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1 Projection-specific integration of convergent 2 thalamic and retrosplenial signals in the 3 presubicular head direction cortex

4 5 Authors

6 Louis Richevaux^{1*}, Dongkyun Lim¹, Mérie Nassar¹, Léa Dias Rodrigues¹, Constanze Mauthe¹,
7 Ivan Cohen³, Nathalie Sol-Foulon², Desdemona Fricker^{1,4*}

8 ¹Université Paris Cité, CNRS, Integrative Neuroscience and Cognition Center, F-75006 Paris,
9 France, ²Sorbonne Université, INSERM, CNRS, Paris Brain Institute, ICM, Pitié-Salpêtrière
10 Hospital, F-75013 Paris, France, ³Sorbonne Université, INSERM, CNRS, Neuroscience Paris
11 Seine, Institut de Biologie Paris Seine, F-75005 Paris, France, ⁴Lead contact.

12 Correspondence: desdemona.fricker@parisdescartes.fr, louis.richevaux@parisdescartes.fr

13 14 Summary

15 Head-direction (HD) signals function as the brain's internal compass. They are
16 organized as an attractor, and anchor to the environment via visual landmarks. Here we
17 examine how thalamic HD signals and visual landmark information from the retrosplenial
18 cortex combine in the presubiculum. We find that monosynaptic excitatory connections
19 from anterior thalamic nucleus and from retrosplenial cortex converge on single layer 3
20 pyramidal neurons in the dorsal portion of mouse presubiculum. Independent dual
21 wavelength photostimulation of these inputs in slices leads to action potential generation
22 preferentially for near-coincident inputs, indicating that layer 3 neurons can transmit a
23 visually matched HD signal to medial entorhinal cortex. Layer 4 neurons, which innervate the
24 lateral mammillary nucleus, form a second step in the association of HD and landmark
25 signals. They receive little direct input from thalamic and retrosplenial axons. We show that
26 layer 4 cells are excited di-synaptically, transforming regular spiking activity into bursts of
27 action potentials, and that their firing is enhanced by cholinergic agonists. Thus, a coherent
28 sense of orientation involves projection specific translaminar processing in the
29 presubiculum, where neuromodulation facilitates landmark updating of HD signals in the
30 lateral mammillary nucleus.

31

32

33 Keywords

34 Keywords: postsubiculum, presubiculum, head direction, multisensory, dendritic integration,
35 circuit mechanism, patch clamp, brain slices, layer specific, projection specific

36

37

38 Introduction

39 The head direction (HD) system functions as the brain's compass system. It is
40 distributed across several interconnected brain structures, and hierarchically organized from
41 the brainstem to the lateral mammillary nucleus (LMN; Stackman and Taube, 1998), the
42 anterior thalamic nuclei (ATN; Taube, 1995), and the dorsal Presubiculum (PrS; Ranck, 1984;
43 Taube et al., 1990a). Modeling work suggests the HD system functions as a ring attractor,
44 where the population of HD neurons is arranged in a one dimensional manifold (Blair and
45 Sharp, 1995; McNaughton et al., 2006; Skaggs et al., 1995). The activity of thalamic and PrS
46 HD neurons is correlated across different brain states, even during sleep, independently of
47 sensory cues (Peyrache et al., 2015). The bump-like activity dynamics of an attractor
48 network can maintain HD signals based on excitatory-excitatory or excitatory-inhibitory
49 interactions (Knierim and Zhang, 2012; Simonnet et al., 2017). However, HD signals may drift
50 in the dark (Taube et al., 1990b; Zugaro et al., 2003). For precise navigation, internally
51 generated information on head direction must be combined with awareness of location in an
52 environment. Mechanisms underlying the combination of egocentric head direction signals
53 with anchoring to allocentric landmarks remain to be clarified.

54
55 Head direction and visual landmark signals may be integrated in the presubiculum
56 (Jeffery et al., 2016; Yoder et al., 2019). Head direction cells are found in the superficial and
57 deep layers of dorsal presubiculum, also termed postsubiculum (Boccaro et al., 2010), and
58 most layer 3 neurons in the dorsal presubiculum are head direction cells (Tukker et al., 2015;
59 Preston-Ferrer et al., 2016). These cells receive monosynaptic head direction inputs from the
60 anterior thalamus (Nassar et al., 2018; Peyrache et al., 2015; Balsamo et al., 2022). Lesions
61 of the PrS impair the visual landmark control of a cell's preferred direction in ATN and in
62 LMN (Goodridge and Taube, 1997; Yoder et al., 2015). Presubicular lesions also induce place
63 field instability in the hippocampus (Calton et al., 2003), suggesting this region may be
64 crucial to the anchoring of directionally modulated neurons to environmental landmarks.

65
66 Landmark based navigation depends on reliable visual cues. The PrS appears to
67 receive direct projections from the primary visual cortex and indirect visual input via the
68 retrosplenial cortex (Van Groen & Wyss 2003; Vogt & Miller 1983). The retrosplenial cortex
69 encodes angular head velocity (Alexander & Nitz 2015; Keshavarzi et al. 2022) and visual
70 landmark information (Auger et al., 2012; Clark et al., 2010; Sit and Goard, 2023). In
71 particular, neurons in the dysgranular retrosplenial cortex encode landmark-dominated
72 head-direction signals (Jacob et al., 2017). Presubicular layer 3 cells receiving projections
73 from both the anterior thalamus and the retrosplenial cortex (Kononenko and Witter 2012)
74 could update the compass and bind landmarks to head direction signals. Presubicular
75 neurons could then broadcast integrated HD-landmark signals directly to the MEC, via deep
76 layers to the ADN and via layer 4 neurons to the LMN (Huang et al., 2017; Yoder et al., 2015;
77 Yoder and Taube, 2011). The different target-specific presubicular projection neurons are
78 well positioned to integrate anterior thalamic and retrosplenial inputs, but technical
79 constraints have limited understanding of how known anatomical inputs are transformed
80 into functional integrated output signals.

81

82 This work was therefore designed to examine integration of visual landmarks and head
83 direction signals in the PrS. Retrograde tracing was used to confirm inputs to the PrS from
84 the ATN and the RSC. The spatial distribution of ATN and RSC targets in the dorsal and
85 ventral PrS was investigated by stereotaxic injection of viral vectors inducing anterograde
86 expression of light-gated ion channels fused to fluorescent reporters. We found that
87 superficial layers of the dorsal PrS are major targets of ATN and RSC projections. We
88 analyzed functional convergence of these inputs in the PrS using dual-wavelength
89 optogenetic stimulations in ex vivo brain slices, while recording from layer 3 and 4 pyramidal
90 neurons. Both ATN and RSC projections made mono-synaptic excitatory connections with
91 single layer 3 principal cells and mostly di-synaptic ones with layer 4 cells. We show that
92 EPSPs induced with close to coincident timing by ATN and RSC fibers summed non-linearly in
93 layer 3 neurons. Layer 4 cell firing is facilitated by cholinergic activation. These data provide
94 insights into the integration of landmark and head direction inputs and their distribution to
95 downstream targets by PrS pyramidal cells.

96

97 **Results**

98 **ATN and RSC send strong axonal projections to the dorsal Presubiculum**

99 The origins of the main afferents projecting to the presubiculum were explored by
100 injecting retrogradely transported fluorescent beads into the PrS (Figure 1A). After 4 days,
101 coronal slices were prepared to examine the injection site and transport of the beads. Some
102 beads remained close to the PrS injection site, due in part to local projections. Strong bead
103 signals were detected within the two subnuclei that form the anterior thalamus, the
104 anterodorsal (AD) and anteroventral (AV) part of ATN. Neurons in AD were labeled most
105 strongly in the medial portion of the AD, while labeled neurons in AV were found in its
106 lateral portion (Figure 1B). Many neurons in the RSC were labeled. Cells with somata in
107 layers 2 and 5 of the dysgranular dRSC were labeled, while mostly layer 5 cells of granular
108 gRSC contained beads (Figure 1C). Regions adjacent to the presubiculum including the
109 subiculum (Sub), parasubiculum (PaS) and the medial and lateral entorhinal cortices (MEC,
110 LEC) were labeled as was the contralateral PrS. Beads were also detected in the laterodorsal
111 thalamic nucleus (LD). Figure 1—figure supplement 1 shows an example of a series of
112 labeled coronal sections, indicating some labeling also in the visual cortices, perirhinal
113 cortex, the nucleus reuniens of the thalamus, the dorsolateral geniculate nucleus, and the
114 claustrum. Some beads were observed in the CA1 region, which has not previously been
115 reported. Potentially though, this labeling could derive from a bead leak into the nearby PaS
116 which is innervated by CA1 (Van Groen et Wyss 1990a). In summary, the ATN and RSC are
117 the major sources of afferents projecting to the presubiculum with lesser inputs from other
118 sites.

119 Projections from the ATN or RSC to the presubiculum were explored by injecting at
120 these sites an anterogradely transported viral construct which expressed the modified
121 Channelrhodopsin Chronos fused to GFP (Figure 1D). After 4 weeks, horizontal slices were
122 prepared to verify the ATN injection site. Chronos-GFP labeling was mostly confined to the
123 ATN, occasionally extending to medial thalamic and reticular nuclei nearby, possibly as
124 bundles of projecting fibers (Figure 1E). After injection at the RSC site, coronal sections
125 (Figure 2F) showed strong expression in layer 5, especially deeper zones, and layer 6 of both
126 dysgranular and granular RSC, while labeled dendrites were evident in layer 1. Chronos-GFP
127 expression extended throughout the RSC, from -1.7 to -3.7 posterior to Bregma on the

128 antero-posterior axis.

129 Projections of the ATN to the presubiculum were examined in horizontal sections
130 (Figure 1G). ATN axons expressing the Chronos-GFP construct targeted layers 1 and 3 of the
131 presubiculum, avoiding layer 2 (Simonnet et al., 2017; Nassar et al., 2018). Labeling was
132 precisely limited to the presubiculum with a sharp reduction of green fluorescence at the
133 posterior border with the parasubiculum, the anterior border with the subiculum and the
134 lateral border with deep layers. Axon terminals in layer 3 were not homogeneously
135 distributed. Patches of higher density were evident in deep layer 3 (Figure 1G). Along the
136 dorso-ventral axis, ATN axons projected from -2 to -4 mm ventral to Bregma. Fluorescence
137 was not detected in adjacent regions including the Sub, PaS, and DG. ATN axons avoided
138 layer 2 of the PrS, including anterior regions with patches of calbindin positive neurons that
139 project to contralateral PrS (Preston-Ferrer et al. 2016; Figure 1K).

