulation of basal forebrain cholinergic neurons. We expressed either a
265 DREADD activator or a DREADD inhibitor in ChAT-Cre mice to systemically increase or decrease cholinergic
266 activity. Interestingly, we noticed that the systemic increase or decrease of cholinergic release resulted in
267 behavioral changes in the mice. Upon chemogenetic activation mice increased their locomotion velocity,
268 and upon chemogenetic inhibition, mice decreased their time spent locomoting, resulting in a
269 bidirectional modulation of the total distance traveled by the two manipulations (**Figure S7**). Importantly,
270 chemogenetic manipulations bidirectionally modulated the decorrelation effect on locomotion in layer 5
271 excitatory neurons in visual cortex. DREADD activation resulted in an increased strength of the locomotion
272 driven decorrelation, while DREADD inhibition resulted in a reduced strength of locomotion driven
273 decorrelation in layer 5 (**Figure 6H**). This effect was absent in layer 2/3 where neither manipulation
274 influenced the locomotion driven decorrelation (**Figure 6G**). Thus, cholinergic activation is likely sufficient
275 to explain the locomotion induced decorrelation of activity in visual cortex.

276 Finally, an effect of locomotion on the responses of layer 5 excitatory neurons, which to the best of our
277 knowledge is unreported, is a decrease in latency to response. In layer 5 excitatory neurons, responses to
278 grating stimuli appeared earlier during locomotion than they did while the mouse was stationary (**Figures**
279 **4B and 7B**). This decrease in response latency during locomotion was absent in layer 2/3 neurons (**Figures**
280 **4A and 7A**). Optogenetic activation of cholinergic axons was able to recapitulate the decrease in response
281 latency in layer 5 neurons (**Figures 4D and 7B**), while leaving layer 2/3 neuron response latency unaffected
282 (**Figures 4C and 7A**). Thus, cholinergic input decreases the response latency of layer 5 excitatory neurons.

283 **DISCUSSION**

284 We set out to address the question of why acetylcholine is released in visual cortex by answering two
285 simpler, intermediate questions: what drives acetylcholine release, and what immediate effects does the
286 released acetylcholine have on the cortical circuits. We found that locomotion was the main driver of
287 ChAT axons activity (**Figure 1**) and acetylcholine release (**Figure S2**) in visual cortex, and cholinergic activity
288 depended on locomotion velocity in a non-linear, step-like manner (**Figure 2**).The acute effects of this
289 increase in acetylcholine on cortical responses were layer-specific: acetylcholine release enhanced
290 responses specifically in layer 5 excitatory neurons, but not in layer 2/3 (**Figure 4**). Using chemogenetic
291 manipulations of basal forebrain cholinergic neurons, we found that we could bidirectionally influence
292 the activity correlations of layer 5 excitatory neurons. Cholinergic activation resulted in a decrease of
293 pairwise correlations while inhibition resulted in an increase (**Figure 6H**). We observed a similar decrease
294 in pairwise correlations during optogenetic stimulation of cholinergic axons locally in visual cortex, arguing
295 that this effect is mediated, in part locally (**Figure 6F**). Similarly, optogenetic stimulation of cholinergic
296 axons in visual cortex drove a decrease in pairwise correlation of layer 2/3 (**Figure 6E**), while global
297 manipulation of the basal forebrain cholinergic system using chemogenetic tools did not significantly
298 change correlations on activation or inhibition (**Figure 6G**). Our results would be consistent with the
299 interpretation that the acute effects of acetylcholine are stronger in layer 5 where they act to increase
300 responsiveness to inputs from outside of the local layer 5 network.

301 While the optogenetic activation of cholinergic axons in visual cortex that we used here likely recapitulates
302 the local effects of acetylcholine more directly than that observed with basal forebrain stimulation, which
303 likely results in a more systemic release of acetylcholine, the method comes with of the following caveats.
304 First, we don't know how the pattern of acetylcholine release induced by optogenetic activation of
305 cholinergic axons compares to that observed endogenously during locomotion. The pattern of cholinergic

306 axon activation was not uniform across axons (**Figures 1E and 2D**), consistent with different modes of
307 activity in cholinergic subpopulations observed in basal forebrain (Laszlovszky et al., 2020). It is unclear
308 whether the exact pattern of cholinergic axon activation substantially influences the observed effects in
309 cortex. However, given that the effects on changes in the correlation of activity induced by optogenetic
310 activation of cholinergic axons was at least as strong as that observed during locomotion, we estimate
311 that optogenetic activation of cholinergic axons can induce levels and patterns of acetylcholine release
312 similar to those observed endogenously. Second, the activation of cholinergic axons likely antidromically
313 activates cholinergic neuron soma in basal forebrain. Thus, we cannot exclude the possibility of a systemic
314 contribution to the effects we observe through shared projections between different cortical and
315 subcortical targets. However, the projection specificity of basal forebrain cholinergic neurons (Kim et al.,
316 2016; Pinto et al., 2013) would argue against this. Finally, it is important to note that here we labeled basal
317 forebrain cholinergic neurons with targeted viral injection. Thus, we cannot exclude the possibility that
318 our labelling of cholinergic axons was incomplete. The alternative approach to label these neurons by
319 crossing ChAT-Cre mice to a reporter line expressing a calcium indicator comes with the caveat of the
320 presence of a substantial fraction of VIP interneurons in visual cortex that also are ChAT positive (Granger
321 et al., 2020). Being unable to separate basal forebrain axons from those of local VIP interneurons
322 complicates the interpretation of results.

323 Given our results, what are the implications for the computational role of acetylcholine in visual cortex?
324 There are likely two aspects to this, the acute effect on cortical activity and the effect on plasticity. We
325 find that acetylcholine was released in visual cortex as a locomotion state signal. This state signal could
326 be used to gate visuomotor plasticity. In systems engaged in sensorimotor learning, plasticity related to
327 feedback prediction errors should occur primarily during self-motion and not when passively experiencing
328 sensory input. Visuomotor development is critically dependent on coupling of locomotion and sensory
329 feedback (Attinger et al., 2017; Held and Hein, 1963; Kaneko and Stryker, 2014), and depends on local
330 plasticity mechanisms in visual cortex (Widmer et al., 2022). Acetylcholine is known to gate plasticity in
331 visual cortex (Bear and Singer, 1986; Greuel et al., 1988; Gu and Singer, 1993; Kirkwood et al., 1999) and
332 could function to gate visuomotor plasticity to phases of self-motion. Given that acetylcholine release is
333 associated with other types of movement as well (Eggermann et al., 2014; Harrison et al., 2016; Lohani et
334 al., 2022; Nelson and Mooney, 2016; Zhu et al., 2023), this is consistent with acetylcholine functioning
335 more generally as a movement state signal. We would predict that in visual cortex, this would primarily
336 relate to movements that result in changes to visual input.

The absence of an acute effect of optogenetically stimulating cholinergic axons locally on activity of visual cortex neurons is consistent with the idea that locomotion driven neuronal activity in visual cortex (Keller et al., 2012; Saleem et al., 2013) is likely not driven by acetylcholine, but is the consequence of a combination of motor-related top-down input (Leinweber et al., 2017) and noradrenaline (Polack et al., 2013), another neuromodulator that is known to accompany locomotion onset (Jordan and Keller, 2023; Reimer et al., 2016). Consistent with previous reports (Goard and Dan, 2009), we find that acetylcholine acutely increases visuomotor responses more strongly in layer 5 excitatory neurons than in layer 2/3 excitatory neurons and that it decorrelates neuronal activity. One of the key questions here is why these effects are induced during locomotion. There are a number of explanations that have been proposed previously that often focus on increasing sensory gain to counteract the noisier visual input during locomotion (Minces et al., 2017; Pinto et al., 2013). We propose a slightly more specific explanation. This is based on the idea that the observed effects of acetylcholine on layer 5 can be explained by assuming the layer 5 network exhibits attractor dynamics and that acetylcholine modifies these attractor dynamics (Kanamaru and Aihara, 2019). The observed differences in the effect of acetylcholine on layer 5, where response strength increased, and layer 2/3, where they did not, are consistent with the interpretation that attractor dynamics are stronger in layer 5 than in layer 2/3. This would, also agree with a proposed microcircuit for predictive processing (Keller and Mrsic-Flogel, 2018), where layer 5 is postulated to maintain an internal representation (Heindorf and Keller, 2022). Thus, acetylcholine could act to increase the sensitivity and speed of response of the internal representation neurons to inputs arriving from outside the local network at the expense of reducing the influence among neurons in the local network. Assuming the internal representation in cortex is a substrate of conscious perception, as proposed by predictive processing, this would be consistent with the finding that anesthesia decouples layer 5 neurons from their inputs onto apical dendrites by cholinergic mechanisms (Suzuki and Larkum, 2020).

What makes this hypothesis all the more tantalizing is the observation that a decrease in cortical cholinergic activation is correlated with the severity of dementia in Alzheimer's disease (Perry et al., 1978). All three drugs currently approved for the management of dementia in Alzheimer's disease are cholinesterase inhibitors, drugs that increase acetylcholine levels. If cholinergic activation alters network dynamics to make the putative layer 5 attractor network more sensitive to external inputs – and hence more easily influenced by long-range cortical inputs – this could explain why cholinergic activation enhances memory retrieval. A decrease in cholinergic tone in Alzheimer's disease is also consistent with the increases of correlation of neuronal activity in cortex observed in mouse models of the disease (Korzhova et al., 2021). Thus the cholinergic gating of the sensitivity of layer 5 to external inputs could

369 constitute a possible mechanism for the cholinergic hypothesis for Alzheimer's disease (Davies and
370 Maloney, 1976; Francis et al., 1999; Hampel et al., 2019; Terry and Buccafusco, 2003).

371 In summary, we show that cholinergic activity in visual cortex conveys a locomotion state signal, that
372 preferentially augments the response of layer 5 excitatory neurons to external inputs and decorrelates
373 activity across neurons, possibly to both sculpt the receptive field tuning of neurons to sensory stimuli as
374 well as to regulate the influence of external input on internal representation to affect our conscious
375 percept.

376

377 **METHODS**

378 **Key Resources Table**

REAGENT or RESOURCE	Source	Identifier
Bacteria and Virus Strains		
AAV2/5-hSyn1-FLEx-axon-GCaMP6s (10^{13}-10^{15} GC/ml)	FMI vector core	vector.fmi.ch
AAV PHP.eB-Ef1α-DIO-GCaMP6s (10^{11} GC/ml)	FMI vector core	vector.fmi.ch
AAV2/1-hSyn-DIO-ChrimsonR-tdTomato (10^{11}-10^{13} GC/ml)	FMI vector core	vector.fmi.ch
AAV2/1-Ef1α-GCaMP6f-WPRE (10^{11}-10^{14} GC/ml)	FMI vector core	vector.fmi.ch
AAV2/1-Ef1α-DIO-hM3D(Gq)-mCherry (10^{11} GC/ml)	FMI vector core	vector.fmi.ch
AAV2/1-Ef1α-DIO-hM4D(Gi)-mCherry (10^{11} GC/ml)	FMI vector core	vector.fmi.ch
AAV2/9-hSyn-GRAB-ACh3.0 (10^{13} GC/ml)	FMI vector core	vector.fmi.ch
AAV2/1-Ef1α-DIO-tdTomato-WPRE (10^{14} GC/ml)	FMI vector core	vector.fmi.ch
AAV2/1-Ef1α-GFP-WPRE (10^{12} GC/ml)	FMI vector core	vector.fmi.ch
Chemicals, Peptides, and Recombinant Proteins		
CNO	Tocris	Cat# 4936-10mg
Fentanyl citrate	Actavis	CAS 990-73-8
Midazolam (Dormicum)	Roche	CAS 59467-96-8
Medetomidine (Domitor)	Orion Pharma	CAS 86347-14-0
Ropivacaine	Presenius Kabi	CAS 132112-35-7
Lidocaine	Bichsel	CAS 137-58-6
Buprenorphine	Reckitt Benckiser Healthcare	CAS 52485-79-7
Ophthalmic gel (Humigel)	Virbac	-
Flumazenil (Anexate)	Roche	CAS 78755-81-4
Atipamezole (Antisedan)	Orion Pharma	CAS 104054-27-5
N-Butyl-2-cyanoacrylate (Histoacryl)	Braun	CAS 6606-65-1
Dental cement (Paladur)	Heraeus Kulzer	CAS 9066-86-8
Deposited data		
All data and code used to generate manuscript figures	This paper	data.fmi.ch
Experimental Models: Organisms/Strains		
Mus musculus: C57BL/6	Charles River	-
Mus musculus: B6J.129S6-Chat$^{tm2(Cre)Lowl}$/MwarJ	Jackson Laboratories	RRID:IMSR_JAX:028861
Software and algorithms		
MATLAB (2020b)	The MathWorks	RRID:SCR_001622
LabVIEW	National Instruments	RRID:SCR_014325
Two-photon acquisition software	Keller laboratory	sourceforge.net/p/iris-scanning/
Image data processing software	Keller laboratory	sourceforge.net/p/iris-scanning/calliope
Python	python.org	RRID:SCR_008394
Panda3D	panda3d.org	N/A
Other		

Virtual reality and two-photon setup	(Leinweber et al., 2014, 2017)	DOI: 10.3791/50885, 10.1016/j.neuron.2017.08.036
Optogenetic stimulation laser (OBIS 673 nm LX)	Coherent	Cat#1187194
Sham stimulation LED	Prizmatix	UHP-T-595
Titanium headplate	FMI/ETHZ workshop	N/A
Dental drill	Meisinger	N/A

379

380 **Mice.** All mice used in experiments described in the paper were ChAT-IRES-Cre (Rossi et al., 2011)
381 heterozygotes, kept on a C57BL/6 background. A total of 64 mice, both male and female, 6-16 weeks old
382 at the start of the experiment, were used. See **Table S2** for details of mouse inclusion for the different
383 figures. Between experiments, mice were group-housed in a vivarium (light/dark cycle: 12/12 hours). All
384 animal procedures were approved by and carried out in accordance with the guidelines laid by the
385 Veterinary Department of the Canton of Basel-Stadt, Switzerland.