140 RSC projections to the presubiculum (Figure 1H), traced with the fluorescent
141 Chronos-GFP construct, also projected to layers 1 and 3. Unlike ATN axons, they were
142 restricted to dorsal regions of the presubiculum (from -2 to -3 mm ventrally to Bregma on
143 the dorso-ventral axis; Figure 1H, I, J). RSC axon labeling tended to avoid patches of high-
144 density ATN projections (Figure 1G, K. Figure 3). Overall, these data show ATN and RSC
145 afferents innervate overlapping dorsal regions of the PrS, in layer 1 and deep parts of layer 3.
146 Different spatial patterns of fiber terminations suggest distinct local connectivities.

147

148 **Layer 3 neurons are directly excited by both ATN and RSC afferents**

149 We next examined physiological effects of ATN and RSC afferents on PrS pyramidal
150 cells (Rees et al., 2017). Responses to photo-stimulation of the Chronos-GFP construct
151 injected either in the thalamus or in the retrosplenial cortex (Figure 2A) were recorded in
152 layer 3 neurons (Figure 2B). These cells had robust regular firing patterns in response to
153 depolarizing current injections (Figure 2C). Intrinsic properties of layer 3 cells were rather
154 uniform (Figure 2—figure supplement 1) and an unsupervised cluster analysis suggested
155 they formed a single population (Figure 2D).

156 Blue light activation of either ATN or RSC axons expressing Chronos-GFP reliably
157 evoked synaptic responses in whole-cell patch-clamp records from layer 3 cells. High
158 intensity stimulation (1 - 3 mW) evoked EPSCs or EPSPs with a probability of 84% (70/83) for
159 ATN fibers and 85% (104/123) for RSC fibers (Figure 2E). Excitatory postsynaptic currents or
160 potentials evoked by optical stimulation of thalamic and retrosplenial inputs will be referred
161 to as oEPSCs and oEPSPs. Mean oEPSC amplitudes (Figure 2F) were similar for ATN ($-305.0 \pm$
162 61.0 pA, $n = 24$) and RSC fiber stimuli (-305.8 ± 59.1 pA, $n = 27$). Response latency (Figure
163 2G) to ATN axon stimulation was 2.45 ± 0.19 ms ($n = 24$) and that for RSC axon stimulation
164 was 2.93 ± 0.23 ms ($n = 27$). The variability in latency of oEPSCs (Figure 2—figure
165 supplement 2A, B) induced by RSC afferent stimuli was 0.35 ± 0.09 ms ($n = 27$) and that for
166 oEPSCs induced by ATN stimuli was 0.19 ± 0.06 ms ($n = 24$). These short oEPSC latencies
167 suggest monosynaptic transmission. We confirmed this point by showing that oEPSCs were
168 maintained in presence of TTX ($1\mu\text{M}$) and 4AP ($100\mu\text{M}$) (Figure 2H, $n = 2$ and 12 for ATN and
169 RSC respectively). EPSCs induced by stimulation of ATN and RSC fibers were reduced in
170 amplitude and decayed more quickly in the presence of the NMDA receptor antagonist APV
171 ($50 \mu\text{M}$) and were completely abolished by further application of the AMPA receptor
172 antagonist NBQX (1 mM) (Figure 2I). The shape of oEPSCs induced by light stimulation of
173 ATN and RSC fibers was comparable. Rise times were 1.38 ± 0.15 ms for ATN EPSCs ($n = 15$)
174 and 1.38 ± 0.08 ms for RSC-mediated EPSCs ($n = 15$) and mean half-widths were 4.00 ± 1.02

175 ms for ATN-induced EPSCs ($n = 15$) and 4.34 ± 1.18 ms for RSC EPSCs ($n = 15$). The mean time
176 constant (tau decay) for ATN-mediated EPSCs was 3.01 ± 0.21 ($n = 15$) and for RSC-induced
177 EPSCs 3.17 ± 0.27 ($n = 15$) (Figure 2—figure supplement 2C-E.).

178 Optical stimulation of ATN and RSC fibers at lower light intensities elicited sub-
179 threshold synaptic events in layer 3 cells. oEPSP amplitude for ATN mediated events was
180 4.09 ± 1.18 mV ($n = 11$) and for RSC events it was 4.80 ± 0.84 mV ($n = 11$). Maximal rising
181 slopes were 2.93 ± 0.82 mV/ms for ATN-mediated events ($n = 11$) and 3.02 ± 0.53 mV/ms for
182 RSC EPSPs ($n = 11$), and decay time constants were 81.4 ± 11.6 ms for ATN EPSPs ($n = 11$) and
183 86.9 ± 11.2 ms for RSC EPSPs ($n = 11$). Rise times were shorter for subthreshold EPSPs
184 induced by ATN afferents at 3.00 ± 0.24 ms ($n = 11$) while those for RSC initiated EPSPs were
185 3.59 ± 0.34 ($n = 11$, $p = 0.042$, Wilcoxon test; Figure 2—figure supplement 2F-J). These data
186 show that ATN and RSC axons make monosynaptic, glutamatergic excitatory contacts on
187 layer 3 PrS cells.

188 The dynamics of responses to repetitive stimulation of layer 3 PrS cells were tested
189 using 20 Hz trains of light stimuli to activate either ATN or RSC afferents. oEPSCs for both
190 inputs followed depressing kinetics. Amplitude decreased for both inputs (Figure 2J, K),
191 significantly after the fourth pulse (Friedman's test and Dunn's multiple comparison test).
192 The $10/1$ ratio was significantly lower than the paired-pulse ratio (PPR) in both cases (ATN
193 $10/1$ 0.56 ± 0.05 vs PPR 0.86 ± 0.05 , $n = 15$, $p < 0.0001$, Wilcoxon test, RSC $10/1$ 0.64 ± 0.05
194 vs PPR 0.83 ± 0.03 , $n = 15$, Wilcoxon test) (Fig 2L). ATN fiber stimulation induced PrS cell
195 firing in current-clamp with a high probability for initial stimuli and lower stable probabilities
196 for later stimuli in the train. Similarly, 20 Hz stimulation of RSC afferents evoked spikes with
197 high probability for initial stimuli and decreasing probabilities for later stimuli (on fourth and
198 7th-10th pulses, Friedman's and Dunn's multiple comparison tests; Figure 2M, N). Responses
199 to repeated subthreshold stimuli had similar dynamics (Figure 2O). Stimulation with lower
200 light intensities (< 1 mW) to initiate sub-threshold oEPSPs elicited different dynamic
201 responses. ATN afferent trains elicited responses with depressing dynamics: the amplitude
202 of the fifth oEPSP was significantly less than the first one (3.18 ± 0.88 vs 5.11 ± 1.64 mV, $p =$
203 0.0185 , Friedman's and Dunn's tests). In contrast, subthreshold 20 Hz stimulation of RSC
204 afferents evoked little or no oEPSP depression (Figure 2—figure supplement 2K-M). These
205 data reveal distinct patterns of integration of repetitive activity in glutamatergic ATN and RSC
206 afferents terminating on PrS layer 3 pyramidal cells.

207

208 **Convergence of ATN and RSC inputs on single layer 3 neurons**

209 Are single layer 3 pyramidal cells innervated by both ATN and RSC afferents? It
210 seemed likely since the probability of connection to a given cell was 84% for ATN fibers and
211 85% for RSC afferents, and cluster analysis (Figure 2D) provided no evidence for
212 subpopulations of layer 3 cells (but see Balsamo et al., 2022).

213 We tested the hypothesis using photostimulation at two different light frequencies to
214 activate ATN and RSC afferents independently. However this approach may be compromised
215 since there is an overlap in the excitation spectra of blue-light activated Chronos (400-600
216 nm) and red-light activated Chrimson (450-700 nm; Klapoetke et al., 2014). Excitation of
217 Chronos with high intensities of blue light might also excite Chrimson. Stimulating with red
218 light at 630 nm should elicit neurotransmitter release in Chrimson-containing but not
219 Chronos-expressing fibers, which we found was indeed the case. Blue light stimuli at 470 nm
220 and intensities higher than 0.01 mW elicited oEPSPs in layer 3 cells of animals with Chronos-
221 expressing fibers. In contrast, blue light intensities higher than 0.25 mW were needed to

222 induce oEPSPs in different animals with Chrimson-expressing axons (Figure 3—figure
223 supplement 1A-D). Moreover, the amplitude of these events was smaller in Chrimson-
224 injected than in Chronos-injected mice. Thus, in our experimental conditions, blue light
225 intensities up to 0.25 mW could be used to excite Chronos-positive axons with confidence
226 that Chrimson-expressing fibers would not be stimulated.

227 We also tested the inverse, red light component of the dual stimulus strategy.
228 Intensities were tested in mice injected either with AAV5-Chronos to label ATN fibers or
229 AAV5-Chrimson to label RSC afferents. The strategy was validated by showing that red light
230 induced oEPSPs in Chrimson-containing, but not Chronos-expressing fibers, while blue light
231 evoked synaptic events in Chronos-, but not Chrimson-expressing fibers (Figure 3—figure
232 supplement 1E-K).

233 Injection in the same animal of the Chronos construction to the thalamus and that
234 for Chrimson to the retrosplenial cortex let us visualize ATN and RSC afferents to the
235 presubiculum (Figure 3A, B). Axon terminal fields of ATN and RSC projections were evident in
236 superficial layers of dorsal PrS (cf. Figure 1). Layer specificities, and labeling inhomogeneities
237 were apparent as were high density ATN patches below layer 2 with no RSC axon innervation
238 (Figure 3B). Layer 3 pyramidal cells could be innervated by ATN and RSC contacts both with
239 apical dendrites in layer 1 and basilar dendrites of layer 3 (Figure 3D). We found optical
240 stimulation of ATN and RSC axons, evoked synaptic responses in 14 out of 17 layer 3
241 pyramidal cells tested (current-clamp and voltage-clamp; Figure 3E, F).

242 In order to quantify substrates of these innervations, we counted numbers of labeled
243 ATN or RSC terminals located at less than $1\mu\text{m}$ from dendrites of biocytin filled layer 3
244 pyramidal cells ($n = 6$; Figure 3G). The distribution of these potential contact sites on
245 dendritic trees of layer 3 neurons was diverse. Some neurons exhibited highly segregated
246 domains receiving either ATN or RSC inputs. For instance, cell 1 had a high proportion of
247 clustered RSC putative synapses on its apical tuft while basilar dendrites were mostly
248 surrounded by potential thalamic terminals. In cell 2, RSC terminals were clustered close to
249 apical dendrites and also together with ATN terminals close to basilar dendrites. In contrast,
250 many ATN terminals were located closer to apical dendrites of cell 3. Segregation of cortical
251 RSC inputs to apical dendrites and thalamic ATN inputs to basal dendrites might favor
252 supralinear EPSP summation. However, afferent terminal distributions differed and we found
253 no clear pattern of sub- or supralinear summation (cell 1, supralinear summation
254 (Dual/summed EPSP, 1.39); cell 2, roughly linear (0.91); cell 3, sublinear (0.69), Figure 3G, H).
255 Furthermore, we noted certain potential contacts were located with separations less than 20
256 μm on the same dendrite (Figure 3I). Such proximity might underly local dendritic
257 computations and supra-linearities such as NMDA-mediated dendritic spikes which are
258 suggested to contribute to non-linear summation of synaptic events (Larkum et al., 1999,
259 2007, 2009).

260

261 **Supralinear integration and amplification of ATN and RSC excitatory postsynaptic** 262 **potentials**

263 We explored interactions between excitatory inputs to layer 3 neurons by stimulating
264 ATN and RSC axons separately with blue or red light. Excitatory synaptic potentials evoked by
265 ATN or RSC axon stimulation, were compared to responses elicited when both sets of axons
266 were activated at short time intervals (dual; Figure 4A). The mean amplitude of dual oEPSPs
267 (synchronous light onset, Figure 4A-C) was significantly larger than calculated summed
268 amplitudes of single oEPSPs ($n = 11$ cells, dual/summed amplitude 2.06 ± 0.55 mV, $p =$

269 0.0068, Wilcoxon test). Charge transfer, quantified as the surface under the dual oEPSP, was
270 greater than the summed values for single oEPSPs (Dual/summed surface 2.21 ± 0.54 mV.s, n
271 = 11, $p = 0.0098$, Wilcoxon test). These mean values cover variability between individual
272 neurons. Summation of responses to ATN and RSC axon stimuli was supralinear in 7 out of 11
273 cells tested, linear for 3 cells and sublinear for 1 cell.

274 Dynamic responses to repetitive stimulations were also transformed by dual
275 photostimulation. Amplitudes of 5 dual oEPSPs elicited at 20 Hz were higher than for a linear
276 summation (Amplitudes in mV, Summed vs. Dual: Stim 1, 7.7 ± 1.9 vs. 11.1 ± 1.9 ; Stim 2, 6.7
277 ± 1.6 vs. 9.5 ± 1.5 ; Stim 3, 6.5 ± 1.4 vs. 9.5 ± 1.5 ; Stim 4, 6.1 ± 1.3 vs. 9.2 ± 1.5 ; Stim 5, $5.9 \pm$
278 1.3 vs. 8.6 ± 1.4 , $p = 0.0117$, Two-way ANOVA; Figure 4E) and charge transfer, quantified as
279 the surface under the 5 oEPSPs, also typically summed supralinearly (20 Hz, Surface in mV.s,
280 Summed vs. Dual: Stim 1, 136.9 ± 30.6 vs. 221.2 ± 41.5 ; Stim 2, 143.3 ± 25.7 vs. 242 ± 43.9 ;
281 Stim 3, 147.3 ± 22.5 vs. 259.2 ± 44 ; Stim 4, 145.1 ± 22 vs. 263.5 ± 49.2 ; Stim 5, 145.6 ± 23.2
282 vs. 258.7 ± 45.2 , $p = 0.0128$, Two-way ANOVA; Figure 4F). Trains of dual ATN and RSC fiber
283 stimulations also showed increased oEPSP integrals, compared to the stimulation of either
284 one set of fibers alone, indicating stronger excitation over time (Figure 4E, F). Taken
285 together, these data show supralinear summation of ATN and RSC inputs in most single layer
286 3 pyramidal cells, and also a facilitation of dynamic responses to repetitive inputs at 20 Hz.

287 We next asked how efficiently action potentials were induced by combined ATN and
288 RSC inputs. Firing probability of layer 3 cells was higher for dual than for single input
289 stimulation at a membrane potential of -65 mV (ATN 0.008 vs Dual 0.268, $n = 5$, $p = 0.0216$,
290 RSC 0.024 vs Dual 0.268, $n = 5$, $p = 0.1195$, Friedman's and Dunn's tests) and higher still at -
291 55 mV (Figure 4G, H). Firing was induced over a narrow window of time delays between
292 stimulation of the two inputs (Figure 4I, J). It occurred for delays of -2 to +5 ms (RSC
293 preceding ATN stimulus) reaching a maximum at a delay of +1 ms.

294 Supralinear summed responses of excitatory synaptic events may be mediated via
295 the activation of NMDA receptors or voltage dependent intrinsic currents that amplify EPSPs
296 (Fricker and Miles, 2000), especially if synaptic inhibition is reduced. We assessed these
297 mechanisms in records made from layer 3 pyramidal neurons using a Cesium-based internal
298 solution to favor cationic amplifying currents, and also containing QX314 to suppress action
299 potentials (Figure 5). In 3 cells photostimulation of ATN fibers evoked oEPSPs of amplitude
300 10.9 ± 2.0 mV and RSC fiber stimulation induced events of 13.7 ± 2.5 mV. The response
301 dynamics to 5 light pulses at 20 Hz were moderately facilitating (2nd vs 1st amplitude ratio:
302 ATN 1.39, RSC 1.17; 5th vs 1st: ATN 1.18, RSC 1.20; $n = 3$). Coincident activation of ATN and
303 RSC afferents induced supralinear oEPSP summation. Furthermore, repetitive stimulation
304 generated large all-or-none depolarizations on the second or third stimulus at 20 Hz (2nd vs
305 1st amplitude ratio: 1.52; 5th vs 1st: 2.90; $n = 3$ cells, Figure 5A). It may be mediated by
306 VGCC or a QX-314 resistant Na^+ inward current (Fricker et al., 2009). This component
307 appeared with higher probability and shorter latency in the presence of GABA_A receptor
308 blocker gabazine (Figure 5B). The NMDA receptor antagonist APV largely abolished the dual
309 EPSP amplification initially (Figure 5C, black trace; 2nd vs 1st integral ratio: 1.22; 5th vs 1st:
310 1.32), although amplification was partially restored by increasing RSC stimulus intensity (pale
311 pink trace). NMDA receptor activation thus assists depolarization towards the threshold of a
312 voltage-dependent process contributing to supra-linear EPSP summation, but is not the only
313 charge carrier involved.

314

315 **Cholinergic modulation and recruitment of presubicular layer 4 neurons by ATN and RSC**
316 **afferents**

317 Presubicular layer 4 neurons are intrinsic bursting pyramidal neurons that project to
318 the lateral mammillary nucleus (Huang et al., 2017). This pathway is critical for the
319 coordinated stable landmark control of HD cells in the thalamus and throughout the HD cell
320 circuit (Yoder et al., 2015; Yoder et al., 2017) To investigate the input connectivity of layer 4
321 principal cells, we recorded responses of these neurons to stimulation of ATN and RSC
322 afferents.

323 Layer 4 neurons labeled by retrograde tracers injected in the lateral mammillary
324 nucleus were located in the *lamina dissecans* of the presubiculum, below layer 3, where
325 thalamic axons ramify (Figure 6A-D). The apical dendrites of layer 4 pyramidal neurons
326 extended towards presubicular layer 1 as previously described (Huang et al., 2017), but
327 tended to circumvent layer 3 and avoid thalamic afferents, by swerving towards the
328 subiculum. Apical dendrites of some neurons crossed layer 3 obliquely, while others avoided
329 thalamic afferents in layer 3 (Figure 6C, D, Figure 6—figure supplement 1).

330 Layer 4 neurons had a more depolarized resting membrane potential, lower input
331 resistance and time constant than layer 3 neurons (Figure 6—figure supplement 2 compares
332 active and passive properties). A characteristic voltage sag in responses to hyperpolarizing
333 steps, indicated the presence of an I_h current. Layer 4 neurons discharged bursts of two or
334 three action potentials at the onset of a depolarizing step current injection and also after the
335 offset of hyperpolarizing steps (Figure 6E). These bursts were abolished by the T-type Ca^{2+}
336 channel blocker TTA-P2 (Figure 6F).

337 We next recorded responses of layer 4 neurons to optical activation of ATN or RSC
338 afferents together with effects of the same stimuli on layer 3 pyramidal cells (Figure 6G).
339 Overall, latencies of oEPSCs in layer 4 neurons were longer than for layer 3 (ATN layer 4, 6.2
340 ± 0.6 ms, $n = 8$, vs. layer 3, 2.4 ± 0.2 ms, $n = 24$; RSC layer 4, 5.6 ± 0.4 ms, $n = 9$, vs. layer 3,
341 2.9 ± 0.2 ms, $n = 27$; Figure 6H), indicating possible polysynaptic excitation of layer 4
342 neurons. Bath application of TTX-4AP did not abolish oEPSPs entirely, leaving a low
343 amplitude component with short, potentially monosynaptic latencies (latency ATN 3.9 ± 0.6
344 ms, RSC 2.9 ± 0.2 ms, $n = 5$; Figure 6I).

345 Comparison of the timing of synaptic events and firing (Fig 7A-C) showed that oEPSP
346 onset in a layer 4 neuron occurred after firing in the layer 3 neuron, for either ATN (4/5) or
347 RSC (1/1) afferent stimuli. Depolarization of the layer 3 cell via the patch pipette, initiated
348 firing, but EPSPs were not elicited in any simultaneously recorded layer 4 neuron (0 out of 6
349 cell pairs tested). These data suggest that excitation of layer 3 cells by ATN and RSC afferents
350 is transmitted to layer 4 neurons, even in the absence of direct evidence for mono-synaptic
351 coupling between cell pairs.

352 In records from pairs of layer 3 and 4 neurons ($n = 3$; Figure 7D, E) layer 3 cells
353 responded with precisely timed action potentials to low intensity stimulation of ATN
354 afferents, while only very small oEPSPs were initiated in layer 4 neurons. Increasing the
355 excitatory drive by stimulating RSC afferents elicited larger oEPSPs more reliably in layer 4
356 neurons. Higher intensity stimulation of both ATN and RSC axons, could evoke bursts of
357 action potentials, with the activation of an underlying Ca^{2+} current in layer 4 cells (Figure 7E).
358 We hypothesize that increasing the activity of layer 3 neurons by strong (and non-specific)
359 stimulation of ATN and RSC afferents is needed to induce discharges in layer 4 cells. We
360 should also note that strong photostimulation of a single set of afferent fibers could initiate

361 discharges in layer 4 pyramidal cells, as was shown in records from mice where only one
362 afferent brain area, ATN or RSC, expressed an opsin (Figure 7—figure supplement 1).