386 **Surgery.** For all surgical procedures, mice were anesthetized with a mixture of fentanyl (0.05 mg/kg;
387 Actavis), midazolam (5.0 mg/kg; Dormicum, Roche), and medetomidine (0.5 mg/kg; Domitor, Orion)
388 injected intraperitoneally. Analgesics were applied perioperatively (2% lidocaine gel, meloxicam 5 mg/kg)
389 and postoperatively (buprenorphine 0.1 mg/kg, meloxicam 5 mg/kg). Eyes were covered with ophthalmic
390 gel (Virbac Schweiz AG). Cranial windows were implanted as previously described (Keller et al., 2012;
391 Leinweber et al., 2014). Briefly, using a dental drill, a 4 mm craniotomy was made over the right visual
392 cortex, centered 2.5 mm lateral and 0.5 mm anterior to lambda. After injection of AAV vectors, the
393 exposed cortex was sealed with a 4 mm circular glass coverslip and glued in place using gel superglue
394 (Ultra Gel, Pattex). The remaining exposed surface of the skull was covered with Histoacryl (B. Braun), and
395 a titanium head bar was fixed to the skull using dental cement (Paladur, Heraeus Kulzer). After surgery,
396 anesthesia was antagonized by a mixture of flumazenil (0.5 mg/kg; Anexate, Roche) and atipamezole (2.5
397 mg/kg; Antisedan, Orion Pharma) injected intraperitoneally.

398 **Axonal labeling.** To image the activity of basal forebrain cholinergic axons in visual cortex, we expressed
399 a calcium indicator in basal forebrain cholinergic neurons. Surgery was performed as described above,
400 and either an AAV2/5-hSyn1-FLEx-axon-GCaMP6s (10^{13} GC/ml) or an AAV-PHP.eB-Ef1α-DIO-GCaMP6s
401 (10^{12} GC/ml) was injected at four locations ipsilateral to the imaging site (AP, ML, DV (in mm): +1.1, +0.1,
402 -4.1; +1.1, +0.1, -3.7; +0.8, +0.1, -4.2; +0.6, +0.6, -4.9) in ChAT-IRES-Cre mice.

403 **GRAB imaging.** We injected an AAV vector carrying GRAB-ACh 3.0, AAV2/9-hSyn-GRAB-ACh3.0 (10^{13}
404 GC/ml), in visual cortex. A 4 mm craniotomy was made over right visual cortex and sealed with a glass
405 coverslip as described above.

406 **Chemogenetic experiments.** To manipulate the activity of the basal forebrain cholinergic system, we used
407 chemogenetic DREADD inhibitor AAV2/1-EF1α-DIO-hM4D(Gi)-mCherry (10^{11} GC/ml) or DREADD activator
408 AAV2/1-EF1α-DIO-hM3D(Gq)-mCherry (10^{11} GC/ml). AAVs were injected in anterior basal forebrain with
409 coordinates as used for axonal imaging described above. DREADDs were stimulated using Clozapine N-
410 oxide (CNO), which was dissolved in DMSO, diluted with saline, and injected intraperitoneally at a dose of
411 0.5 mg/kg body weight. Activity from each imaging site in the DREADD series was acquired starting 30 min
412 - 60 min after CNO injection.

413 **Optogenetic experiments.** We injected AAV2/1-hSyn-DIO-ChrimsonR-tdTomato (10^{11} GC/ml) into basal
414 forebrain at the same coordinates as for axonal imaging described above, and injected AAV2/1-Ef1α-
415 GCaMP6f-WPRE (10^{11-14} GC/ml) into visual cortex for imaging excitatory neurons in layer 2/3 and layer 5.
416 In no-opsin control mice, instead of ChrimsonR, we injected an AAV2/1-DIO-tdTomato-WPRE (10^{14} GC/ml)
417 virus. ChrimsonR stimulation and functional imaging of GcaMP6f-expressing neurons was done as
418 previously described (Attinger et al., 2017). For imaging, we used a modified Thorlabs B-Scope with a 12
419 kHz resonance scanner (Cambridge Technology). The illumination source for the optogenetic stimulation
420 was a 637nm laser (OBIS LX, Coherent). We used a dichroic mirror (ZT775sp-2p, Chroma) to combine the
421 two-photon laser and stimulation laser. A second long-pass dichroic mirror (F38-555SG, Semrock) was
422 used to split the GCaMP emission from both illumination light sources. Light leak from the 637 nm
423 stimulation laser was reduced by synchronizing the stimulation laser to the turnaround times of the
424 resonant scanner (during which imaging data were not acquired). Lastly, amplified PMT signals were
425 digitally bandpass filtered at 80 MHz to reduce the effect of ringing in the amplifier. This allowed for near
426 stimulation-artifact free synchronous imaging and optogenetic stimulation. For all experiments with
427 stimulation of cholinergic axons locally in visual cortex, we used a square-wave pulse of 1s duration and
428 10 mW/mm^2 power (measured at objective). To measure correlation changes, we employed a longer laser
429 stimulation window of 1 minute, at 40 Hz (50% duty cycle), with an average power output of 20 mW/mm^2
430 (measured at objective). For sham stimulation, we used an optical guide coupled to a high-power LED
431 (UHP-T-595, Prizmatix) to diffusely illuminate the mouse and surrounding virtual reality setup.

432 **Virtual reality environment.** The virtual reality setup is based on the design of Dombeck and colleagues
433 (Dombeck et al., 2007). Briefly, mice were head-fixed and free to run on an air-supported spherical

434 treadmill. The rotation of the ball was restricted around the vertical axis with a pin. The virtual reality
435 environment was projected onto a toroidal screen covering approximately 240 degrees horizontally and
436 100 degrees vertically of the mouse's visual field, using a projector (Samsung SP-F10M) synchronized to
437 the resonant scanner of the two-photon microscope. The virtual environment consisted of an infinite
438 corridor with walls patterned with vertical sinusoidal gratings with a spatial frequency of approximately
439 0.04 cycles per degree (Leinweber et al., 2014). In closed loop condition, the locomotion of the mouse
440 was coupled to movement along a virtual tunnel. In open loop condition, we uncoupled the two and
441 replayed the visual flow from a preceding closed loop condition. In grating condition, we presented full
442 field drifting gratings (0°, 45°, 90°, 270°, moving in either direction) in a pseudo-random sequence. Grating
443 stimuli were presented for between 2 s and 3 s. In the inter-stimulus interval (between 2 s and 3 s), mice
444 were shown a gray screen with average luminance matched to that of the grating stimuli.

445 **Eye tracking and facial movement measurement.** During all experiments, we recorded the mouse's left
446 eye (contralateral to the imaged hemisphere) with a CMOS infrared camera at 30 Hz frame rate. The pupil
447 was backlit by the 930 nm laser used for two-photon imaging. We calculated pupil diameter offline by
448 fitting a circle to the pupil. Frames with occluded pupil were excluded from analysis. To extract facial
449 movements, we computed the average image displacement in different image patches on the face of the
450 mouse.

451 **Two-photon microscopy.** Functional two-photon calcium imaging was performed using custom-built two-
452 photon microscopes (Leinweber et al., 2014). The illumination source was a tunable femtosecond laser
453 (Insight, Spectra Physics or Chameleon, Coherent) tuned to 930 nm. Emission light was band-pass filtered
454 using a 525/50 filter for GCaMP and a 607/70 filter for tdTomato/mCherry (Semrock) and detected using
455 a GaAsP photomultiplier (H7422, Hamamatsu). Photomultiplier signals were amplified (DHPCA-100,
456 Femto), digitized (NI5772, National Instruments) at 800 MHz, and band-pass filtered at 80 MHz using a
457 digital Fourier-transform filter implemented in custom-written software on an FPGA (NI5772, National
458 Instruments). The scanning system of the microscopes was based either on a 12 kHz or an 8 kHz resonant
459 scanner (Cambridge Technology). Images were acquired at a resolution of 750 x 400 pixels (60 Hz or 40
460 Hz frame rate, respectively), and a piezo-electric linear actuator (P-726, Physik Instrumente) was used to
461 move the objective (Nikon 16x, 0.8 NA) in steps of 15 µm between frames to acquire images at 4 different
462 depths. This resulted in an effective frame rate of 15 Hz or 10 Hz, respectively. The field of view was 375
463 µm x 300 µm.

464 **Extraction of neuronal activity.** Calcium imaging data were processed as previously described (Keller et
465 al., 2012) and all data analysis was done in MATLAB (MathWorks). Briefly, raw images were full-frame
466 registered to correct for lateral brain motion. Neurons and axons were manually selected based on mean
467 and maximum fluorescence images. For GRAB imaging, neuropil and blood vessels were manually
468 selected. For a subset of mice, the apical dendrites of layer 5 neurons were marked in layer 2/3 by
469 manually tracing them to their soma in layer 5 using a z-stack acquired at the end of an experiment. Raw
470 fluorescence traces were corrected for slow drift in fluorescence using an 8th-percentile filtering with a 66
471 s (or 1000 frames) window (Dombeck et al., 2007). ΔF/F traces were calculated as mean fluorescence in a
472 selected region of every imaging frame, subtracted and normalized by the overall median fluorescence.
473 All neuronal calcium activity data was acquired at 15 Hz. For axonal imaging, 9 (of 25 sites) were imaged
474 at 10 Hz, and analytically resampled at 15 Hz using a polyphase antialiasing filter to make the time-base
475 compatible with the rest of the axonal imaging data.

476 **Data analysis.** All data analysis was done using custom scripts written in MATLAB (MathWorks). To
477 quantify the average population response traces, we first calculated the average event-triggered
478 fluorescence trace for each region of interest (ROI). The responses of all ROIs was then averaged and
479 baseline-subtracted.

480 Locomotion onset was defined as locomotion velocity crossing a threshold of 0.25 cm/s for at least 1 s,
481 while having been below the threshold for 1 s before. The same criteria were used to define visual flow
482 onsets in the open loop condition using visual flow speed. Visuomotor mismatch responses were probed
483 by presenting brief 1 s full field visual flow halts in the closed loop condition. For a mismatch event to be
484 included in analysis, mice had to be locomoting uninterrupted above threshold (0.25 cm/s) from -0.5 s to
485 +1 s after the event onset. Additionally, for a ROI to be included for analysis of the response to a particular
486 event, it had to have at least 5 onsets to the event.

487 In **Figure 1E**, the calcium responses were baseline subtracted using a -1 s to -0.5 s window relative to
488 locomotion onset and sorted based on the average response in the +0.5 s to +1.5 s activity window. In
489 **Figures 1F-1H,** the activity was averaged over all axons, with a baseline subtraction window of -1 s to -0.5
490 s relative to onset. In **Figure 1I**, the baseline window was -0.5 s to 0 s relative to mismatch onset (to include
491 the locomotion related response in the baseline), and a 95% confidence interval was calculated from
492 randomly drawn timepoints during locomotion. In **Figure 1J**, the locomotion onset response is plotted
493 across different visuomotor conditions, and the baseline window was -1 s to -0.5 s. In **Figure 1K**,
494 cholinergic axons ware classified as responsive to a particular stimulus if the mean activity in a window

495 +0.5 s to +1.5 s after stimulus onset was significantly different (paired two-sample t-test over onsets, $p <$
496 0.05) from the mean of the baseline window (-1 s to -0.5 s).

497 Locomotion offset was defined as the threshold crossing at which locomotion velocity decreased below
498 0.25 cm/s for at least 1 s after mice ran above the threshold for at least 1 s. To isolate locomotion bouts,
499 we identified consecutive locomotion onset and offset events that were separated by at least 4 s. In **Figure**
500 **2C,** we computed correlations in average calcium activity (or locomotion velocity) between locomotion
501 onset and offset across locomotion bouts. Onset responses were defined as the average population
502 activity (or locomotion velocity) +0.5 s to +1.5 s after locomotion onset with a baseline of -1 s to -0.5 s.
503 Offset responses were similarly defined as the average population activity (or locomotion velocity) over -
504 1.5 s to -0.5 s before locomotion offset with the same baseline as for bout onset. In **Figure 2D,** axons were
505 sorted based on their average activity at locomotion onset (defined as above), and the same sort order
506 was preserved for plotting the average activity during the bout and at bout offset. In **Figure 2E**, locomotion
507 velocity was binned into 100 evenly spaced bins in the range of 0 cm/s to 6.25 cm/s, and the activity of
508 cholinergic axons in each bin was averaged. In **Figure 2F**, the correlation of the activity of each cholinergic
509 axon was computed against the mouse locomotion velocity, and this was compared against the
510 correlation computed with a binarized version of locomotion velocity, binarized using a threshold of 0.25
511 cm/s.

512 In **Figures 3B, 3C and S4B** with pupil data, we excluded frames with blinks. For variability and correlation
513 analysis involving pupil diameter, sites with less than 30 s of pupil data in a visuomotor condition were
514 excluded.

515 In **Figures 4A-4H**, the visual response is to a grating onset (2 s moving sinusoidal grating with a 3 s gray
516 inter stimulus interval). To probe for the influence of cholinergic stimulation on responses in visual cortex,
517 the optogenetic stimulation laser was turned on for 1 s coincident with the onset of mismatch or grating
518 stimuli. For stimulation on locomotion onset, we detected locomotion onsets based on locomotion
519 velocity in real-time. The average delay between locomotion onsets and laser stimulation onsets was 406
520 ms ± 41 ms (mean ± SEM).

521 In **Figures 6A-6F**, to compute the average pairwise correlation in activity of neurons, we first split the
522 activity trace based on the locomotion state of the mouse and the presence of optogenetic stimulation.
523 Then, for each condition, we computed the average over all pairwise correlations of a given neuron with
524 every other neuron from the same imaging site. For the chemogenetic experiment, we further compared

525 the change in average pairwise correlation before and after injection of the DREADD ligand. We only
526 included those sites in this analysis which had at least 15 s behavior for each of the two compared
527 conditions.

528 We defined latency to activation on locomotion onset (**Figure S1**) for a neuron or axon as the time after
529 onset when the calcium activity, averaged across onsets, first was 2 standard deviations above baseline,
530 and remained above this threshold thereafter for at least 1 s. Neurons that did not reach threshold within
531 the analysis window -2 s to +3 s and were not significantly responsive to locomotion onset (paired two-
532 sample t-test over onsets, $p < 0.05$, between baseline -1 s to -0.5 s and response +0.5 s to +1.5 s), were
533 not included in the analysis.