363 Overall, it seems probable that layer 3 neurons relay activity projected by ATN and
364 RSC inputs onto layer 4 pyramidal neurons. However, evidence on this point could be
365 improved. In dual records, we did not detect functional synapses between layer 3 and layer 4
366 neurons (n = 9). Anatomically, putative synaptic contacts between filled axons of layer 3 and
367 dendrites of layer 4 cells, were not evident (n = 5 filled layer 4 cells; see also Peng et al.,
368 2017). We did find a possible synaptic connection between two neighboring L4 neurons in
369 one case (Figure 6—figure supplement 1D), with a close apposition between axon and
370 dendrite. Recurrent excitation may promote bursting in a positive feedback loop in layer 4
371 neurons, and NMDA receptor related EPSP amplification favors burst firing (Figure 7F).

372 Neuromodulatory factors may help drive layer 4 cells to fire action potentials. As
373 Presubiculum is rich in acetylcholinesterase (Slomianka and Geneser 1991), we examined
374 excitability changes in the presence of a broad cholinergic agonist. The application of
375 Carbachol (10 μ M; Figure 7G) led to a depolarization of the recorded layer 4 neurons'
376 membrane potential by 13 ± 4 mV, with increased action potential firing during a positive
377 step current injection, and from the baseline (Figure 7Gi,ii). The depolarizing effect of
378 carbachol was mimicked by muscarine (10 μ M; n = 3), and persisted in the presence of TTX
379 (1 μ M; n = 2), indicating a direct effect on layer 4 neurons.

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385 Discussion

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Understanding the anchoring of HD signals with visual landmarks provides a useful
framework to ask how external information is integrated to revise and improve brain
representations. Here we show that thalamic ATN afferents signaling HD data and cortical
RSC projections that carry visual landmark information converge on layer 3 pyramidal cells of
the dorsal presubiculum. Independent optogenetic stimulation of these two glutamatergic
afferents in slices was used to define mechanisms of their integration in the presubiculum.
Most layer 3 cells were innervated by both ATN and RSC fibers, sometimes close on the same
dendritic branches, sometimes on different dendrites. Nearly coincident EPSPs (2-5 ms)
induced independently by ATN and RSC afferents, evoked non-linear membrane responses
to trigger layer 3 cell firing, transmitted to the MEC. In a second processing step, layer 3
neurons also excite layer 4 pyramidal cells which receive little direct ATN and RSC
innervation. These burst firing neurons, project a distinct, di-synaptically mediated, visually-
updated HD signal to the LMN. Our data thus suggest that the presubiculum integrates HD
and landmark signals producing two distinct output signals which are transmitted to
different regions. Cholinergic modulation may facilitate responses to salient stimuli, for
flexible anchoring to landmarks.

404 *Anatomical convergence of ATN and RSC projections in the dorsal Presubiculum*

405 Retrograde tracing in this study confirmed strong projections to the presubiculum
406 from the ATN and the RSC to the presubiculum (Figure 1). Anterior thalamic fibers

407 (Van Groen et Wyss 1990c; 1990a; 1990b; 1992; Shibata et Honda 2012; Vogt et Miller 1983)
408 ramify in and delimit the anatomical borders of the presubiculum (Liu et al., 2021; Simonnet
409 et al., 2017). HD signals from HD cells of the anterior thalamus project to the presubiculum
410 (Goodridge and Taube, 1997) where they form synapses directly with layer 3 pyramidal cells
411 (Nassar et al., 2018). The retrosplenial cortex was the most strongly labeled cortical region
412 innervating the presubiculum. We detected retrogradely transported beads in cells of layers
413 2 and 5 of dysgranular RSC, and layer 5 of the granular RSC across its antero-posterior axis
414 (Sugar and Witter, 2016).

415 We examined the anatomy and physiology of these two afferent systems to ask how visual
416 landmark signals from the RSC are combined with HD signals relayed via the ATN.
417 Anterograde fiber tracing, with an AAV5 construct expressing Chronos-GFP, confirmed that
418 axons from both regions project to superficial layers of the dorsal presubiculum. No RSC
419 projections were found in ventral PrS for injections in rostral RSC, as previously noted (Jones
420 and Witter 2007; Kononenko and Witter 2012). Both RSC and ATN innervated superficial
421 presubicular layers 1 and 3, while sparing layer 2 containing somata of calbindin positive
422 neurons (Figure 1K and cf. Balsamo et al. 2022). We detected microzones containing a high
423 density of ATN-positive fibers, but no RSC fibers, in upper layer 3.

424

425 *Pathway specific functional connectivity onto PrS layer 3 neurons.*

426 Photostimulation of GFP-Chronos expressing axons let us compare synaptic events
427 initiated in layer 3 pyramidal cells by fibers from the ATN or the RSC in dorsal presubiculum
428 slices (Figure 2). Both ATN and RSC afferents formed mono-synaptic glutamatergic
429 connections with components mediated by NMDA and AMPA receptors. The amplitudes of
430 synaptic currents varied, with an overall similar amplitude distribution for ATN and RSC
431 stimulation. This may be due to variable expression levels (Hooks et al. 2015), or may imply
432 variations in coupling weights across cells. ATN and RSC fiber mediated EPSCs depressed
433 during repetitive activation at 20 Hz. EPSPs induced firing early during repetitive stimulation.
434 RSC inputs tended to produce more sustained EPSP dynamics and slower rise times than ATN
435 afferents for low intensity stimulation. ATN synapses with presubicular pyramidal cells
436 resemble those made by thalamic afferents in the somatosensory cortex - high release with
437 depressing dynamics, (Gil et al., 1999) possibly due to presynaptic expression of VGLut2 (Liu
438 et al., 2021).

439 Dual wavelength optogenetic stimulation was used for independent activation of
440 intermingled afferents expressing blue-light sensitive Chronos in ATN fibers and red-shifted
441 Chrimson in RSC fibers (Klapoetke et al., 2014). Precautions were taken to avoid cross-
442 stimulation since all channelrhodopsin variants are somewhat sensitive to blue light. With
443 the fast, sensitive opsin Chronos expressed in ATN fibers, synaptic events were induced by
444 very short (0.5 ms), low intensity, 0.25 mW, stimuli. For RSC fibers expressing Chrimson,
445 using light stimuli of duration 2 ms, adjusting intensity up to 2 mW initiated synaptic events
446 of comparable amplitude. Calibration experiments (Figure 3—figure supplement 1) provided
447 strong evidence for the independence of responses. While Chrimson has slower dynamics
448 than Chronos (Klapoetke et al., 2014), synaptic events induced by stimulating either opsin
449 were similar, as we showed by swapping the two opsin variants injected in ATN and RSC
450 fibers respectively (Figure 4).

451 This dual opsin approach permitted independent stimulation with blue and red light
452 pulses. Most (76%) recorded layer 3 neurons generated synaptic events in response to
453 stimulation of both ATN and RSC fibers providing a substrate for integration of landmark

454 information from the RSC with thalamic HD signals. Layer 3 cell firing was most effectively
455 triggered by nearly coincident inputs (-2 to +5 ms separation, Figure 4I, J). This is of interest
456 since HD representations in the AD and RSC are highly coherent (Fallahnezhad et al., 2023),
457 with short intervals between spikes in these two regions (< 5ms; van der Goes et al. 2022).
458 Converging inputs from thalamic and retrosplenial axons can therefore excite common
459 postsynaptic presubicular layer 3 neurons with very short delays, such that coincidence
460 detection by these cells will tend to enhance HD signals transmitted to the MEC. Response
461 dynamics to combined stimulation of both inputs at 20 Hz were maintained or facilitating, in
462 contrast to the depressing dynamics of repetitive stimulation of one set of afferent fibers.
463 Combined and temporally precise inputs from ATN and RSC may thus help maintain HD
464 signaling during immobility.

465

466 *Nonlinear signal integration in layer 3 thalamo-recipient neurons*

467 Convergence of ATN and RSC axons onto single layer 3 pyramidal cells provides the
468 anatomical basis of synaptic integration. Putative synaptic contacts from both afferent fiber
469 systems were found on the basal dendrites of pyramidal cells (Figure 3), sometimes on the
470 same branch. Photostimulation centered on the soma of recorded neurons predominantly
471 activated synapses on basal dendrites. Supralinear summation could result from local spike-
472 generating mechanisms if the activated synapses were located on a same dendritic branch
473 (Makarov et al., 2023; Poirazi and Papoutsi, 2020; Polsky et al., 2004). Clustered synapses
474 could also guide the formation of new spines and synapses during integration of landmark
475 information in the HD signal. Learning might bind new inputs into functional synaptic
476 clusters (Hedrick et al., 2022). Our small sample of layer 3 pyramidal cells suggest that both
477 ATN and RSC axons target basal dendrites, and more RSC than ATN axons contact apical
478 dendrites (Figure 3). Tests on the effects of precise, near coincident activation of basal and
479 apical synapses could be revealing but were not technically possible in this study. Distinct
480 dendritic inputs to layer 3 cells may improve spatial perception (Takahashi et al., 2016)
481 enhancing HD signal quality by integration with landmark information.

482

483 Our data show that EPSPs elicited by nearly coincident ATN and RSC inputs which
484 exceed a threshold are amplified by NMDA receptor activation and voltage gated inward
485 dendritic currents (Figure 5; Fricker et al. 2009). EPSP amplification is facilitated by a
486 reduction in synaptic inhibition (Figure 5), indicating that disinhibition may be permissive for
487 supralinearity and gate firing by dynamic modulation of the balance between inhibition and
488 excitation (Milstein et al., 2015). VIP expressing interneurons, which are excited by
489 cholinergic modulation could provide such disinhibition of the presubicular microcircuit
490 (Porter et al., 1999; Slomianka and Geneser, 1991). A ‘when-to-learn’ signal for HD updating
491 in the presubiculum might function analogously to the promotion by dopamine of
492 associations between sensory cues and head direction cells in the fly compass neurons
493 (Fisher et al., 2022). It is however unclear whether LTP type learning takes place in the
494 mammalian head direction circuit. Presubicular layer 3 cells do not express the GluR1
495 subunit of AMPA receptors (Martin et al 1993; Ishihara et al., 2016) that is critical for LTP
496 expression (Boehm et al., 2006). The absence of GluR1 might indicate that the thalamo-
497 presubicular synapses in layer 3 function without classical long-term plasticity.