534 Response latency to grating stimuli (**Figure 7**) was defined for a neuron as the time after onset when the
535 calcium activity, averaged across onsets, first was 2 standard deviations above baseline, and remained
536 above this threshold for at least 1/3 s. Neurons that did not reach threshold within the analysis window
537 of 0 s to +2 s (equal to the duration of grating presentation) were not included in the analysis.

538 **Statistical Analysis.** All statistical information for the tests performed in the manuscript is provided in
539 **Table S1**. Unless stated otherwise, the shading indicates the standard error of the mean across axons or
540 neurons. For analysis where the experimental unit was axon, neuron, or pair of neurons, we used
541 hierarchical bootstrap (Saravanan et al., 2020) for statistical testing due to the nested nature
542 (axons/neurons and mice) of the data. Briefly, we first resampled the data (with replacement) at the level
543 of imaging sites, and then, from the selected sites, resampled for axons (or neurons or neuron pairs). We
544 then computed the mean of this bootstrap sample and repeated this 10 000 times (or 1000 times when
545 comparing response traces in **Figures 4 and S5**) to generate a bootstrap distribution of the mean estimate.
546 For paired tests, the p-value was defined as the proportion of bootstrap samples higher (or lower,
547 depending on the hypothesis) than zero. For unpaired tests, the distribution of the mean for the two
548 variables were compared as the proportion of values higher (or lower, depending on the hypothesis). For
549 analysis where the experimental unit was imaging site, we first tested for normality using Kolmogorov-
550 Smirnov test at a significance level of 5%. For datasets that did not pass the test for normality, the medians
551 were compared with Wilcoxon rank-sum test or signed rank test, as applicable. Otherwise, the means
552 were compared using paired or unpaired t-test, as applicable.

553

554 **Supplemental tables**

555 **Table S1. Statistical information on all analysis.**

556 For statistical comparisons we used paired or unpaired t-tests, or hierarchical bootstraps as indicated in
557 the table below. The units (ROIs, sites, or mice) over which testing was done are boldfaced. For
558 hierarchical bootstraps the two levels of units used are boldfaced.

Figure panel	Value compared	Mean 1	SD 1	Mean 2	SD 2	Test	P-value	N (ROIs)	N (Sites)	N (Mice)
1K	Fraction of responsive cholinergic axons responsive to locomotion onsets *vs* grating onsets	0.523	0.220	0.063	0.075	t-test	$<10^{-5}$	-	**25**	14
	Fraction of responsive cholinergic axons responsive to locomotion onsets *vs* mismatch onsets	0.523	0.220	0.062	0.051	t-test	$<10^{-5}$	-	**25**	14
	Fraction of responsive cholinergic axons responsive to locomotion onsets	0.523	0.220	-	-	t-test	$<10^{-5}$	-	**25**	14
	Fraction of responsive cholinergic axons responsive to grating onsets	0.063	0.075	-	-	t-test	0.40	-	**25**	14
	Fraction of responsive cholinergic axons responsive to mismatch onsets	0.062	0.051	-	-	t-test	0.25	-	**25**	14
2A	Locomotion velocity at locomotion bout onset *vs* offset	0.020	0.008	0.014	0.005	Hierarchical bootstrap	0	**5048**	**25**	14
2B	Cholinergic axon activity [% ΔF/F] at locomotion bout onset *vs* offset	1.47	2.03	1.39	1.80	Hierarchical bootstrap	0.22	**5048**	**25**	14
2C	Correlation between locomotion bout onset and offset for cholinergic axon activity *vs* locomotion velocity	0.554	0.215	0.135	0.258	t-test	$<10^{-5}$	-	**25**	14
2E	Average cholinergic axon activity [% ΔF/F] for low *vs* high velocity locomotion velocity	1.38	2.26	1.57	2.24	Hierarchical bootstrap	0.19	**5048**	**25**	14
2F	Correlation of activity of cholinergic axons with raw *vs* binarized velocity	0.083	0.082	0.099	0.092	Hierarchical bootstrap	0	**5048**	**25**	14
3A	Correlation of average cholinergic axon activity with locomotion velocity in closed loop *vs* open loop	0.286	0.170	0.322	0.199	t-test	0.15	-	**25**	14
	Correlation of average cholinergic axon activity with locomotion velocity in dark *vs* grating	0.319	0.200	0.311	0.167	t-test	0.77	-	**25**	14
	Correlation of average cholinergic axon activity with locomotion velocity in closed loop *vs* dark	0.286	0.170	0.319	0.200	t-test	0.27	-	**25**	14
	Correlation of average cholinergic axon activity with locomotion velocity in open loop *vs* grating	0.322	0.199	0.311	0.167	t-test	0.75	-	**25**	14
	Correlation of average cholinergic axon activity with locomotion velocity in open loop *vs* dark	0.322	0.199	0.319	0.200	t-test	0.93	-	**25**	14
	Correlation of average cholinergic axon activity with locomotion velocity in closed loop *vs* grating	0.286	0.170	0.311	0.167	t-test	0.33	-	**25**	14

3B	Correlation of average cholinergic axon activity with pupil diameter in closed loop *vs* open loop	0.165	0.150	0.277	0.170	t-test	0.0001	-	**25**	14
	Correlation of average cholinergic axon activity with pupil diameter in dark *vs* grating	0.002	0.141	0.300	0.136	Unpaired t-test	0.0000	-	**21, 25**	13, 14
	Correlation of average cholinergic axon activity with pupil diameter in closed loop *vs* dark	0.165	0.150	0.002	0.141	Unpaired t-test	0.0005	-	**25, 21**	14, 13
	Correlation of average cholinergic axon activity with pupil diameter in open loop *vs* grating	0.277	0.170	0.300	0.136	t-test	0.61	-	**25**	14
	Correlation of average cholinergic axon activity with pupil diameter in open loop *vs* dark	0.277	0.170	0.002	0.141	Unpaired t-test	0.0000	-	**25, 21**	14, 13
	Correlation of average cholinergic axon activity with pupil diameter in closed loop *vs* grating	0.165	0.150	0.300	0.136	t-test	0.0028	-	**25**	14
3C	Correlation of pupil diameter with locomotion velocity in closed loop *vs* open loop	0.324	0.217	0.457	0.215	t-test	0.0001	-	**25**	14
	Correlation of pupil diameter with locomotion velocity in dark *vs* grating	0.060	0.232	0.435	0.239	Unpaired t-test	0.0000	-	**21, 25**	13, 14
	Correlation of pupil diameter with locomotion velocity in closed loop *vs* dark	0.324	0.217	0.060	0.232	Unpaired t-test	0.0003	-	**25, 21**	14, 13
	Correlation of pupil diameter with locomotion velocity in open loop *vs* grating	0.457	0.215	0.435	0.239	t-test	0.70	-	**25**	14
	Correlation of pupil diameter with locomotion velocity in open loop *vs* dark	0.457	0.215	0.060	0.232	Unpaired t-test	0.0000	-	**25, 21**	14, 13
	Correlation of pupil diameter with locomotion velocity in closed loop *vs* grating	0.324	0.217	0.435	0.239	t-test	0.045	-	**25**	14
4A	Grating response difference in layer 2/3 neurons [% ΔF/F] while locomoting *vs* not locomoting	-0.12	10.66	-1.97	6.78	Hierarchical bootstrap	0	**6529**	**21**	14
4B	Grating response difference in layer 5 neurons [% ΔF/F] while locomoting *vs* not locomoting	3.13	8.10	0.88	5.50	Hierarchical bootstrap	0	**6798**	**25**	14
4C	Grating response difference in layer 2/3 neurons [% ΔF/F] on optogenetic stimulation *vs* no stimulation while not locomoting	-2.43	8.10	-2.44	7.44	Hierarchical bootstrap	0.46	**3992**	**13**	13
4D	Grating response difference in layer 5 neurons [% ΔF/F] on optogenetic stimulation *vs* no stimulation while not locomoting	1.21	6.05	0.62	6.06	Hierarchical bootstrap	0.0060	**4715**	**16**	13
4E	Mismatch response difference in layer 2/3 neurons [% ΔF/F] on optogenetic stimulation vs no stimulation	2.00	10.14	2.25	8.36	Hierarchical bootstrap	0.66	**2506**	**9**	9
4F	Mismatch response difference in layer 5 neurons [% ΔF/F] on optogenetic stimulation *vs* no stimulation	2.72	7.26	1.77	5.30	Hierarchical bootstrap	0.028	**2801**	**9**	6
4G	Locomotion onset response difference in closed loop in layer 2/3 neurons [% ΔF/F] on optogenetic stimulation *vs* no stimulation	1.66	9.36	1.23	5.76	Hierarchical bootstrap	0.13	**2747**	**10**	10
4H	Locomotion onset response difference in closed loop in layer 5	2.63	5.54	1.86	4.26	Hierarchical bootstrap	0	**3623**	**11**	8

	neurons [% ΔF/F] on optogenetic stimulation *vs* no stimulation									
5A	Mean response in layer 2/3 neurons [% ΔF/F] on locomotion onset *vs* zero	1.82	7.37	-	-	Hierarchical bootstrap	0	**6154**	**20**	14
5B	Mean response in layer 5 neurons [% ΔF/F] on locomotion onset *vs* zero	2.00	4.28	-	-	Hierarchical bootstrap	0	**6032**	**22**	12
5C	Mean response in layer 2/3 neurons [% ΔF/F] to optogenetic stimulation of ChAT axons *vs* zero	-0.07	6.63	-	-	Hierarchical bootstrap	0.62	**6154**	**20**	14
5D	Mean response in layer 5 neurons [% ΔF/F] on optogenetic stimulation of ChAT axons *vs* zero	-0.16	3.21	-	-	Hierarchical bootstrap	0.86	**6032**	**22**	13
6E	Difference in average pairwise correlation of layer 2/3 neurons while locomoting *vs* not locomoting	0.035	0.020	0.038	0.021	Hierarchical bootstrap	0.045	**7826**	**25**	15
	Difference in average pairwise correlation of layer 2/3 neurons on optogenetic stimulation *vs* no stimulation while not locomoting	0.021	0.018	0.029	0.022	Hierarchical bootstrap	0	**1688**	**4**	4
6F	Difference in average pairwise correlation of layer 5 neurons while locomoting *vs* not locomoting	0.030	0.017	0.036	0.017	Hierarchical bootstrap	$<10^{-3}$	**7132**	**26**	14
	Difference in average pairwise correlation of layer 5 neurons on optogenetic stimulation *vs* no stimulation while not locomoting	0.009	0.010	0.014	0.009	Hierarchical bootstrap	0.0017	**100**	**4**	4
6G	Layer 2/3 neuron difference in the change in average pairwise correlation on locomotion following ligand injection for DREADD activation *vs* inhibition	-0.0005	$<10^{-3}$	-0.002	$<10^{-3}$	Hierarchical bootstrap	0.32	**2429, 3103**	**7, 10**	7, 9
6H	Layer 5 neuron difference in the change in average pairwise correlation on locomotion following ligand injection for DREADD activation *vs* inhibition	-0.003	$<10^{-3}$	0.007	$<10^{-3}$	Hierarchical bootstrap	0.032	**1651, 1762**	**7, 7**	7, 7
7A	Latency to grating response onset in layer 2/3 neurons [ms] while locomoting *vs* not locomoting	629	474	620	443	Hierarchical bootstrap	0.44	**1739**	**24**	15
	Latency to grating response onset in layer 2/3 neurons [ms] on optogenetic stimulation *vs* no stimulation while not locomoting	650	498	575	425	Hierarchical bootstrap	0.13	**782**	**14**	14
7B	Latency to grating response onset in layer 5 neurons [ms] while locomoting *vs* not locomoting	672	421	758	415	Hierarchical bootstrap	0.027	**1021**	**25**	14
	Latency to grating response onset in layer 5 neurons [ms] on optogenetic stimulation *vs* no stimulation while not locomoting	692	444	808	429	Hierarchical bootstrap	0.036	**688**	**16**	13
S1D	Latency to response on locomotion onset [ms] for cholinergic axons *vs* layer 2/3 neurons	477	457	366	614	Hierarchical bootstrap	0.040	**1182, 1166**	**23, 25**	12, 15
	Latency to response on locomotion onset [ms] for cholinergic axons *vs* layer 5 neurons	477	457	635	503	Hierarchical bootstrap	0.014	**1182, 1299**	**23, 26**	12, 14
	Latency to response on locomotion onset [ms] for layer 2/3 neurons *vs* layer 5 neurons	366	614	635	503	Hierarchical bootstrap	0	**1166, 1299**	**25, 26**	15, 14
S2G	Correlation of cholinergic axon population activity vector between	0.735	0.187	0.007	0.088	t-test	$<10^{-5}$	-	**25**	14