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498 Layer 3 pyramidal cells may be described as multi-compartmental computational
499 devices (Häusser and Mel, 2003; Mel, 1993; Poirazi et al., 2003; Spruston, 2008) which
500 integrate HD and landmark information. ATN axons drive presubicular HD neurons (Peyrache
et al., 2015) and RSC mixed selectivity neurons contribute visual landmark information and

501 allocentric spatial references (Jacob et al., 2017; Mitchell et al., 2018; Vann et al., 2009).
502 NMDA mediated dendritic spikes enhance tuning selectivity in visual cortex (Smith et al.,
503 2013; Wilson et al., 2016) and barrel cortex (Lavzin et al., 2012). Dendritic events in Layer 3
504 PrS cells may enable binding of visual landmarks with HD tuning. It is tempting to speculate
505 that nonlinear synaptic integration and inhibitory gating may be involved in flexibly updating
506 the allocentric direction of HD cells based on the integration of visual landmark information
507 to the current HD signal. In the primary sensory cortex, nonlinearities act to increase
508 perceptual threshold of sensory information (Takahashi et al., 2016; 2020). The attractor
509 network in the PrS could thus be either stabilized or flexibly reset to external spatial cues.

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513 *Functional significance of two-layer processing preceding projection to LMN*

514 Burst-firing layer 4 PrS cells project a distinct version of HD-landmark signals to the
515 LMN. The dendrites of these LMN projecting neurons overlap little with direct ATN and RSC
516 inputs granting partial isolation from direct excitation, and permitting a second level of
517 integration of information from layer 3 neurons. Layer 3 axons may innervate layer 4
518 neurons basal dendrites, which may be driven to fire by sufficiently strong excitatory inputs
519 from layer 3, especially if combined with cholinergic activation.

520 There are advantages to segregating the integration of converging input signals from
521 the updating signal across layers. This separation permits both a fast transmission of an
522 integrated signal to the medial entorhinal cortex, and the conditional transmission of a
523 updating signal mediated by burst firing to upstream HD circuit elements. The synaptic
524 threshold implicit in this two-stage system permits a gated updating. Functionally layer 4
525 neurons are uniquely positioned to update the HD signal in the LMN with visual landmark
526 information (Yoder et al., 2015). In this way, cell-type specific cholinergic facilitation may
527 help idiothetic cue based navigation (Yoder et al., 2017). These cellular and synaptic circuit
528 data support and expand the findings of Yoder et al. (2015 and 2017). They clarify how the
529 thalamic HD signal integrated with visual landmark information is relayed to the grid cell
530 system in the medial entorhinal cortex and to the lateral mammillary nuclei.

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532 *Limitations*

533 Burst firing may play a role for learning in hierarchical circuits (Naud et al., 2023; Payeur et
534 al., 2021). In the HD circuit, visual landmark information contained in bursts might reset or
535 anchor the HD attractor in the LMN and beyond. The effects of burst firing signals
536 transmitted to the LMN remain to be assessed. Potentially HD signals may be updated with
537 visual signals at several sites including the PrS, but this region uniquely provides a feed-back
538 projection to the lateral mammillary nucleus. Further work on layer 3 to layer 4 transmission
539 is warranted, and the link to spatial perception and behavioural updating needs to be
540 strengthened.

541

542 **Methods**

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544 **Animals**

545 Experiments were performed on wild-type and transgenic C57Bl6 mice, housed on a
546 12 hours light/dark cycle with food and water available *ad libitum*. Animal care and use

547 conformed to the European Community Council Directive (2010/63/EU) and French law
548 (87/848). Our study was approved by the local ethics committee (CEEA - 34) and the French
549 Ministry for Research 01025.02.

550

551 **Viral vectors and beads**

552 Projecting neurons were labeled with retrograde fluorescent tracers (Retrobeads,
553 Lumafluor, and pAAV-CAG-tdTomato, Addgene 59462P). Fluorescent beads were stored at
554 4°C before use. Channelrhodopsin expression was achieved by injecting Adeno-associated
555 viral constructions. AAV5.Syn.Chronos-GFP.WPRE.bGH (AAV5-Chronos, Penn Vector Core,
556 Addgene 59170P) was used to induce neuronal expression of the blue light induced
557 channelrhodopsin Chronos fused to the GFP marker and under the control of the Synapsin
558 promoter. AAV5.Syn.ChrimsonR-tdTomato.WPRE.bGH (AAV5-Chrimson, Penn Vector Core,
559 Addgene 59171P) induced neuronal expression of the red light gated channelrhodopsin
560 Chrimson fused to the tdTomato marker, under the control of the Synapsin promoter. Viral
561 vectors were stored at -80°C before use.

562

563 **Stereotaxic surgery**

564 Mice at ages of 4-5 weeks were anesthetized by intraperitoneal (i.p.) injection of a
565 mixture of ketamine hydrochloride and xylazine (100 and 15 mg/kg respectively, in NaCl
566 0.9%). They were placed in a stereotaxic frame for injections. Fluorescent beads for
567 retrograde tracing were injected (300-500 nl) into the PrS at the coordinates: -4.06 antero-
568 posterior (AP), 2.00 medio-lateral (ML) and -2.15 mm dorso-ventral (DV) and into the LMN (-
569 2.8 AP, 0.75 ML, -5.35 DV) with respect to the bregma.

570 Viral injections were performed unilaterally (Mathon et al., 2015; Richevaux et al., 2019) at
571 the coordinates -0.82 AP, 0.75 ML and -3.2 mm DV for the ADN, and at -2.1 to -2.15 AP, 0.65
572 ML and -0.65 mm DV for the RSC. Volumes of 200 to 250 nl were injected with a 10 µL
573 Hamilton syringe equipped with 33ga needle over a time of 10 min. The needle was slowly
574 removed after a delay of 10 min to avoid leakage from the injection site. Best expression of
575 AAV5 serotypes was achieved after 3 to 4 weeks.

576

577 **Tissue fixation and slicing for retrograde tracing**

578 Brains were removed for anatomy at 4 days after retrobead injection. Mice were
579 anesthetized by i.p. injection of the ketamine/xylazine mixture. An intracardiac perfusion
580 with 0.1M PBS was followed by perfusion with 4% paraformaldehyde. Brains were stored
581 overnight in paraformaldehyde at 4°C and then washed in PBS. Coronal or horizontal
582 sections were cut at 100 µm with a vibratome and stored in sucrose at 4°C.

583

584 **Preparation of brain slices for physiology**

585 Slices of the temporal lobe were prepared 3-4 weeks after injection of AAV5 viral
586 constructions. Mice were anesthetized by i.p. injection of the ketamine/xylazine mixture.
587 They were then perfused intracardially with a cutting solution containing (in mM): 125 NaCl,
588 25 sucrose, 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 2.5 D-glucose, 0.1 CaCl₂, 7 MgCl₂, cooled to
589 4°C, and oxygenated with a 5% CO₂ / 95% O₂. The brain was removed and a vibratome was
590 used to cut horizontal slices at 300 µm in the same solution. Slices were stored for 15 min at
591 34°C in an ACSF containing (in mM): 124 NaCl, 2.5 KCl, 26 NaHCO₃, 1 NaH₂PO₄, 2 CaCl₂, 2
592 MgCl₂, and 11 D-glucose, bubbled with 5% CO₂ / 95% O₂. They were then kept in the same
593 solution at room temperature until recording.

594

595 **Whole-cell patch-clamp recordings**

596 Slices were transferred to a recording chamber perfused with oxygenated, warmed
597 (~32 °C) ACSF mounted on an epifluorescence microscope. Patch-clamp records were made
598 from neurons with borosilicate glass pipettes of external diameter 1.5 mm (Clark Capillary
599 Glass, Harvard Apparatus) pulled with a Brown-Flaming electrode puller (Sutter
600 Instruments). Electrodes, filled with a potassium-gluconate based solution containing (in
601 mM): 135 K-gluconate, 1.2 KCl, 10 HEPES, 0.2 EGTA, 2 MgCl₂, 4 MgATP, 0.4 Tris-GTP and 10
602 Na₂-phosphocreatine, had a resistance of 4-8 MΩ. An alternative, cesium-gluconate based
603 solution facilitated neuronal depolarization to examine synaptic inhibition. It contained (in
604 mM): 125 Cs-gluconate, 10 HEPES, 0.2 EGTA, 2 MgCl₂, 4 MgATP, 0.4 Tris-GTP and 10 Na₂-
605 Phosphocreatine, together with 5 mM QX-314 to block Na⁺ channels. Pipette solutions also
606 contained 3 mM biocytin to reveal morphology after recording. They were adjusted to pH
607 7.3 and osmolarity 290 mOsm. Whole-cell, patch-clamp signals were filtered at 3kHz,
608 amplified with a MultiClamp 700B amplifier and acquired with pCLAMP software (Molecular
609 Devices). Monosynaptic excitation induced by optical stimulation was tested in the presence
610 of TTX (1 μM) and 4-AP (100 μM). NBQX (10 μM) and APV (100 μM) were used to block
611 AMPA and NMDA receptors respectively. Gabazine (10 μM) was used to block GABA_A
612 receptors. Carbachol (10 μM) was used to activate acetylcholine receptors. All drugs were
613 bath applied.

614

615 **Optical stimulation**

616 LED illumination (Cairn Research, OptoLED) was used to visualize the fluorescent
617 reporters GFP and tdTomato, and to stimulate opsin-expressing axons, using a 470 nm LED
618 for Chronos and a 627 nm LED for Chrimson. Illuminated spots had a diameter of 200 μm
619 with a 60x objective and were centered on the recorded cell soma. Photostimulation thus
620 covered most of the basilar dendrites of layer 3 pyramidal neurons (typical distance from tip
621 of apical to tip of basilar dendrite <400μm). Stimuli consisted of light pulses of 0.5 to 5 ms
622 duration, repeated 5-10 times at 20 Hz.

623

624 A multiband filter allowed simultaneous stimulation by blue and red LEDs of axons
625 containing Chronos and Chrimson (Simonnet et al., 2021). Stimulus power intensity (set in
626 mV) was calibrated as light intensity. The response probability of layer 3 cells was calibrated
627 to blue or red illumination of ATN or RSC afferents expressing Chronos or Chrimson to avoid
628 stimulus overlap (Figure 3—figure supplement 1). Chronos was targeted to the ATN and
629 Chrimson to the RSC after testing the reverse configuration. Projections from the thalamus
630 are larger and Chronos is more sensitive to blue light, so this configuration assured reliable
631 activation of thalamic fibers at minimal blue-light intensities. For experiments investigating
632 the integration of ATN and RSC inputs, we aimed to give similar weights to both inputs:
633 Chrimson-expressing fibers were stimulated with light of intensity adjusted to initiate
634 optically evoked excitatory postsynaptic potentials (oEPSPs) of amplitude similar to those
635 induced by blue light Chronos-fiber stimuli.

635

636 **Data analysis**

637 ATN and RSC projections to the PrS, were analyzed from Chronos and Chrimson
638 expression, using the ImageJ Plot Profile plug-in to quantitate normalized plot profiles (2000
639 pixels) of horizontal presubicular sections. Dorso-ventral differences were derived by
640 dividing differences in labeling intensity between the PrS and the dentate gyrus (DG)

641 molecular layer by DG intensity in slices from 5 dorso-ventral PrS levels. Values from all
642 animals were averaged and then normalized.

643 Cells were qualified as synaptically 'connected' when they responded to light
644 stimulation of afferent axons with a delay < 8 ms. Slices with very low or absent expression
645 of the fluorescent reporter were excluded. Cells were qualified as 'non-connected'
646 synaptically, if they did not respond to light stimulation, but at least one neighboring cell in
647 the same slice did.

648 Intrinsic neuronal properties were analyzed with custom MATLAB routines to derive
649 15 electrophysiological parameters (Huang et al., 2017). Parameters were standardized and
650 unsupervised cluster analysis performed with MATLAB was used to compare different
651 neurons (Huang et al., 2017; Simonnet et al., 2013).

652 Axograph was used to analyze responses to optical stimulation of ATN and RSC fibers.
653 Layer 3 neurons were recorded at potentials near -65 mV. Responses to light were averaged
654 from 10 stimulus trains at 20 Hz. Amplitudes and latencies of initial light evoked EPSCs, of
655 latency shorter than 10ms, were quantified from voltage-clamp records. Latency was
656 measured from stimulus onset to 10% of the peak amplitude of the first optically induced
657 EPSC. Amplitude was measured with respect to the pre-stimulus baseline. Paired-pulse ratio
658 (PPR) was defined as the ratio of the amplitude of the second to the first EPSC and 10/1 ratio
659 as that between the 10th and the 1st EPSC, in responses to 20 Hz stimulations. Spike
660 probability was counted over 5 to 10 trials per cell and then averaged over all cells. EPSPs
661 induced by dual wavelength stimulation were analyzed using Axograph and a custom-made
662 software. Events evoked by light stimuli were detected in a window of 1-10 ms after
663 stimulation. EPSP amplitude and integrated area were calculated over 50 ms after
664 stimulation. Baseline suppression was applied using an average of membrane potential
665 during 50 ms before stimulation. Summation of ATN and RSC evoked EPSCs in layer 3
666 neurons was determined from the amplitude and integral of averaged events. Summation
667 was considered to be supralinear, if values were more than 10% higher than a linear
668 addition, linear for values within $\pm 10\%$, and sublinear for values more than 10% lower.

669

670 **Biocytin revelation and morphology**

671 Recorded neurons were filled with biocytin to visualize their morphology and
672 location. Slices were fixed in 4% PFA in 0.1 M PBS at 4°C overnight, washed 3 times in 0.1
673 PBS and cryoprotected in 30% sucrose. Cell membranes were permeabilized by three freeze-
674 thaw cycles over dry ice and rinsed in PBS. Slices were agitated in a saturation buffer (2%
675 milk, 1% Triton X-100 in 0.1 M PBS) for 2 hours at room temperature. They were then
676 incubated with Streptavidin-Cy5 conjugate (1:500) and DAPI (1:1000) overnight at 4°C.
677 Sections were washed 3 times with PBS and mounted on coverslips with ProLong gold
678 antifade mountant. For anatomical study they were mounted in Mowiol medium. Cell, bead
679 and virus expression were visualized with an epifluorescence microscope. Higher resolution
680 of morphology was obtained with confocal microscopy. Cell morphologies were
681 reconstructed using IMARIS.

682

683 **Quantification and statistical analysis**

684 Data was analyzed with AxoGraphX and custom-written software (MATLAB, The
685 MathWorks). Results are given as mean \pm SEM. Statistical analysis was performed with Prism
686 (GraphPad Software). The Mann-Whitney unpaired t-test was used to compare two groups.
687 The Wilcoxon or Kruskal-Wallis test was used to compare paired groups. Evolution of

688 parameters evoked by optical stimulation (current or potential amplitudes, spike probability)
689 was analyzed with Friedman's test followed by multiple comparison Dunn's test. Šidák's
690 multiple comparison test was also used to compare linear and observed responses to ATN
691 and RSC stimulations. Significance levels are given as p values.

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696

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707

708 **Author contributions**

709 L.R. and D.F. conceived experiments, designed study, and interpreted data. D.F.
710 acquired funding, L.R. and D.F. managed the project. L.R., D.L., M.N., C.M., L.D-R. and N.S-F.
711 performed experiments and analyzed data, I.C. helped with analysis software, L.R. and D.L.
712 prepared figures, L.R. and D.F. wrote the manuscript.

713

714 **Declaration of Interests**

715 The authors declare no competing financial interests.

716

717 **Figure titles and legends**

718 **Figure 1** with 1 supplement.

719 **ATN and RSC send strong axonal projections to the superficial layers of dorsal**
720 **Presubiculum**

721 A, Retrograde labeling of cortical and subcortical regions projecting to the presubiculum
722 (PrS) with retrobeads.

723 B, Ipsilateral anterodorsal and anteroventral thalamic nuclei are labeled with beads.

724 C, Ipsilateral granular (gRSC) and dysgranular (dRSC) retrosplenial cortex labeling. Scale bars
725 B, C, 200 μ m.

726 D, Anterograde labeling of thalamic and retrosplenial projections to PrS with AAV-Chronos-
727 GFP.

728 E, F, Injection sites in ATN (E) and RSC (F). AD: anterodorsal thalamic nucleus, AV:
729 anteroventral thalamic nucleus, CA1: field of the hippocampus, cc: corpus callosum, cg:
730 cingulum, CPu: caudate putamen, DG: dentate gyrus, MD: thalamic medial dorsal nucleus,

731 MEC: medial entorhinal cortex, PaS: parasubiculum, PVA: paraventricular thalamic nucleus,
 732 anterior, Rt: thalamic reticular nucleus, sm: stria medullaris, Sub: subiculum, VL: thalamic
 733 ventrolateral nucleus.
 734 G, H, Five sequential 100- μ m horizontal slices of the parahippocampal region show ATN (G)
 735 and RSC (H) axons expressing Chronos-GFP (green). Dashed lines show limits of the
 736 parahippocampal region to the left and of the dentate gyrus to the right. Numbers show
 737 dorsoventral level with respect to bregma. Scale bars 100 μ m.
 738 I, Normalized profiles of fluorescent intensity for ATN and RSC projections to the
 739 presubiculum, from white matter (WM) to pia (PIA). Mean (green) \pm SEM (grey), n = 8.
 740 J, Ventral (V) to dorsal (D) normalized distribution of ATN and RSC projections to the
 741 presubiculum, n = 3 mice.
 742 K, ATN axon labeling (green) is segregated from calbindin labeling (white) in the PrS. Scale
 743 bar 100 μ m.
 744 See also Figure 1—figure supplement 1.

745

746 **Figure 1—figure supplement 1. Brain regions providing input to the Presubiculum.**

747 Semi-quantitative estimate of retrobeads labelling in serial coronal slices following injection
 748 in the presubiculum. Color intensity represents beads density (mouse #69).

- 749 - Thalamic nuclei: anterodorsal (AD) +++, anteroventral (AV) +++, laterodorsal (LD)
 750 +++, lateroposterior ++, dorsal lateral geniculate ++, reuniens ++, anteromedial -,
 751 ventrolateral -
- 752 - Retrosplenial cortex: dysgranular (dRSC) +++, granular +++, dysgranular
 753 contralateral ++
- 754 - Visual cortex: primary (V1) ++, secondary (V2L) +++
- 755 - Entorhinal cortex: lateral (LEnt) +++, medial (MEnt) +++
- 756 - Hippocampus/parahippocampus: parasubiculum (PaS) +++, subiculum (Sub) +++,
 757 controlateral PrS ++, CA1 ++, CA3 -, dentate gyrus -
- 758 - Other: claustrum ++, perirhinal cortex ++, temporal association cortex ++

759

760 ADN: anterodorsal thalamic nucleus; AM: anteromedial thalamic nucleus; APT(V): anterior
 761 pretecal nucleus (ventral part); AVDM: anteroventral thalamic nucleus, dorsomedial part;
 762 AVVL: anteroventral thalamic nucleus, ventrolateral part; CA: cornu ammonis; cc: corpus
 763 callosum; Cl: Claustrum; Cg1,2: cingular cortex area 1,2; coll. sup: colliculus superior; CPu:
 764 caudate putamen (striatum); dhc: dorsal hippocampal commissure; DG: dentate gyrus; DLG:
 765 dorsal lateral geniculate nucleus; LEnt: lateral entorhinal cortex; LDDM: laterodorsal thalamic
 766 nucleus, dorsomedial part; LDVL: laterodorsal thalamic nucleus, ventrolateral part; LMN:
 767 lateral mammillary nucleus; LS: lateral septal nucleus; M1,2: primary, secondary motor
 768 cortex; MEnt: medial entorhinal cortex; MMN: medial mammillary nucleus; PAG:
 769 periaqueductal gray; PaS: Parasubiculum; PF: parafascicular thalamic nucleus; PH: posterior
 770 hypothalamic area; PMCo: posteromedial cortical amygdaloid nucleus; Po: posterior
 771 thalamic nuclear group; PRh: perirhinal cortex; PrS: presubiculum; PVA: paraventricular
 772 thalamic nucleus, anterior part; S1BF, S1FL, S1ULp, S1Tr: primary somatosensory cortex,
 773 barrel field, forelimb region, upper lip region, trunk region; S2: secondary somatosensory
 774 cortex; sm: stria medullaris of the thalamus; Sub: subiculum; Subst. Nigra: substantia nigra;
 775 Re/Reuniens: reuniens thalamic nucleus; RSA = dRSC, dysgranular retrosplenial cortex; RSC:
 776 retrosplenial cortex; RSG = gRSC: granular retrosplenial cortex; Rt: reticular thalamic nucleus;
 777 V1: primary visual cortex; V2M: secondary visual cortex, medial part; V2L: secondary visual

778 cortex, lateral part; VA: ventral anterior thalamic nucleus; VDB: nucleus of the vertical limb
779 of the diagonal band; VL: ventrolateral thalamic nucleus; VPL: ventral posterolateral thalamic
780 nucleus; VPM: ventral posteromedial thalamic nucleus

781

782

783 **Figure 2** with 2 supplements.