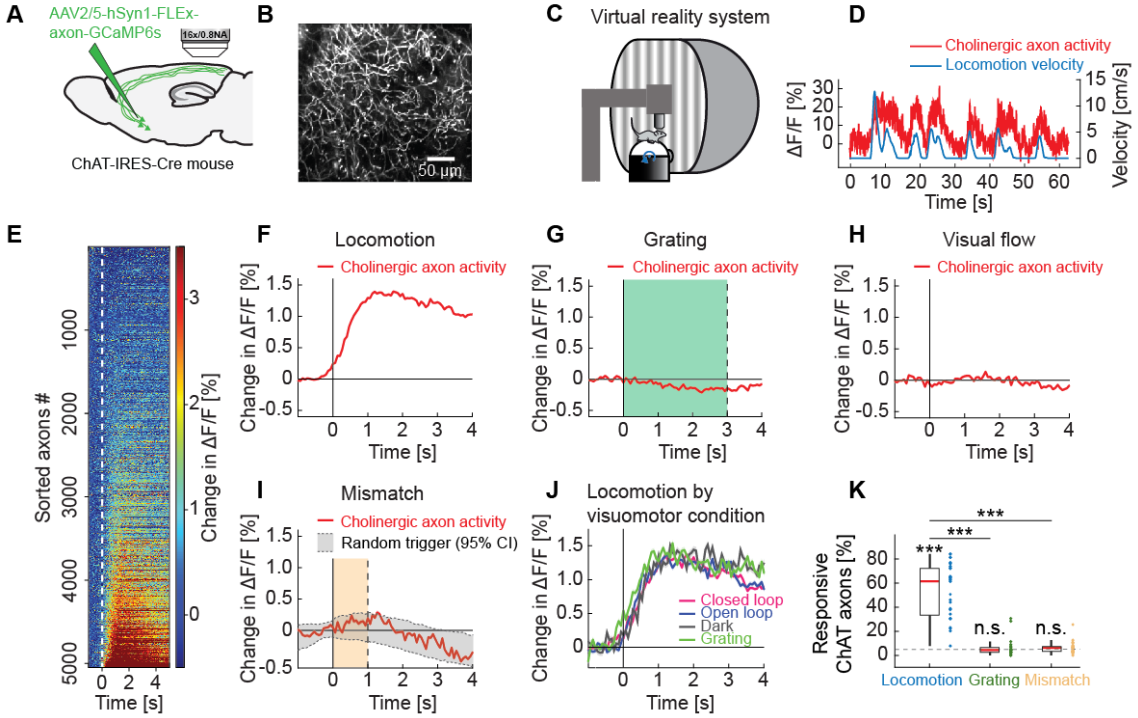
	bout onset and during the bout for actual *vs* random triggers									
	Correlation of cholinergic axon population activity vector between during the bout and at bout onset for actual *vs* random triggers	0.759	0.194	0.036	0.120	t-test	$<10^{-5}$	-	**25**	14
	Correlation of cholinergic axon population activity vector between bout onset and offset for actual *vs* random triggers	0.721	0.196	0.012	0.103	t-test	$<10^{-5}$	-	**25**	14
S2H	Average GRAB-ACh activity [% ΔF/F] in visual cortex during low *vs* high velocity	0.013	0.005	0.003	0.006	Hierarchical bootstrap	0.097	**178**	**8**	8
S4A	Variability in pupil diameter [SD] in closed loop *vs* open loop	21.32	8.47	18.68	5.48	t-test	0.15	-	**25**	14
	Variability in pupil diameter [SD] in dark *vs* grating	25.52	17.45	16.54	7.16	Unpaired t-test	0.023	-	**21, 25**	13, 14
	Variability in pupil diameter [SD] in closed loop *vs* dark	21.32	8.47	25.52	17.45	Unpaired t-test	0.29	-	**25, 21**	14, 13
	Variability in pupil diameter [SD] in open loop *vs* grating	18.68	5.48	16.54	7.16	t-test	0.10	-	**25**	14
	Variability in pupil diameter [SD] in open loop *vs* dark	18.68	5.48	25.52	17.45	Unpaired t-test	0.070	-	**25, 21**	14, 13
	Variability in pupil diameter [SD] in closed loop *vs* grating	21.32	8.47	16.54	7.16	t-test	0.041	-	**25**	14
S4B	Correlation of average cholinergic axon activity with locomotion velocity *vs* facial movement in closed loop	0.286	0.170	0.095	0.172	t-test	$<10^{-4}$	-	**25**	14
	Correlation of average cholinergic axon activity with locomotion velocity *vs* facial movement in open loop	0.322	0.199	0.148	0.195	t-test	0.0001	-	**25**	14
	Correlation of average cholinergic axon activity with locomotion velocity *vs* facial movement in dark	0.319	0.200	0.090	0.224	t-test	$<10^{-4}$	-	**25**	14
	Correlation of average cholinergic axon activity with locomotion velocity *vs* facial movement in grating condition	0.311	0.167	0.130	0.214	t-test	0.0001	-	**25**	14
S5F	Change in average pairwise correlation of layer 2/3 neuron on control stimulation *vs* no stimulation while not locomoting	0.025	0.030	0.025	0.022	Hierarchical bootstrap	0.46	**1047**	**3**	3
	Change in average pairwise correlation of layer 5 neurons on control stimulation *vs* no stimulation while not locomoting	0.017	0.021	0.012	0.006	Hierarchical bootstrap	0.18	**88**	**3**	3
S7B	Locomotion velocity before *vs* after DREADD activation	0.022	0.005	0.028	0.005	t-test	$<10^{-3}$	-	**14**	7
	Locomotion velocity before *vs* after DREADD inhibition	0.017	0.004	0.016	0.005	t-test	0.46	-	**17**	9
S7D	Time spent locomoting before *vs* after DREADD activation	0.683	0.143	0.699	0.154	t-test	0.64	-	**14**	7
	Time spent locomoting before *vs* after DREADD inhibition	0.572	0.156	0.360	0.164	t-test	$<10^{-5}$	-	**17**	9
S7E	Total distance run before *vs* after DREADD activation	5.086	1.964	6.511	2.457	t-test	0.0067	-	**14**	7
	Total distance run before *vs* after DREADD inhibition	3.314	1.713	2.144	1.413	t-test	$<10^{-4}$	-	**17**	9
	Change in total distance run on DREADD activation *vs* inhibition	1.425	1.657	-1.170	0.913	Unpaired t-test	$<10^{-5}$	-	**14, 17**	7, 9

560 **Table S2. Number of mice in different experimental groups**

Genotype	Virus	Site of injection	Nbr. Mice	Nbr. ROIs	Figures
ChAT-IRES-Cre	AAV2/5-hSyn1-FLEx-axon-GCaMP6s	Basal forebrain – MS/vDB/hDB	12	4451	Figures 1, 2, 3, S1, S2G, S3, S4
ChAT-IRES-Cre	AAV PHP.eB-Ef1α-DIO-GCaMP6s	Basal forebrain – MS/vDB/hDB	2	597	Figures 1, 2, 3, S1, S2G, S3, S4
ChAT-IRES-Cre	AAV2/1-hSyn-DIO-ChrimsonR-tdTomato	Basal forebrain – MS/vDB/hDB	15	15 103	Figures 4, 5, 6A-6F, 7, S1, S5A and S5B, S5F, S6
	AAV2/1-Ef1α-GCaMP6f-WPRE	Primary visual cortex			
ChAT-IRES-Cre	AAV2/1-Ef1α-DIO-hM3D(Gq)-mCherry	Basal forebrain – MS/vDB/hDB	7	4080	Figures 6G-6H, S7
	AAV2/1-Ef1α-GCaMP6f-WPRE	Primary visual cortex			
ChAT-IRES-Cre	AAV2/1-Ef1α-DIO-hM4D(Gi)-mCherry	Basal forebrain – MS/vDB/hDB	9	4865	Figures 6G-6H, S7
	AAV2/1-Ef1α-GCaMP6f-WPRE	Primary visual cortex			
ChAT-IRES-Cre	AAV2/9-hSyn-GRAB-ACh3.0	Primary visual cortex	8	609	Figures S2A-S2C, S2H
ChAT-IRES-Cre	AAV2/1-Ef1α-DIO-tdTomato-WPRE	Basal forebrain – MS/vDB/hDB	5	2409	Figures S5C-S5E
	AAV2/1-Ef1α-GCaMP6f-WPRE	Primary visual cortex			
ChAT-IRES-Cre	AAV2/1-Ef1α-eGFP-WPRE	Primary visual cortex	6	2640	Figures S2D-S2F

561

562 **FIGURES**



563

564 **Figure 1. Cholinergic axons in visual cortex are activated during locomotion independent of visual**
565 **stimuli.**

566 (**A**) GCaMP6s was expressed by injecting an AAV2/5-hSyn1-FLEx-axon-GCaMP6s vector in basal forebrain
567 of ChAT-IRES-Cre mice. We then imaged calcium activity of cholinergic axons projecting to visual cortex.

568 (**B**) Example two-photon image of cholinergic axons in visual cortex.

569 (**C**) Schematic of the virtual reality system. Mice were head-fixed on a spherical treadmill and surrounded
570 by a toroidal screen on which we presented visual stimuli in different visuomotor conditions.

571 (**D**) Example trace of the calcium activity of one cholinergic axon in visual cortex (red) and the
572 corresponding locomotion velocity of the mouse (blue).

573 (**E**) Average locomotion onset activity of all cholinergic axons (5048 axons in 14 mice), sorted by their
574 average response during locomotion onset.

575 (**F**) Average response of cholinergic axons to locomotion onset over all the cholinergic axons, of the data
576 shown in **E**. Shading indicates SEM over axons. Due to SEM being very small, the shading is fully obscured
577 by the red line.

578 (**G**) As in **F**, but for grating onsets. Green shading marks the duration of grating presentation.

579 (**H**) As in **F**, but for visual flow onset during the open loop condition.

580 (**I**) As in **F**, but for visuomotor mismatch onset. Orange shading marks the duration of visuomotor
581 mismatch. As mismatch events occur only during times of locomotion, and locomotion itself is a strong
582 driver of cholinergic activity, we would expect to find an increase in cholinergic activity by chance at

583 mismatch. To correct for this, we quantified the distribution of cholinergic activity on random triggers
584 during locomotion (95% confidence interval (CI), gray shading).

585 (**J**) Locomotion onset activity in different visuomotor conditions.
586
587 (**K**) The fraction of cholinergic axons responsive to locomotion, grating, and visuomotor mismatch onset,
588 quantified for each imaging site. Each datapoint is one imaging site. Boxes show 25th and 75th percentile,
589 central mark is the median, and the whiskers extend to the most extreme datapoints not considered
590 outliers. Dashed line marks chance level. n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; see
591 **Table S1** for all statistical information.

592

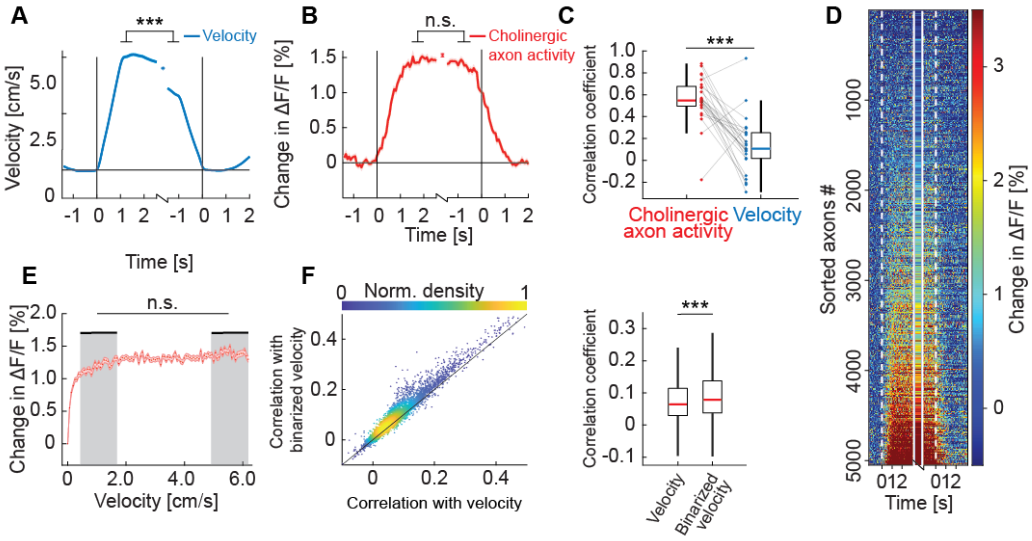


Figure 2. Activity in cholinergic axons was better explained by a binary locomotion state signal than by locomotion velocity.

(**A**) Average locomotion velocity profiles aligned to locomotion onset and offset. The isolated datapoint between the two traces is the average locomotion velocity over the locomotion bout. Shading marks SEM. Here and elsewhere, n.s.: not significant; *: p<0.05; **: p<0.01; ***: p<0.001; see **Table S1** for all statistical information.

(**B**) As in **A**, but for activity of all cholinergic axons in visual cortex, for the data shown in **D**. Shading marks SEM over axons.

(**C**) Bout-by-bout correlation coefficient between the locomotion onset and offset changes for average cholinergic activity and locomotion velocity. Each datapoint is one imaging site. Boxes show 25th and 75th percentile, central mark is the median, and the whiskers extend to the most extreme datapoints not considered outliers.

(**D**) Average onset and offset responses for all cholinergic axons sorted by strength of locomotion onset response.

(**E**) Average activity of cholinergic axons as a function of locomotion velocity. We found no evidence of a difference in activation between low and high locomotion velocities (indicated by gray shading). Red shading marks SEM.

(**F**) Left: For all axons, the correlation coefficient of their calcium activity with locomotion velocity plotted against the correlation coefficient with a binarized version of the locomotion velocity. Right: Distributions of correlation coefficients between calcium activity and locomotion velocity and binarized velocity. Same data as shown on the left. Boxes show 25th and 75th percentile, central mark is the median, and the whiskers extend to the most extreme datapoints not considered outliers.

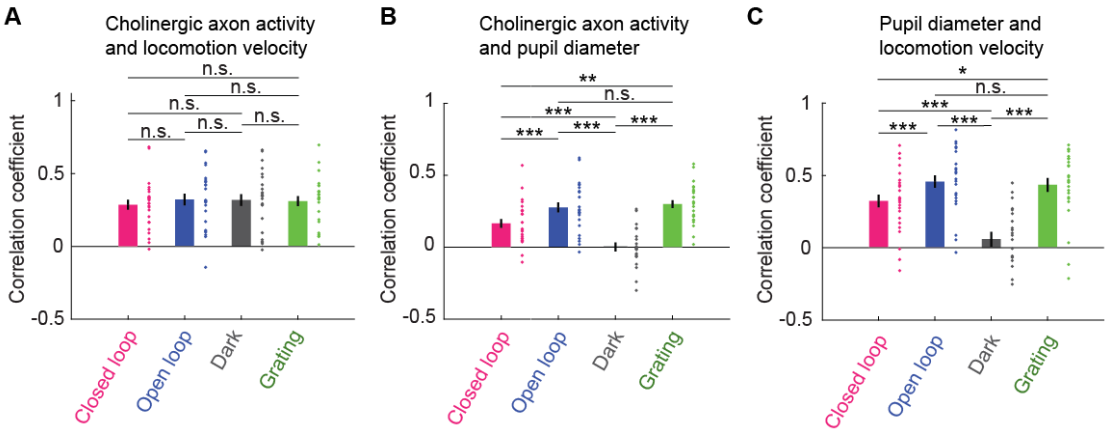


Figure 3. Average activity of cholinergic axons was more strongly correlated with locomotion velocity than with pupil diameter.

(**A**) Correlation of average activity of cholinergic axons activity with locomotion velocity in closed loop, open loop, dark, and grating conditions. Each point represents data from one imaging site, error bars indicate SEM. Here and elsewhere, n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; see **Table S1** for all statistical information.

(**B**) As in **A**, but for correlation between average activity of cholinergic axons and pupil diameter.

(**C**) As in **A**, but for correlation between locomotion velocity and pupil diameter.

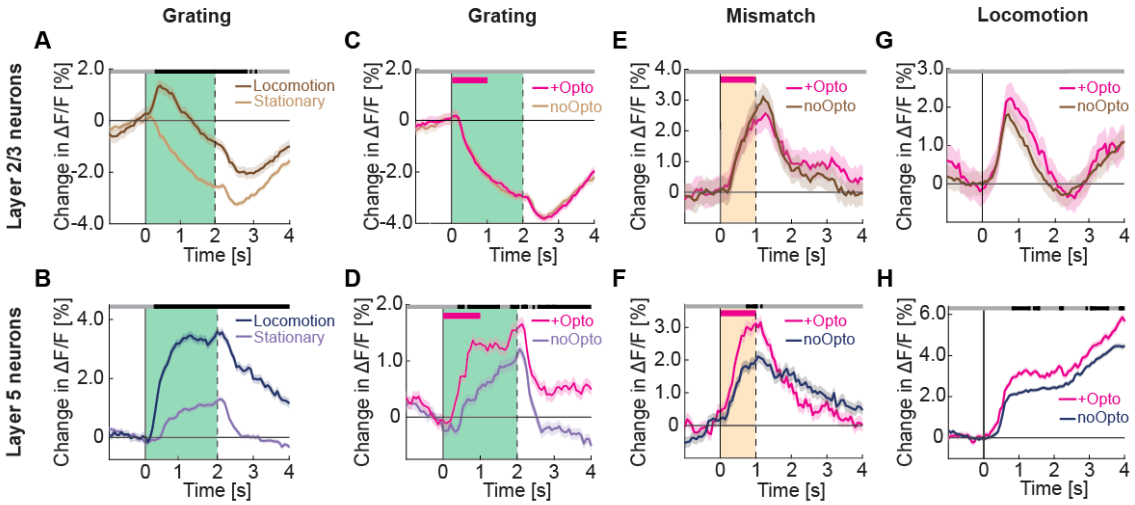


Figure 4. Optogenetic activation of cholinergic axons in visual cortex primarily enhances responses of layer 5, but not layer 2/3 neurons.

(**A**) Average calcium response of layer 2/3 neurons in visual cortex to full field drifting gratings in the absence (light brown) or presence of locomotion (brown). Green shading indicates duration of grating stimulus. The responses are compared bin-by-bin using a nested hierarchical bootstrap test. Here and in subsequent panels, bins with a significant difference ($p < 0.05$) are indicated by a black line above the plot; those with $p > 0.05$ are marked gray. Shading marks SEM.

(**B**) As in **A**, but for layer 5 neurons in visual cortex in the absence (light blue) or presence (dark blue) of locomotion.

(**C**) Average calcium response of layer 2/3 neurons in visual cortex to full field drifting gratings while the mice were stationary, without (light brown) or with (pink) optogenetic activation of cholinergic axons in visual cortex. Duration of optogenetic stimulation is marked by a pink bar. Green shading indicates duration of the grating stimulus.

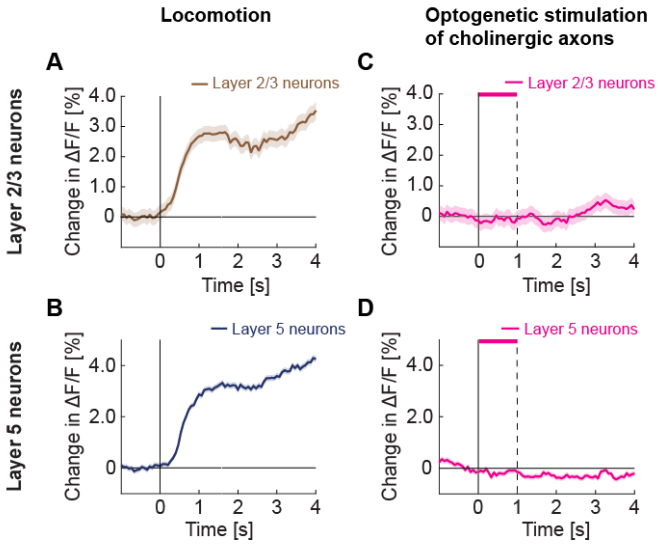
(**D**) As in **C**, but for layer 5 neurons in visual cortex.

(**E**) Average calcium response to visuomotor mismatch in layer 2/3 neurons in visual cortex, without (brown) and with (pink) optogenetic stimulation of cholinergic axons in visual cortex. Orange shading indicates mismatch duration, and pink bar indicates optogenetic stimulation.

(**F**) As in **E**, but for layer 5 neurons in visual cortex.

(**G**) Average calcium response of layer 2/3 neurons in visual cortex to locomotion onset in closed loop, without (brown) or with (pink) optogenetic stimulation of cholinergic axons in visual cortex.

(**H**) As in **G**, but for layer 5 neurons in visual cortex.



649

650 **Figure 5. Optogenetic activation of cholinergic axons in visual cortex did not drive responses in either**
651 **layer 2/3 or layer 5 excitatory neurons.**

652 (**A**) Average calcium response of layer 2/3 neurons in visual cortex to locomotion onset in open loop.
653 Shading indicates SEM.

654 (**B**) As in **A**, but for layer 5 neurons in visual cortex.

655 (**C**) Average calcium response of layer 2/3 neurons in visual cortex during local optogenetic stimulation of
656 cholinergic axons while the mice were stationary. Pink bar marks duration of the optogenetic stimulus.

657 (**D**) As in **C**, but for layer 5 neurons in visual cortex.

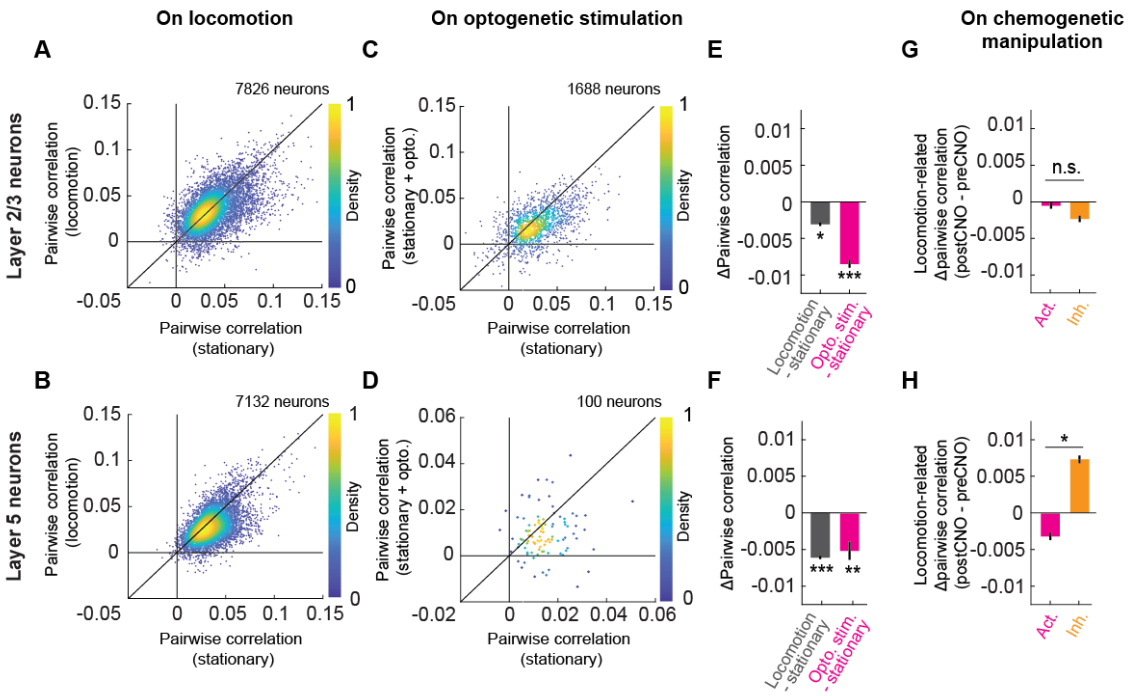


Figure 6. Cholinergic axon activation reduced pairwise correlations between neurons in visual cortex.

(**A**) Average pairwise correlation in calcium activity of layer 2/3 neurons while the mouse was stationary and while the mouse was locomoting. Each dot is the mean of the pairwise correlations for one neuron to all others in the same field of view.

(**B**) As in **A**, but for layer 5 neurons.

(**C**) As in **A**, but comparing the correlation while the mice were stationary, to that while cholinergic axons were stimulated optogenetically.

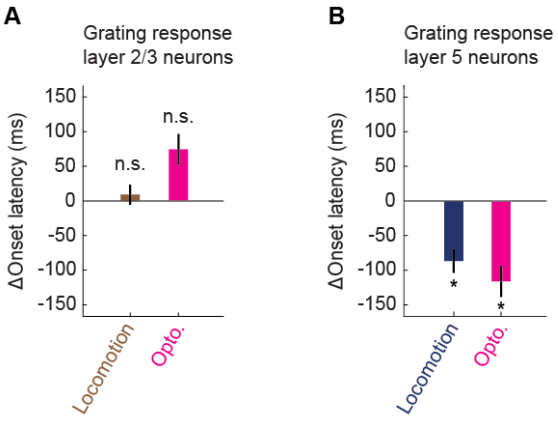
(**D**) As in **C**, but for layer 5 neurons. Note, for this experiment the imaging plane was in L2/3 to simultaneously image layer 2/3 neurons and apical dendrites of layer 5. The latter were traced to their soma in layer 5 offline and were used to compute layer 5 activity correlations.

(**E**) Average change in pairwise correlations of layer 2/3 neurons on locomotion (gray) or on optogenetic stimulation of cholinergic axons during stationary periods (pink), compared to the correlations during stationary (stat.) periods at baseline. Here and elsewhere, n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; see **Table S1** for all statistical information.

(**F**) As in **E**, but for layer 5 neurons of visual cortex.

(**G**) Change in the average pairwise correlations of layer 2/3 neurons during locomotion, upon DREADD activation (pink) or inhibition (orange) of basal forebrain cholinergic neurons.

(**H**) As in **G**, but for layer 5 neurons.



678

679 **Figure 7. Cholinergic input reduced response latency of layer 5 neurons in visual cortex to grating**
680 **stimuli.**

681 (**A**) Locomotion (brown) or optogenetic stimulation of cholinergic axons (pink) induced change in the
682 response latency of layer 2/3 neurons in visual cortex to full field drifting grating onset. Here and
683 elsewhere, n.s.: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; see **Table S1** for all statistical
684 information.

685 (**B**) As in **A**, but for layer 5 neurons.

686

SUPPLEMENTARY FIGURES

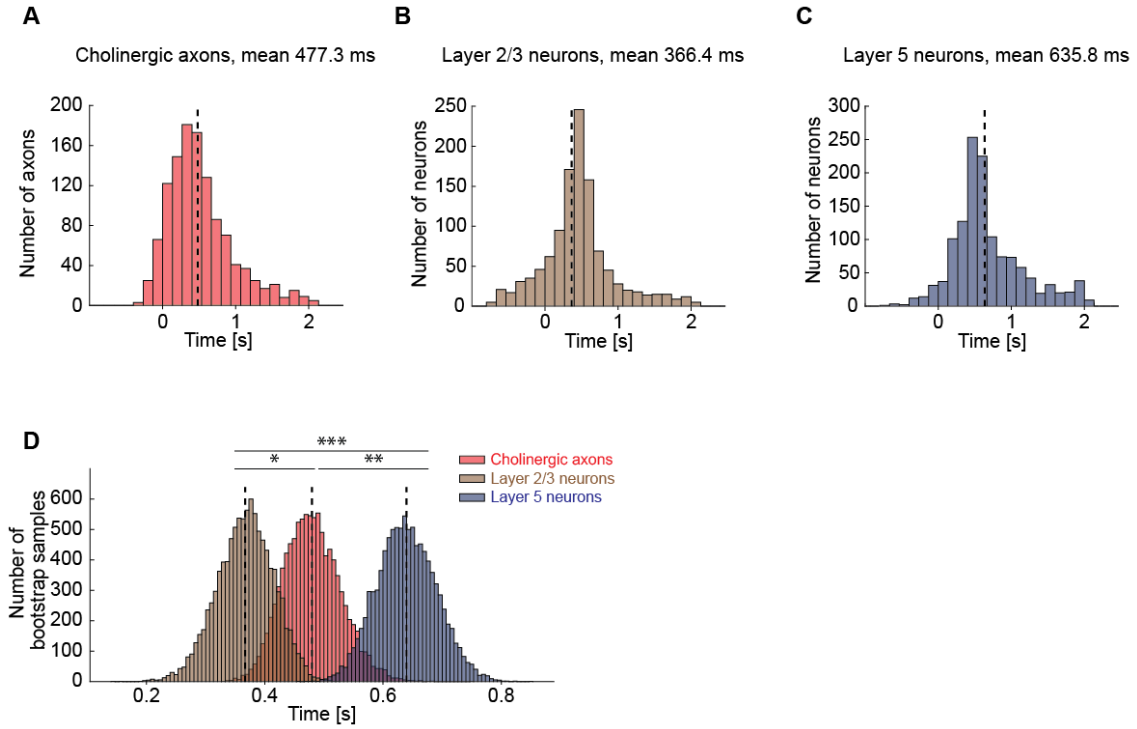


Figure S1. Cholinergic axons in visual cortex were activated after layer 2/3 but before layer 5 visual cortex neurons. Related to Figure 1.