784 **Responses of layer 3 presubicular cells to photoactivation of ATN and RSC fibers.**

785 A, Expression of AAV5-Chronos in ATN or RSC.

786 B, Biocytin labeled layer 3 cells (white) and GFP positive axons (green) from the ATN or RSC.

787 Scale bar 10 μ m.

788 C, Firing pattern of two layer 3 cells receiving inputs from the ATN (*cerulean*) and the RSC

789 (*purple*). Insets show current commands.

790 D, Cluster analysis of physiological parameters for cells tested by stimulating ATN or RSC

791 fibers.

792 E, Representative EPSCs evoked in layer 3 cells by light stimulation (blue bar) of ATN or RSC

793 inputs. Right, proportion of cells receiving ATN or RSC inputs.

794 F, Average amplitudes of ATN and RSC induced synaptic currents.

795 G, Latency of EPSCs evoked by light-stimulation of ATN (n = 24 cells) or RSC fibers (n = 27

796 cells).

797 H, EPSPs induced in layer 3 cells (single traces) by stimulating ATN or RSC inputs in absence

798 and presence of 1 μ M TTX and 100 μ M 4-AP.

799 I, EPSCs induced by stimulating ATN or RSC fibers in the absence and presence of 100 μ M

800 APV and APV + 10 μ M NBQX. Holding potential +40 mV.

801 J, Voltage-clamp responses of layer 3 cells to 20 Hz train stimulations of ATN (*left*) and RSC

802 (*right*) inputs. Insets show EPSCs in response to the first two stimuli.

803 K, oEPSC amplitudes for 10 ATN or RSC fiber stimuli at 20 Hz. n = 15 neurons. Short middle

804 line, mean; Min to max and quartiles.

805 L, Paired-pulse ratio (PPR) and 10/1 ratio (ratio between 10th and 1st event amplitudes) for

806 ATN or RSC inputs. Wilcoxon matched-pairs signed rank test: ATN PPR vs 10/1 **** p <

807 0.0001, RSC PPR vs 10/1 *** p = 0.0001.

808 M, Current clamp traces showing action potentials and EPSPs evoked by 10 stimuli at 20 Hz.

809 N, Spiking probability during trains of 10 stimuli. ATN, n = 6, RSC, n = 12. Full line, median;

810 short line, mean; Min to max and quartiles. In K and N, * p < 0.05, ** p < 0.01, *** p < 0.001,

811 **** p < 0.0001 from Friedman's test followed by Dunn's *post-hoc* test.

812 O, oEPSP amplitudes for trains of 5 stimuli at 20 Hz.

813 See also Figure 2—figure supplements 1 and 2.

814

815

816 **Figure 2—figure supplement 1. Table showing the intrinsic electrophysiological**

817 **physiological properties of layer 3 cells tested for responses to stimulating ATN or RSC**

818 **fibers.**

819

820 **Figure 2—figure supplement 2. Comparison of evoked synaptic events in presubicular layer**

821 **3 neurons following photostimulations of ATN or RSC axons.**

822 A, Variance of latency (jitter) in oEPSP onset following Chronos-expressing ATN or RSC fiber

823 stimuli. RSC input stimulation gives similar variation in latency as ATN inputs.

824 B, Jitter as a function of latency for ATN and RSC stimuli shows a similar coefficient of
825 variation.
826 C, Decay time constants of EPSCs evoked in layer 3 neurons by stimulating ATN or RSC inputs.
827 D, E, First oEPSC amplitude as a function of oEPSC half-width (D) and oEPSC rise time (E) for
828 both inputs. We found no clear segregation of oEPSC shapes according to the origin of
829 afferent fibers.
830 F, oEPSPs in a presubicular layer 3 cell, induced by blue or red light stimulation of Chronos-
831 expressing ATN or Chrimson-expressing RSC afferents, respectively.
832 G-J, Comparison of oEPSPs evoked by stimulating ATN or RSC axons, recorded from a same
833 postsynaptic layer 3 presubicular neuron. G, Mean amplitude. H, Maximum rising slope of
834 oEPSPs. I, oEPSPs rise time. $p < 0.05$, Wilcoxon test. J, oEPSPs decay time constants. oEPSPs
835 following ATN and RSC fiber stimulation recorded from the same cell are connected by lines.
836 Dashed lines indicate reversed experimental design (expressing Chronos in RSC and
837 Chrimson in ATN).
838 K, Responses to 10 light stimuli at 20 Hz for ATN and RSC inputs to the same cell.
839 L, Amplitudes of oEPSPs evoked by trains of ATN (left) or RSC (right) fiber stimuli for 11 cells.
840 Dashed lines indicate the reversed experimental design (expressing Chronos in RSC and
841 Chrimson in ATN).
842 M, Summary data from L. On average, the amplitudes of oEPSPs evoked by ATN fiber
843 stimulation were smaller for the fifth than for the first stimulus. oEPSPs evoked by RSC fiber
844 stimulation were similar across five stimuli. Full line, median; Short line, mean; Min to max
845 and quartiles. * $p < 0.05$, Friedman's test and post-hoc Dunn's test comparing each stimulus
846 with the first.

847
848

849 **Figure 3** with 1 supplement.

850 **ATN and RSC axons converge in dorsal presubiculum and contact single layer 3 pyramidal**
851 **neurons.**

852 A, Expression of the blue light sensitive opsin Chronos in ATN and the red light sensitive
853 opsin Chrimson in RSC.
854 B, Axons from ATN (GFP labeled, green) and RSC (tdTomato labeled, red) overlap in layer 1
855 and 3 of dorsal presubiculum. Layer 2 possesses patches of axon-dense and axon-poor
856 zones. RSC fibers avoid axon-dense microstructures formed by ATN fibers in upper layer 3.
857 Scale bar, 200 μm .
858 C, Independent dual wavelength optogenetic stimulation of light sensitive afferent fibers in
859 presubicular slices.
860 D, Two biocytin labeled PrS layer 3 pyramidal cells surrounded by ATN (green) and RSC (red)
861 axons.
862 E, Patch clamp recording from a layer 3 neuron shows optical EPSCs following
863 photostimulation of ATN axons (blue light) and RSC axons (red light).
864 F, 76% of layer 3 pyramidal neurons tested ($n = 17$) received both ATN and RSC input.
865 G, Distributions of putative synaptic contacts from ATN (green) and RSC (red) on the
866 dendrites of three layer 3 neurons. Scale bar 50 μm . The boxed area on Cell 2 is shown in
867 panel I.
868 H, Normalized number of green and red spots for 6 neurons as a function of the distance
869 from soma. Paired values are indicated by dotted lines for the 3 cells in G.

870 I, Examples of ATN-labeled (left), RSC-labeled (middle) and both (right) synapses closely
871 apposed to dendrites of a biocytin-filled layer 3 pyramidal cell. Scale bar 20 μm . Insets show
872 representative high-magnification images. Scale bar 2 μm .
873 See also Figure 3—figure supplement 1.

874

875 **Figure 3—figure supplement 1. Calibration of blue and red light stimulation of Chronos and**
876 **Chrimson.**

877 A, Blue light stimulation of ATN Chronos-expressing fibers evoked oEPSPs in presubicular
878 layer 3 neurons at an intensity of 0.25mW, and 0.5 ms duration of illumination.

879 B, Blue light stimulation at the same intensity and duration did not evoke oEPSPs from RSC
880 Chrimson-expressing fibers.

881 C, D, Response probabilities of presubicular layer 3 cells to stimulation of fibers expressing
882 Chronos or Chrimson with red (C) or blue (D, left) light.

883 E, Experimental design. Injection of AAV5-Chronos-GFP (green) in the ATN, followed by
884 photostimulation combined with patch recording from a presubicular layer 3 cell.

885 F, Amplitudes of oEPSCs evoked in layer 3 cells by blue (0.5 ms, 0.25 mW) or red light (2 ms,
886 0.6 mW) stimuli.

887 G, Representative responses to light stimulation of ATN inputs.

888 H, Injection of AAV5-Chrimson-tdTomato (red) in the RSC, followed by photostimulation
889 combined with patch recording from a presubicular layer 3 cell.

890 I, Amplitudes of oEPSCs evoked in layer 3 cells by blue (0.5 ms, 0.25 mW) or red light (2 ms,
891 0.6 mW) stimuli.

892 J, Representative responses to light stimulation of RSC inputs

893 K, Values and statistical analysis for different stimulation configurations (Panels F and I).

894

895 **Figure 4. Supralinear summation of EPSPs and action potential firing following**
896 **photostimulation of ATN and RSC axons.**

897 A, Optical EPSPs in layer 3 neurons in response to blue light activation of ATN axons
898 (cerulean traces), red light activation of RSC axons (purple traces). Supralinear summation of
899 EPSPs following coincident activation of both ATN and RSC axons (black traces). The pink
900 broken line indicates the calculated linear sum of oEPSPs evoked by stimulation of either
901 ATN or RSC axons. Records from cell 1, Fig 3D.

902 B, Amplitudes of dual ATN and RSC oEPSPs plotted as a function of the sum of separate ATN
903 and RSC stimulations (*left*). Each circle is a cell (n=11), Pink line ($\pm 10\%$) indicates linearity.
904 Pie charts give the number of tested layer 3 neurons with supralinear (grey), linear (pink) or
905 sublinear (white) summation. oEPSPs normalized to linear sum (*bottom right*). Solid circle,
906 Chronos in ATN/Chrimson in RSC, empty circle, Chronos in RSC/Chrimson in ATN. * $p < 0.05$,
907 ** $p < 0.01$, from Wilcoxon test.

908 C, As in B, for dual ATN and RSC oEPSP integrals.

909 D, oEPSPs induced by 20 Hz stimulation of ATN (cerulean), RSC (purple) or both (black). The
910 pink broken line indicates the calculated linear sum of oEPSPs evoked by separate ATN and
911 RSC photostimulation.

912 E, Amplitudes of dual oEPSPs compared to those of the sum of ATN and RSC oEPSPs show
913 that supralinearity increases across 5 stimulations. Two-way ANOVA, $p = 0.0117$. * $p < 0.05$,
914 **** $p < 0.0001$, Šidák's multiple comparison test. # $p < 0.05$, ## $p < 0.01$, Friedman's and
915 *post-hoc* Dunn's test.