(**A**) Distribution of time to first significant response after locomotion onset for cholinergic axons in visual cortex. Axons without any response to locomotion onset are omitted.

(**B**) As in **A**, but for layer 2/3 neurons in visual cortex.

(**C**) As in **A**, but for layer 5 neurons in visual cortex.

(**D**) Nested bootstrap resampling of the distributions shown in **A-C**. Here and elsewhere, n.s.: not significant; *: p<0.05; **: p<0.01; ***: p<0.001; see **Table S1** for all statistical information.

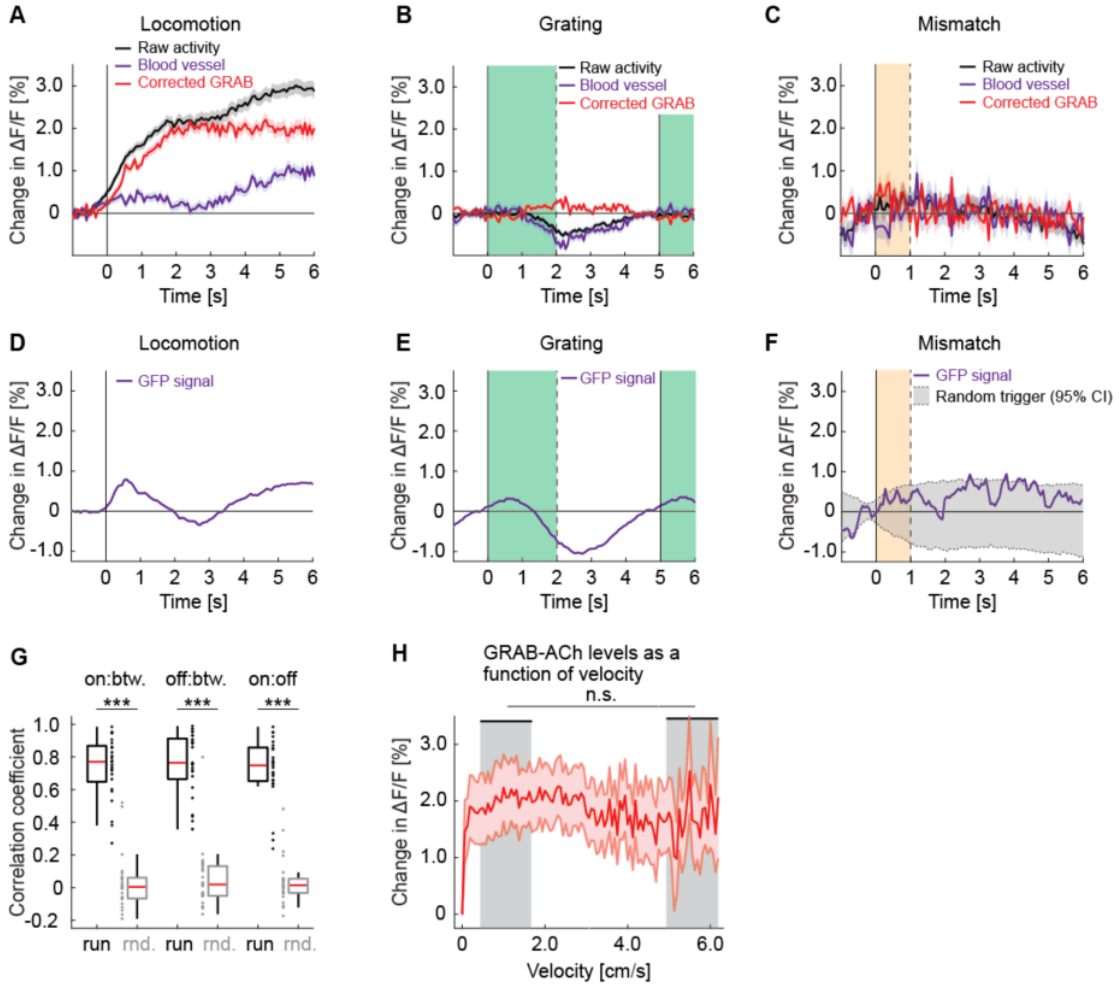


Figure S2. Responses of GRAB-ACh3.0 in visual cortex are similar to the calcium responses in cholinergic axons. Related to Figure 2.

(**A**) Average change in GRAB-ACh3.0 fluorescence during locomotion onset. We used the fluorescence change measured in blood vessels (purple) to estimate hemodynamic occlusion and subtracted this from the raw activity (black) to generate a corrected response (red). Shading marks SEM.

(**B**) As in **A**, but for the GRAB-ACh3.0 response to grating onsets. The green shading marks the duration of the grating stimulus.

(**C**) As in **A**, but for the GRAB-ACh3.0 response to visuomotor mismatch. The orange shading marks the duration of the visuomotor mismatch.

(**D**) To estimate hemodynamic occlusion effects while two-photon imaging, we measured fluorescence changes on locomotion onset in layer 2/3 neurons in visual cortex that express GFP during locomotion onset.

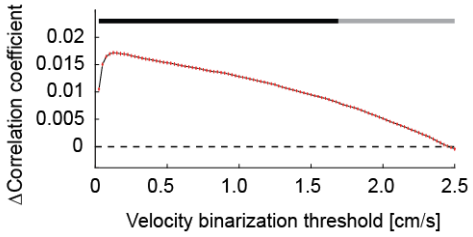
(**E**) As in **D**, but for responses to grating onsets.

(**F**) As in **D**, but for responses to visuomotor mismatch.

712 (**G**) The correlation coefficient between the population activity vectors during locomotion onset (on),
713 offset (off), and during locomotion bout (btw), compared to shuffle (rnd.) controls. Here and elsewhere,
714 n.s.: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; see **Table S1** for all statistical information.

715 (**H**) GRAB-ACh3.0 fluorescence as a function of locomotion velocity. We found no evidence of a difference
716 in fluorescence between low and high locomotion velocities. Red shading indicates SEM.

717

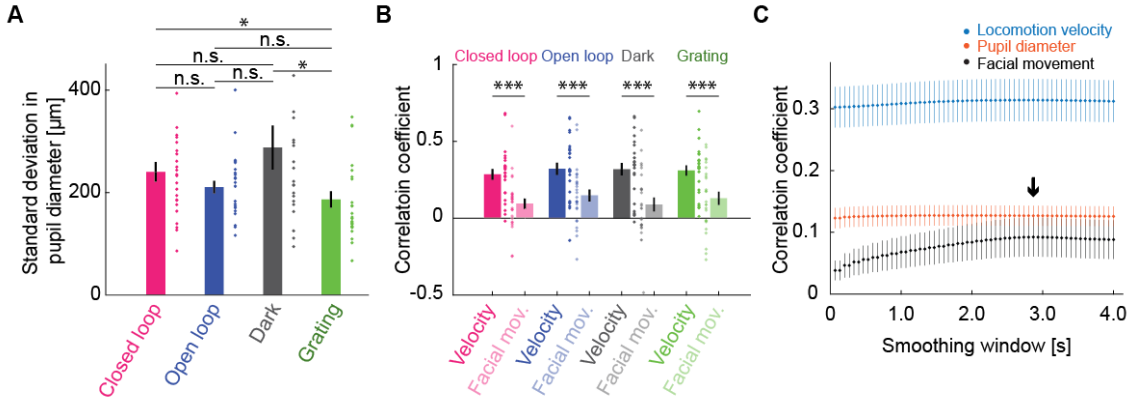


718

719 **Figure S3. Cholinergic axon activity correlated better with a binarized locomotion vector than with the**
720 **unfiltered locomotion vector over a large range of binarization thresholds. Related to Figure 2.**

721 The correlation between activity of cholinergic axons in visual cortex and locomotion velocity was higher
722 when we used a binarized version of the locomotion velocity trace. This difference was present over a
723 large range of thresholds. The black bar at the top of the plot indicates threshold values for which the
724 correlation with binarized locomotion velocity was higher than that with raw locomotion velocity.

725



726

727 **Figure S4. Activity of cholinergic axons correlated better with locomotion velocity than with facial**
728 **movement. Related to Figure 3.**

729 (**A**) Standard deviation of pupil diameter as a function of visuomotor condition. Here and elsewhere, n.s.:
730 not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; see **Table S1** for all statistical information.

731 (**B**) Average correlation of activity of cholinergic axons in visual cortex with facial movements as a function
732 of visuomotor condition, compared with the same activity correlation to locomotion velocity (same data
733 as **Figure 3A**). Error bars indicate SEM. Individual data points are imaging sites.

734 (**C**) Average correlation of activity of cholinergic axons in visual cortex and locomotion velocity (blue), pupil
735 diameter (red), or facial movement (black) as a function of smoothing window. The black arrows marks
736 the smoothing window used for facial movements.

737

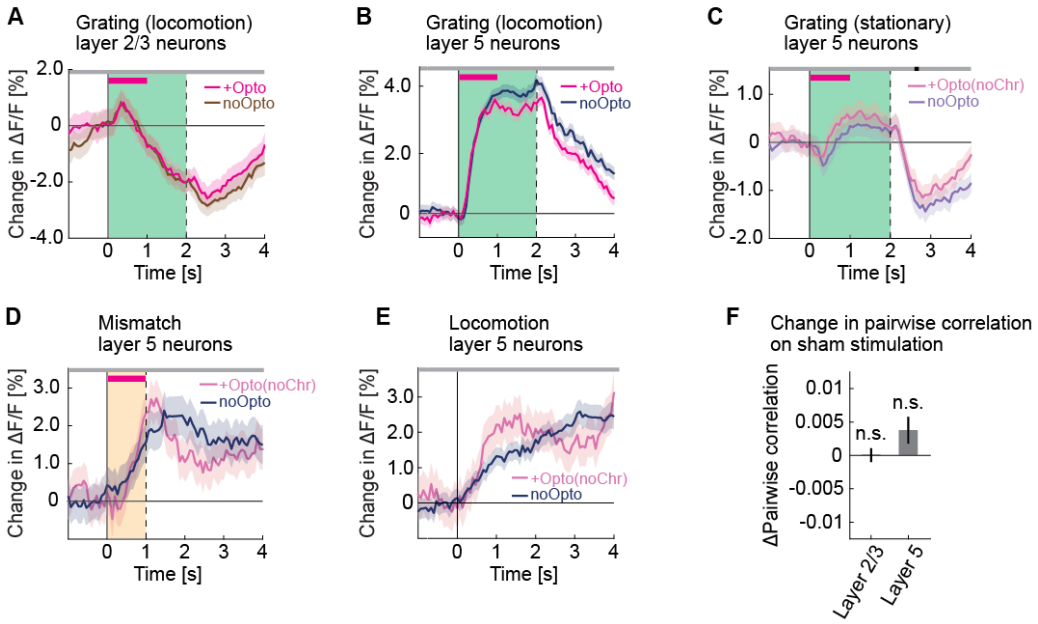


Figure S5. Optogenetic activation did not increase grating responses during locomotion and had no effect in opsin-negative control mice. Related to Figures 4 and 6.

(**A**) Average calcium response of layer 2/3 neurons in visual cortex to full field drifting gratings (green shading) presented while the mice were locomoting, without (brown) and with (pink) optogenetic stimulation of cholinergic axons in visual cortex. The duration of optogenetic stimulation is marked by a pink bar. The responses are compared bin-by-bin using a nested hierarchical bootstrap test. Here and in subsequent panels, bins with a significant difference ($p < 0.05$) are indicated by a black line above the plot; those with $p > 0.05$ are marked gray. Shading marks SEM.

(**B**) As in **A**, but for layer 5 neurons.

(**C**) Average calcium response of layer 5 neurons in visual cortex to full field drifting gratings (green shading) presented while the mice were stationary, without (pale blue) and with (pale pink) optogenetic stimulation in mice that did not express ChrimsonR in cholinergic axons. The duration of stimulation is marked by a pink bar.

(**D**) Average calcium response of layer 5 neurons in visual cortex to visuomotor mismatch (orange shading) without (blue) and with (pale pink) optogenetic light stimulation in mice that do not express ChrimsonR in cholinergic axons. The duration of stimulation light is marked by a pink bar.

(**E**) As in **D**, but for locomotion onset responses. Note, the locomotion onset threshold was relaxed to 0.025 cm/s to increase the number of onsets we could analyze.

(**F**) Change in pairwise correlations in layer 2/3 neurons and in layer 5 neurons in visual cortex during sham stimulation while the mice were stationary. Here and elsewhere, n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; see **Table S1** for all statistical information.

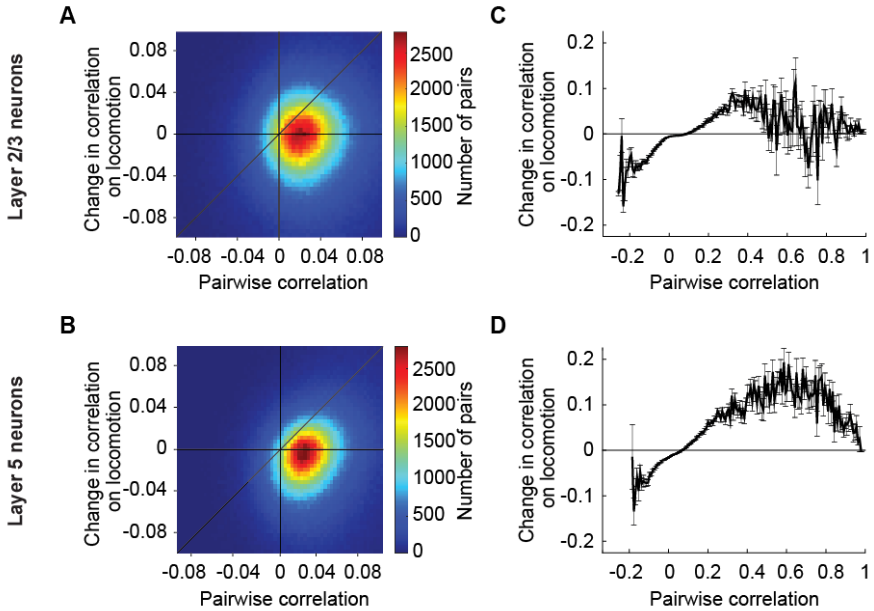


Figure S6. Locomotion increases the correlation between those layer 5 neurons in visual cortex that on average have a higher correlation. Related to Figure 6.

(**A**) Change in the pairwise correlation between pairs of layer 2/3 neurons in visual cortex on locomotion and their average correlation over the entire imaging session.

(**B**) As in **A**, but for layer 5 neurons in visual cortex.

(**C**) Related to **A,** with all neuron pairs binned by their average correlation. Bars indicate SEM.

(**D**) As in **C,** but for layer 5 neurons in visual cortex.

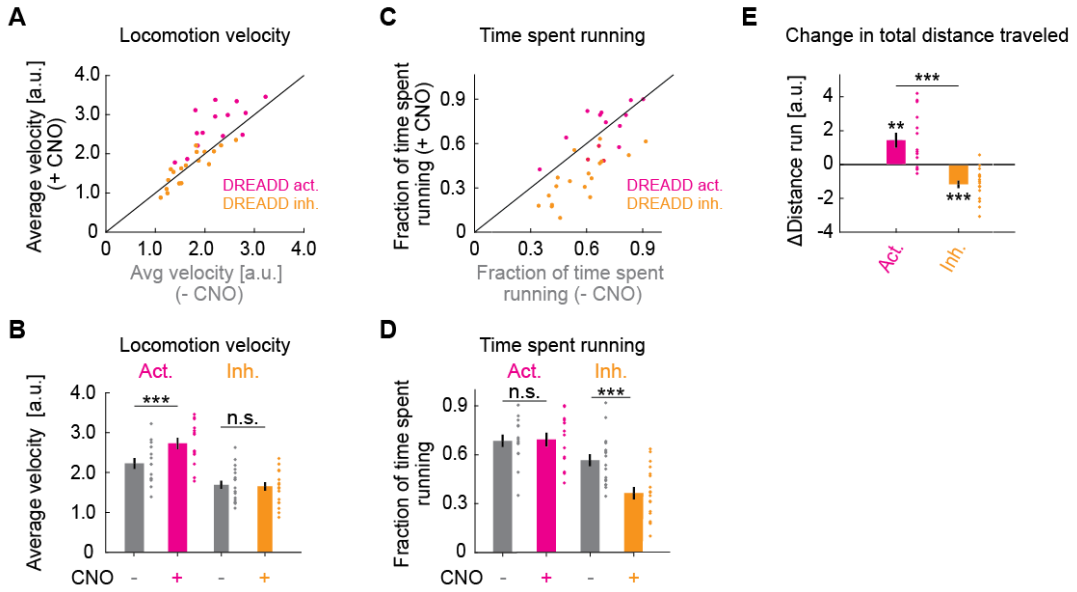


Figure S7. Chemogenetic manipulation of basal forebrain cholinergic neurons affects locomotion behavior of mice. Related to Figure 6.

(**A**) Average locomotion velocity before and after administration of CNO in ChAT-Cre mice that expressed a DREADD activator (pink) or a DREADD inhibitor (orange) in basal forebrain. Each dot is an experimental session.

(**B**) Quantification of the data shown in **A**. Here and elsewhere, n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; see **Table S1** for all statistical information.

(**C**) As in **A**, but for fraction of time spent running.

(**D**) Quantification of the data shown in **C**.

(**E**) CNO induced change in the total distance traveled.

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AUTHOR CONTRIBUTIONS

BY designed and performed the experiments and analyzed the data. All authors wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

REFERENCES

Alitto, H.J., Dan, Y., 2012. Cell-type-specific modulation of neocortical activity by basal forebrain input. Front. Syst. Neurosci. https://doi.org/10.3389/fnsys.2012.00079

Attinger, A., Wang, B., Keller, G.B., Attinger, A., Wang, B., Keller, G.B., 2017. Visuomotor coupling shapes the functional development of mouse visual cortex. Cell 169, 1291–1302. https://doi.org/10.1016/j.cell.2017.05.023

Ayaz, A., Saleem, A.B., Schölvinck, M.L., Carandini, M., 2013. Locomotion Controls Spatial Integration in Mouse Visual Cortex. Curr. Biol. 23, 890–894. https://doi.org/10.1016/J.CUB.2013.04.012

Aydın, Ç., Couto, J., Giugliano, M., Farrow, K., Bonin, V., 2018. Locomotion modulates specific functional cell types in the mouse visual thalamus. Nat. Commun. 9, 4882. https://doi.org/10.1038/s41467-018-06780-3

Bakin, JS., Weinberger, NM., Sharma, J., Perea, G., Petravicz, J., Le, C., Sur, M., 2012. Induction of a physiological memory in the cerebral cortex by stimulation of the nucleus basalis. PNAS 93, 11219–11224. https://doi.org/10.1073/pnas.93.20.11219

Bear, M.F., Singer, W., 1986. Modulation of visual cortical plasticity by acetylcholine and noradrenaline. Nature 320, 172–176. https://doi.org/10.1038/320172a0

Broussard, G.J., Liang, Y., Fridman, M., Unger, E.K., Meng, G., Xiao, X., Ji, N., Petreanu, L., Tian, L., 2018. In vivo measurement of afferent activity with axon-specific calcium imaging. Nat. Neurosci. 21. https://doi.org/10.1038/s41593-018-0211-4

Chatfield, P.O., Dempsey, E.W., 1941. Some effects of prostigmine and acetylcholine on cortical potentials [WWW Document]. URL https://journals.physiology.org/doi/abs/10.1152/ajplegacy.1942.135.3.633 (accessed 3.20.23).

Chen, N., Sugihara, H., Sur, M., 2015. An acetylcholine-activated microcircuit drives temporal dynamics of cortical activity. Nat. Neurosci. 18, 892–902. https://doi.org/10.1038/nn.4002

Colangelo, C., Shichkova, P., Keller, D., Markram, H., Ramaswamy, S., 2019. Cellular, synaptic and network effects of acetylcholine in the neocortex. Front. Neural Circuits 13. https://doi.org/10.3389/fncir.2019.00024

Collins, L., Francis, J., Emanuel, B., McCormick, D., 2022. Cholinergic and noradrenergic axonal activity is both strongly coordinated with behavioral state and heterogeneous across the dorsal cortex. https://doi.org/10.1101/2022.07.12.499792

Dadarlat, M.C., Stryker, M.P., 2017. Locomotion enhances neural encoding of visual stimuli in mouse V1. J. Neurosci. 37, 3764–3775. https://doi.org/10.1523/JNEUROSCI.2728-16.2017

Davies, P., Maloney, A.J., 1976. Selective loss of central cholinergic neurons in Alzheimer's disease. Lancet Lond. Engl. 2, 1403. https://doi.org/10.1016/s0140-6736(76)91936-x

Dombeck, D.A., Khabbaz, A.N., Collman, F., Adelman, T.L., Tank, D.W., 2007. Imaging large-scale neural activity with cellular resolution in awake, mobile mice. Neuron 56, 43–57. https://doi.org/10.1016/j.neuron.2007.08.003

Eggermann, E., Kremer, Y., Crochet, S., Petersen, C.C.H., 2014. Cholinergic Signals in Mouse Barrel Cortex during Active Whisker Sensing. Cell Rep. 9, 1654–1660. https://doi.org/10.1016/j.celrep.2014.11.005

Ego-Stengel, V., Shulz, D.E., Haidarliu, S., Sosnik, R., Ahissar, E., 2001. Acetylcholine-Dependent Induction and Expression of Functional Plasticity in the Barrel Cortex of the Adult Rat. J. Neurophysiol. 86, 422–437. https://doi.org/10.1152/jn.2001.86.1.422

Erisken, S., Vaiceliunaite, A., Jurjut, O., Fiorini, M., Katzner, S., Busse, L., 2014. Effects of Locomotion Extend throughout the Mouse Early Visual System. Curr. Biol. 24, 2899–2907. https://doi.org/10.1016/j.cub.2014.10.045

Forster, F.M., McCARTER, R.H., 1946. The effects of local applications of acetylcholine to the acoustic cortex. J. Neuropathol. Exp. Neurol. 5, 24–28. https://doi.org/10.1097/00005072-194601000-00002

Francis, P.T., Palmer, A.M., Snape, M., Wilcock, G.K., 1999. The cholinergic hypothesis of Alzheimer's disease: a review of progress. J. Neurol. Neurosurg. Psychiatry 66, 137–147. https://doi.org/10.1136/jnnp.66.2.137

Froemke, R.C., Merzenich, M.M., Schreiner, C.E., 2007. A synaptic memory trace for cortical receptive field plasticity. Nature 450, 425–429. https://doi.org/10.1038/nature06289

Fu, Y., Tucciarone, J.M., Espinosa, J.S., Sheng, N., Darcy, D.P., Nicoll, R.A., Huang, Z.J., Stryker, M.P., 2014. A Cortical Circuit for Gain Control by Behavioral State. Cell 156, 1139–1152. https://doi.org/10.1016/j.cell.2014.01.050

Gasselin, C., Hohl, B., Vernet, A., Crochet, S., Petersen, C.C.H., 2021. Cell-type-specific nicotinic input disinhibits mouse barrel cortex during active sensing. Neuron 109, 778-787.e3. https://doi.org/10.1016/j.neuron.2020.12.018

Goard, M., Dan, Y., 2009. Basal forebrain activation enhances cortical coding of natural scenes. Nat. Neurosci. 12, 1444–1449. https://doi.org/10.1038/nn.2402

Granger, A.J., Wang, W., Robertson, K., El-Rifai, M., Zanello, A.F., Bistrong, K., Saunders, A., Chow, B.W., Nuñez, V., Turrero García, M., Harwell, C.C., Gu, C., Sabatini, B.L., 2020. Cortical ChAT+ neurons co-transmit acetylcholine and GABA in a target- and brain-region-specific manner. eLife 9, e57749. https://doi.org/10.7554/eLife.57749

Greuel, J.M., Luhmann, H.J., Singer, W., 1988. Pharmacological Induction of Use-Dependent Receptive Field Modifications in the Visual Cortex. Science 242, 74–77. https://doi.org/10.1126/science.2902687

Gu, Q., Singer, W., 1993. Effects of intracortical infusion of anticholinergic drugs on neuronal plasticity in kitten striate cortex. Eur. J. Neurosci. 5, 475–485. https://doi.org/10.1111/j.1460-9568.1993.tb00514.x

Gulledge, A.T., Stuart, G.J., 2005. Cholinergic Inhibition of Neocortical Pyramidal Neurons. J. Neurosci. 25, 10308–10320. https://doi.org/10.1523/JNEUROSCI.2697-05.2005

Guo, W., Robert, B., Polley, D.B., 2019. The Cholinergic Basal Forebrain Links Auditory Stimuli with Delayed Reinforcement to Support Learning. Neuron 103, 1164-1177.e6. https://doi.org/10.1016/j.neuron.2019.06.024

Hampel, H., Mesulam, M.-M., Cuello, A.C., Khachaturian, A.S., Vergallo, A., Farlow, M.R., Snyder, P.J., Giacobini, E., Khachaturian, Z.S., 2019. Revisiting the Cholinergic Hypothesis in Alzheimer's Disease: Emerging Evidence from Translational and Clinical Research. J. Prev. Alzheimers Dis. 6, 2–15. https://doi.org/10.14283/jpad.2018.43

Hangya, B., Ranade, S.P., Lorenc, M., Kepecs, A., 2015. Central Cholinergic Neurons Are Rapidly Recruited by Reinforcement Feedback. Cell 162, 1155–1168. https://doi.org/10.1016/j.cell.2015.07.057

Harrison, T.C., Pinto, L., Brock, J.R., Dan, Y., 2016. Calcium Imaging of Basal Forebrain Activity during Innate and Learned Behaviors. Front. Neural Circuits 10, 36. https://doi.org/10.3389/fncir.2016.00036

Hay, Y.A., Lambolez, B., Tricoire, L., 2016. Nicotinic Transmission onto Layer 6 Cortical Neurons Relies on Synaptic Activation of Non-α7 Receptors. Cereb. Cortex N. Y. N 1991 26, 2549–2562. https://doi.org/10.1093/cercor/bhv085

Hedrick, T., Waters, J., 2015. Acetylcholine excites neocortical pyramidal neurons via nicotinic receptors. J. Neurophysiol. 113, 2195–2209. https://doi.org/10.1152/jn.00716.2014

Heindorf, M., Keller, G.B., 2022. Reduction of layer 5 mediated long-range cortical communication by antipsychotic drugs. bioRxiv 2022.01.31.478462. https://doi.org/10.1101/2022.01.31.478462

Held, R., Hein, A., 1963. Movement-produced stimulation in the development of visually guided behavior. J. Comp. Physiol. Psychol. 56, 872–6.

Jing, M., Li, Yuexuan, Zeng, J., Huang, P., Skirzewski, M., Kljakic, O., Peng, W., Qian, T., Tan, K., Zou, J., Trinh, S., Wu, R., Zhang, S., Pan, S., Hires, S.A., Xu, M., Li, H., Saksida, L.M., Prado, V.F., Bussey, T.J., Prado, M.A.M., Chen, L., Cheng, H., Li, Yulong, 2020. An optimized acetylcholine sensor for monitoring in vivo cholinergic activity. Nat. Methods 17, 1139–1146. https://doi.org/10.1038/s41592-020-0953-2

Jordan, R., Keller, G.B., 2023. The locus coeruleus broadcasts prediction errors across the cortex to promote sensorimotor plasticity (preprint). elife. https://doi.org/10.7554/eLife.85111.1

Jordan, R., Keller, G.B., 2020. Opposing Influence of Top-down and Bottom-up Input on Excitatory Layer 2/3 Neurons in Mouse Primary Visual Cortex. Neuron 108, 1194-1206.e5. https://doi.org/10.1016/j.neuron.2020.09.024

Kanamaru, T., Aihara, K., 2019. Acetylcholine-mediated top-down attention improves the response to bottom-up inputs by deformation of the attractor landscape. PloS One 14, e0223592. https://doi.org/10.1371/journal.pone.0223592

Kaneko, M., Stryker, M.P., 2014. Sensory experience during locomotion promotes recovery of function in adult visual cortex. eLife 3. https://doi.org/10.7554/eLife.02798

Keller, G.B., Bonhoeffer, T., Hübener, M., 2012. Sensorimotor mismatch signals in primary visual cortex of the behaving mouse. Neuron 74, 809–15. https://doi.org/10.1016/j.neuron.2012.03.040

Keller, G.B., Mrsic-Flogel, T.D., 2018. Predictive Processing: A Canonical Cortical Computation. Neuron 100, 424–435. https://doi.org/10.1016/j.neuron.2018.10.003

Kilgard, M.P., Merzenich, M.M., 1998. Cortical map reorganization enabled by nucleus basalis activity. Science 279, 1714–1718. https://doi.org/10.1126/science.279.5357.1714

Kim, J.-H., Jung, A.-H., Jeong, D., Choi, I., Kim, K., Shin, S., Kim, S.J., Lee, S.-H., 2016. Selectivity of Neuromodulatory Projections from the Basal Forebrain and Locus Ceruleus to Primary Sensory Cortices. J. Neurosci. 36, 5314–5327. https://doi.org/10.1523/JNEUROSCI.4333-15.2016

Kirkwood, A., Rozas, C., Kirkwood, J., Perez, F., Bear, M.F., 1999. Modulation of long-term synaptic depression in visual cortex by acetylcholine and norepinephrine. J. Neurosci. 19, 1599–1609. https://doi.org/10.1523/jneurosci.19-05-01599.1999

Klapoetke, N.C., Murata, Y., Kim, S.S., Pulver, S.R., Birdsey-Benson, A., Cho, Y.K., Morimoto, T.K., Chuong, A.S., Carpenter, E.J., Tian, Z., Wang, J., Xie, Y., Yan, Z., Zhang, Y., Chow, B.Y., Surek, B., Melkonian, M., Jayaraman, V., Constantine-Paton, M., Wong, G.K.-S., Boyden, E.S., 2014. Independent optical excitation of distinct neural populations. Nat. Methods 11, 338–346. https://doi.org/10.1038/nmeth.2836

Korzhova, V., Marinković, P., Njavro, J.R., Goltstein, P.M., Sun, F., Tahirovic, S., Herms, J., Liebscher, S., 2021. Long-term dynamics of aberrant neuronal activity in awake Alzheimer's disease transgenic mice. Commun. Biol. 4, 1368. https://doi.org/10.1038/s42003-021-02884-7

Krnjević, K., Phillis, J.W., 1963. Acetylcholine-sensitive cells in the cerebral cortex. J. Physiol. 166, 296–327. https://doi.org/10.1113/jphysiol.1963.sp007106

Larsen, R.S., Turschak, E., Daigle, T., Zeng, H., Zhuang, J., Waters, J., 2018. Activation of neuromodulatory axon projections in primary visual cortex during periods of locomotion and pupil dilation. bioRxiv 502013–502013. https://doi.org/10.1101/502013

Laszlovszky, T., Schlingloff, D., Hegedüs, P., Freund, T.F., Gulyás, A., Kepecs, A., Hangya, B., 2020. Distinct synchronization, cortical coupling and behavioral function of two basal forebrain cholinergic neuron types. Nat. Neurosci. https://doi.org/10.1038/s41593-020-0648-0

Lee, A.M., Hoy, J.L., Bonci, A., Wilbrecht, L., Stryker, M.P., Niell, C.M., 2014. Identification of a Brainstem Circuit Regulating Visual Cortical State in Parallel with Locomotion. Neuron 83, 455–466. https://doi.org/10.1016/J.NEURON.2014.06.031

Leinweber, M., Ward, D.R., Sobczak, J.M., Attinger, A., Keller, G.B., 2017. A Sensorimotor Circuit in Mouse Cortex for Visual Flow Predictions. Neuron 95, 1420-1432.e5. https://doi.org/10.1016/j.neuron.2017.08.036

Leinweber, M., Zmarz, P., Buchmann, P., Argast, P., Hübener, M., Bonhoeffer, T., Keller, G.B., 2014. Two-photon calcium imaging in mice navigating a virtual reality environment. J. Vis. Exp. JoVE e50885. https://doi.org/10.3791/50885

Li, X., Yu, B., Sun, Q., Zhang, Y., Ren, M., Zhang, X., Li, A., Yuan, J., Madisen, L., Luo, Q., Zeng, H., Gong, H., Qiu, Z., 2018. Generation of a whole-brain atlas for the cholinergic system and mesoscopic projectome analysis of basal forebrain cholinergic neurons. Proc. Natl. Acad. Sci. U. S. A. 115, 415–420. https://doi.org/10.1073/pnas.1703601115

Lohani, S., Moberly, A.H., Benisty, H., Landa, B., Jing, M., Li, Y., Higley, M.J., Cardin, J.A., 2022. Spatiotemporally heterogeneous coordination of cholinergic and neocortical activity. Nat. Neurosci. 25, 1706–1713. https://doi.org/10.1038/s41593-022-01202-6

Metherate, R., Weinberger, N.M., 1990. Cholinergic modulation of responses to single tones produces tone-specific receptive field alterations in cat auditory cortex. Synapse 6, 133–145. https://doi.org/10.1002/syn.890060204

Minces, V., Pinto, L., Dan, Y., Chiba, A.A., 2017. Cholinergic shaping of neural correlations. Proc. Natl. Acad. Sci. U. S. A. 114, 5725–5730. https://doi.org/10.1073/pnas.1621493114

Nelson, A., Mooney, R., 2016. The Basal Forebrain and Motor Cortex Provide Convergent yet Distinct Movement-Related Inputs to the Auditory Cortex. Neuron 90, 635–648. https://doi.org/10.1016/j.neuron.2016.03.031

Niell, C.M., Stryker, M.P., 2010. Modulation of Visual Responses by Behavioral State in Mouse Visual Cortex. Neuron 65, 472–479. https://doi.org/10.1016/j.neuron.2010.01.033

Niell, C.M., Stryker, M.P., 2008. Highly Selective Receptive Fields in Mouse Visual Cortex. J. Neurosci. 28, 7520–7536. https://doi.org/10.1523/JNEUROSCI.0623-08.2008

Obermayer, J., Verhoog, M.B., Luchicchi, A., Mansvelder, H.D., 2017. Cholinergic modulation of cortical microcircuits is layer-specific: Evidence from rodent, monkey and human brain. Front. Neural Circuits 11. https://doi.org/10.3389/fncir.2017.00100

Pafundo, D.E., Nicholas, M.A., Zhang, R., Kuhlman, S.J., 2016. Top-Down-Mediated Facilitation in the Visual Cortex Is Gated by Subcortical Neuromodulation. J. Neurosci. 36, 2904–2914. https://doi.org/10.1523/JNEUROSCI.2909-15.2016

Pakan, J.M., Lowe, S.C., Dylda, E., Keemink, S.W., Currie, S.P., Coutts, C.A., Rochefort, N.L., 2016. Behavioral-state modulation of inhibition is context-dependent and cell type specific in mouse visual cortex. eLife 5. https://doi.org/10.7554/eLife.14985

Perry, E.K., Tomlinson, B.E., Blessed, G., Bergmann, K., Gibson, P.H., Perry, R.H., 1978. Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia. Br. Med. J. 2, 1457–1459.

Pinto, L., Goard, M.J., Estandian, D., Xu, M., Kwan, A.C., Lee, S.-H., Harrison, T.C., Feng, G., Dan, Y., 2013. Fast modulation of visual perception by basal forebrain cholinergic neurons. Nat. Neurosci. 2013 1612 16, 1857–1857. https://doi.org/10.1038/nn.3552

Polack, P.-O., Friedman, J., Golshani, P., 2013. Cellular mechanisms of brain state–dependent gain modulation in visual cortex. Nat. Neurosci. 16, 1331–1339. https://doi.org/10.1038/nn.3464

Rasmusson, D.D., Dykes, R.W., 1988. Long-term enhancement of evoked potentials in cat somatosensory cortex produced by co-activation of the basal forebrain and cutaneous receptors. Exp. Brain Res. 70, 276–286. https://doi.org/10.1007/BF00248353

Reimer, J., Froudarakis, E., Cadwell, C.R., Yatsenko, D., Denfield, G.H., Tolias, A.S., 2014. Pupil Fluctuations Track Fast Switching of Cortical States during Quiet Wakefulness. Neuron 84, 355–362. https://doi.org/10.1016/j.neuron.2014.09.033

Reimer, J., McGinley, M.J., Liu, Y., Rodenkirch, C., Wang, Q., McCormick, D.A., Tolias, A.S., 2016. Pupil fluctuations track rapid changes in adrenergic and cholinergic activity in cortex. Nat. Commun. https://doi.org/10.1038/ncomms13289

Ren, C., Peng, K., Yang, R., Liu, W., Liu, C., Komiyama, T., 2022. Global and subtype-specific modulation of cortical inhibitory neurons regulated by acetylcholine during motor learning. Neuron 110, 2334-2350.e8. https://doi.org/10.1016/j.neuron.2022.04.031

Robert, B., Kimchi, E.Y., Watanabe, Y., Chakoma, T., Jing, M., Li, Y., Polley, D.B., 2021. A functional topography within the cholinergic basal forebrain for encoding sensory cues and behavioral reinforcement outcomes. eLife 10, e69514. https://doi.org/10.7554/eLife.69514

Rossi, J., Balthasar, N., Olson, D., Scott, M., Berglund, E., Lee, C.E., Choi, M.J., Lauzon, D., Lowell, B.B., Elmquist, J.K., 2011. Melanocortin-4-receptors Expressed by Cholinergic Neurons Regulate Energy Balance and Glucose Homeostasis. Cell Metab. 13, 195–204. https://doi.org/10.1016/j.cmet.2011.01.010

Sachdev, R.N.S., Lu, S.-M., Wiley, R.G., Ebner, F.F., 1998. Role of the Basal Forebrain Cholinergic Projection in Somatosensory Cortical Plasticity. J. Neurophysiol. 79, 3216–3228. https://doi.org/10.1152/jn.1998.79.6.3216

Saleem, A.B., Ayaz, A., Jeffery, K.J., Harris, K.D., Carandini, M., 2013. Integration of visual motion and locomotion in mouse visual cortex. Nat. Neurosci. 16, 1864–9. https://doi.org/10.1038/nn.3567

Saravanan, V., Berman, G.J., Sober, S.J., 2020. Application of the hierarchical bootstrap to multi-level data in neuroscience. Neurons Behav. Data Anal. Theory 3, https://nbdt.scholasticahq.com/article/13927-application-of-the-hierarchical-bootstrap-to-multi-level-data-in-neuroscience.

Sato, H., Hata, Y., Masui, H., Tsumoto, T., 1987. A functional role of cholinergic innervation to neurons in the cat visual cortex. J. Neurophysiol. 58, 765–780. https://doi.org/10.1152/jn.1987.58.4.765

Sillito, A.M., Kemp, J.A., 1983. Cholinergic modulation of the functional organization of the cat visual cortex. Brain Res. 289, 143–155. https://doi.org/10.1016/0006-8993(83)90015-X

Soma, S., Shimegi, S., Suematsu, N., Tamura, H., Sato, H., 2013. Modulation-specific and laminar-dependent effects of acetylcholine on visual responses in the rat primary visual cortex. PloS One 8, e68430. https://doi.org/10.1371/journal.pone.0068430

Suzuki, M., Larkum, M.E., 2020. General Anesthesia Decouples Cortical Pyramidal Neurons. Cell 180, 666-676.e13. https://doi.org/10.1016/j.cell.2020.01.024

Terry, A.V., Buccafusco, J.J., 2003. The cholinergic hypothesis of age and Alzheimer's disease-related cognitive deficits: recent challenges and their implications for novel drug development. J. Pharmacol. Exp. Ther. 306, 821–827. https://doi.org/10.1124/jpet.102.041616

Unal, C.T., Golowasch, J.P., Zaborszky, L., 2012. Adult mouse basal forebrain harbors two distinct cholinergic populations defined by their electrophysiology. Front. Behav. Neurosci. 6, 21–21. https://doi.org/10.3389/fnbeh.2012.00021

Vasilevskaya, A., Widmer, F.C., Keller, G.B., Jordan, R., 2023. Locomotion-induced gain of visual responses cannot explain visuomotor mismatch responses in layer 2/3 of primary visual cortex. Cell Rep. 42, 112096. https://doi.org/10.1016/j.celrep.2023.112096

Vinck, M., Batista-Brito, R., Knoblich, U., Cardin, J.A., 2015. Arousal and Locomotion Make Distinct Contributions to Cortical Activity Patterns and Visual Encoding. Neuron 86, 740–754. https://doi.org/10.1016/j.neuron.2015.03.028

Widmer, F.C., O'Toole, S.M., Keller, G.B., 2022. NMDA receptors in visual cortex are necessary for normal visuomotor integration and skill learning. eLife 11, e71476. https://doi.org/10.7554/eLife.71476

Wilson, D.A., Fletcher, M.L., Sullivan, R.M., 2004. Acetylcholine and Olfactory Perceptual Learning. Learn. Mem. 11, 28–34. https://doi.org/10.1101/lm.66404

1026 Xiang, Z., 1998. Cholinergic Switching Within Neocortical Inhibitory Networks. Science 281, 985–988.
1027 https://doi.org/10.1126/science.281.5379.985
1028 Zhu, F., Elnozahy, S., Lawlor, J., Kuchibhotla, K.V., 2023. The cholinergic basal forebrain provides a parallel
1029 channel for state-dependent sensory signaling to auditory cortex. Nat. Neurosci. 1–10.
1030 https://doi.org/10.1038/s41593-023-01289-5
1031 Zolles, G., Wagner, E., Lampert, A., Sutor, B., 2009. Functional expression of nicotinic acetylcholine
1032 receptors in rat neocortical layer 5 pyramidal cells. Cereb. Cortex N. Y. N 1991 19, 1079–1091.
1033 https://doi.org/10.1093/cercor/bhn158
1034