916 F, As in E, for dual ATN and RSC oEPSP integrals. Two-way ANOVA, $p = 0.0128$.
917 G, Excitatory postsynaptic responses to photostimulation of ATN (blue light, cerulean traces)
918 or RSC axons (red light, purple traces) or both (blue and red light, black traces) at resting
919 membrane potential (-65 mV) or at a depolarized holding potential (-55 mV). Synaptic
920 excitation led to action potentials when dual ATN and RSC stimuli reached firing threshold.
921 H, Action potential (AP) probability for either or both stimuli at -65 and -55 mV. Data are
922 presented as mean \pm SEM.
923 I, Action potentials were induced in presubicular layer 3 neurons by near coincident
924 activation of ATN axons (red light) and RSC axons (blue light). Time delays varied from -50 to
925 +50 ms.
926 J, Firing probability was maximal for short delays between -2 to +5 ms (RSC preceding ATN).
927

928 **Figure 5. EPSP amplifications in layer 3 pyramidal neurons.**

929 A, Optical EPSPs evoked in layer 3 pyramidal neurons following the stimulation of ATN axons
930 (blue light, cerulean traces), RSC axons (red light, purple traces) or both (blue and red light,
931 black traces). Recording pipette contained a cesium gluconate based internal solution and
932 the Na⁺ channel blocker QX-314. A large all-or-none EPSP amplification occurred for dual
933 stimuli at 20 Hz, on some trials.
934 B, In the presence of the GABA_A receptor antagonist gabazine (10 μ M), dual EPSP
935 amplification occurred earlier in the train.
936 C, The additional presence of the NMDA receptor antagonist APV (100 μ M) abolished dual
937 EPSP amplification (black trace). EPSP amplification was partially restored by increasing red
938 light intensity x2 (pale pink traces).
939

940 **Figure 6 with 2 supplements.**

941 **Presubicular LMN-projecting layer 4 neurons avoid thalamo-recipient layer 3 and receive**
942 **little direct input from ATN and RSC.**

943 A, Expression of Chronos-GFP in ATN and retrograde labeling of neurons that target LMN.
944 B, Thalamic axons (green) in superficial layers 1 and 3 of presubiculum. Retrobeads label cell
945 bodies of presubicular layer 4 cells (red).
946 C, Retrograde rAAV2-tdTomato label cell bodies and dendrites of layer 4 LMN projecting
947 neurons (red). Apical dendrites of layer 4 pyramidal neurons avoid layer 3 where thalamic
948 axons ramify.
949 D, Presubicular slice containing two layer 3 and two layer 4 neurons filled with biocytin
950 (white) and GFP expressing thalamic axons (green). Scale bar 100 μ m. Inset, retrobeads (red)
951 in the soma of a biocytin filled LMN-projecting layer 4 neuron. Scale bar 10 μ m.
952 E, Layer 3 neurons are regular spiking and layer 4 neurons are burst firing, initially and at
953 rebound, in response to current injection. Black trace, rheobase.
954 F, T-type Ca²⁺ channel blocker TTA-P2 (1 μ M) suppressed burst firing in presubicular layer 4
955 neurons, while single action potentials were preserved.
956 G. Representative oEPSCs in layer 3 (left) and layer 4 (right) pyramidal cells, in response to
957 stimulation of ATN (cerulean) or RSC (purple) inputs.
958 H, oEPSC latencies in layer 3 and layer 4 cells, for ATN inputs (left, cerulean), or RSC inputs
959 (right, purple). Each dot is a cell. Same layer 4 cells are indicated by connecting lines, to
960 show the difference in latency for direct and indirect synaptic responses.
961 I, oEPSPs in layer 4 neurons in response to stimulation of ATN (cerulean) or RSC (purple)
962 inputs in control and in the presence of TTX (1 μ M) and 4-AP (100 μ M). Dashed lines indicate

963 the timing of the large disynaptic component of the responses (i, indirect), and the small
964 monosynaptic response (d, direct), isolated in TTX-4AP.
965 See also Figure 6—figure supplements 1 and 2.

966
967

968 **Figure 6—figure supplement 1. Apical dendrites of presubicular layer 4 neurons avoid the**
969 **thalamorecipient layer 3.**

970 A, Retrograde AAV2retro-tdTom was injected in LMN.
971 B, PrS L4 neuron cell bodies and dendrites were labeled. Scale bar, 50 μm . Full arrowheads,
972 L4 neurons apical dendrites crossing L3; Empty arrowheads, L4 neurons apical dendrites
973 skirting L3.
974 C, Anterograde AAV5-Chronos-GFP was injected in ATN to label thalamic axons, delimiting
975 PrS layer 3.
976 D, Examples of biocytin filled layer 4 presubicular neurons. (1) The apical dendrite of this
977 neuron avoided the densely packed ATN afferents and it didn't show responses to thalamic
978 stimulations. (2) This neuron's apical dendrite crossed through a short stretch of layer 3
979 containing ATN afferents, and it responded with low amplitude indirect events. (3) The apical
980 dendrite of this neuron crossed layer 3 and the zone containing ATN afferents, and was
981 directly recruited. Scale bar 10 μm .
982 E, AAV-Chronos-GFP injected in ATN, and AAV-Chrimson-tdTom injected in RSC.
983 F, Image from Figure 7 panel D. Scale bar 100 μm .
984 G, (Left) Enlarged view of dotted area in A, with two labelled L4 neurons. The apical dendrite
985 of top neuron makes a U-turn to ramify in deep layers and seems to cross path with the axon
986 of the bottom neuron, potentially forming a synaptic contact. (Right) The dendrite of the top
987 neuron and axon of bottom neuron are overlaid with pink and green lines respectively.
988 Scale bar 20 μm .

989

990 **Figure 6—figure supplement 2. Electrophysiological passive and active intrinsic properties**
991 **of layer 3 vs. layer 4 neurons.**

992 Data for layer 3 are summarized in Figure 2—figure supplement 1 and are shown here for
993 comparison. Statistical differences are indicated as levels of p values (* $p < 0.05$, ** $p < 0.01$,
994 *** $p < 0.001$, **** $p < 0.0001$) obtained from Mann-Whitney tests between layer 4 ($n = 19$)
995 and layer 3 ($n = 33$) for each parameter. Box plots indicate maximum and minimum values,
996 quartiles, full line is the median, short line is the mean.

997

998 **Figure 7** with 1 supplement.

999 **Cross-laminar activation of LMN-projecting layer 4 bursting neurons.**

1000 A, Simultaneous records of a layer 4 and a layer 3 neuron during photostimulation of ATN
1001 afferents. EPSP onset was delayed in the layer 4 neuron. Right panel, expanded view of the
1002 boxed area.
1003 B, Simultaneous records of a layer 4 and a layer 3 neuron during photostimulation of RSC
1004 afferents. As in A, layer 4 neurons responded with a delay. Right panel, expanded view of the
1005 boxed area.
1006 C, Latencies of synaptic activation indicated by the dotted lines in A, B (ATN stimulation, $n = 4$
1007 cells; RSC stimulation, $n = 1$). EPSP onset and AP peak from layer 3 neurons ($n = 5$). Dotted
1008 lines link to the EPSP onset in simultaneously recorded layer 4 neurons ($n = 5$). $p < 0.05$,
1009 Kruskal-Wallis multiple comparison test.

1010 D, Biocytin labeled layer 3 and layer 4 pyramidal neurons, in a presubicular slice containing
1011 thalamic (green) and retrosplenial (red) axons. The apical dendrite of one layer 4 neuron
1012 makes a U-turn (arrowhead), away from layer 3, where the thalamic axons ramify. The
1013 neighboring layer 4 neuron's apical dendrite crosses the thalamo-recipient layer 3 for a short
1014 distance before arborizing outside of ATN targeted area, towards the subiculum, on the
1015 right. Another biocytin filled layer 4 neuron's dendrites extend toward the deep layers. Scale
1016 bar, 100 μ m.

1017 E, Simultaneous records from a layer 3 and a layer 4 cell to ATN input stimulation (top, 0.25
1018 mW, blue light), ATN and RSC input stimulation (middle, 0.25 mW blue and 0.25 mW red
1019 light; light intensities compatible with independent photostimulation) or non-specific ATN
1020 and RSC input stimulation (bottom, 3.25 mW blue and 0.25 mW red light). ATN fibers
1021 expressed Chronos-GFP (green) and RSC fibers expressed Chrimson-tdTomato (red).

1022 F, Top, oEPSPs and bursts of action potentials in a layer 4 neuron, evoked by dual wavelength
1023 stimulation of ATN and RSC afferents at 20 Hz. Amplifications of dual oEPSPs led to firing.
1024 Bottom, the NMDA receptor antagonist APV (100 μ M) reduced EPSP amplification and
1025 prevented action potential firing.

1026 G. Layer 4 bursting neurons are sensitive to the acetylcholine receptor agonist carbachol (10
1027 μ M). Action potential firing in response to step current injections in control (black), in the
1028 presence of carbachol (grey), and after wash-out (black). Bottom graph, membrane
1029 potential depolarization during a 2-minute carbachol application. The number of action
1030 potentials increased during the depolarizing steps (i) and on the baseline (ii).

1031 See also Figure 7—figure supplement 1.

1032

1033

1034 **Figure 7—figure supplement 1. Regular firing layer 3 vs. intrinsically bursting layer 4**
1035 **neurons responded to high-intensity light stimulations of ATN or RSC afferents.**

1036 Layer 3 neurons respond with single spikes following each light stimulation while layer 4
1037 neurons respond with bursts of action potentials at the beginning of the stimulation train.

1038

1039 **References**

1040

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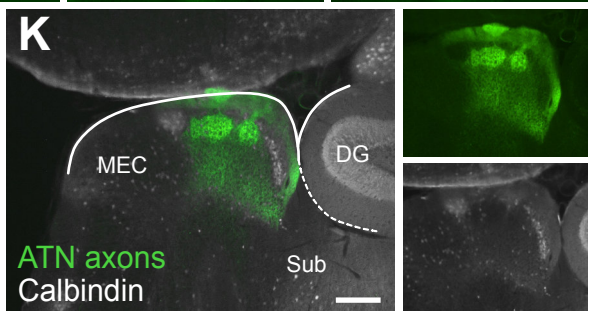
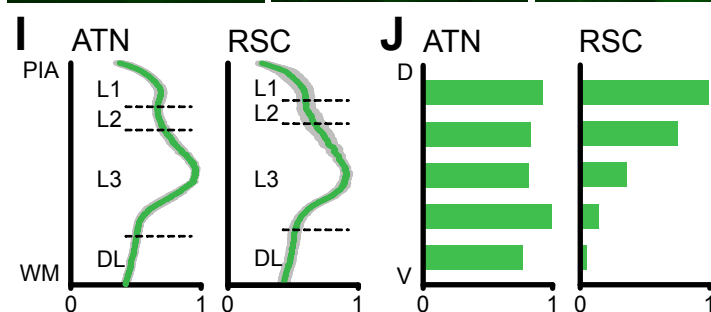
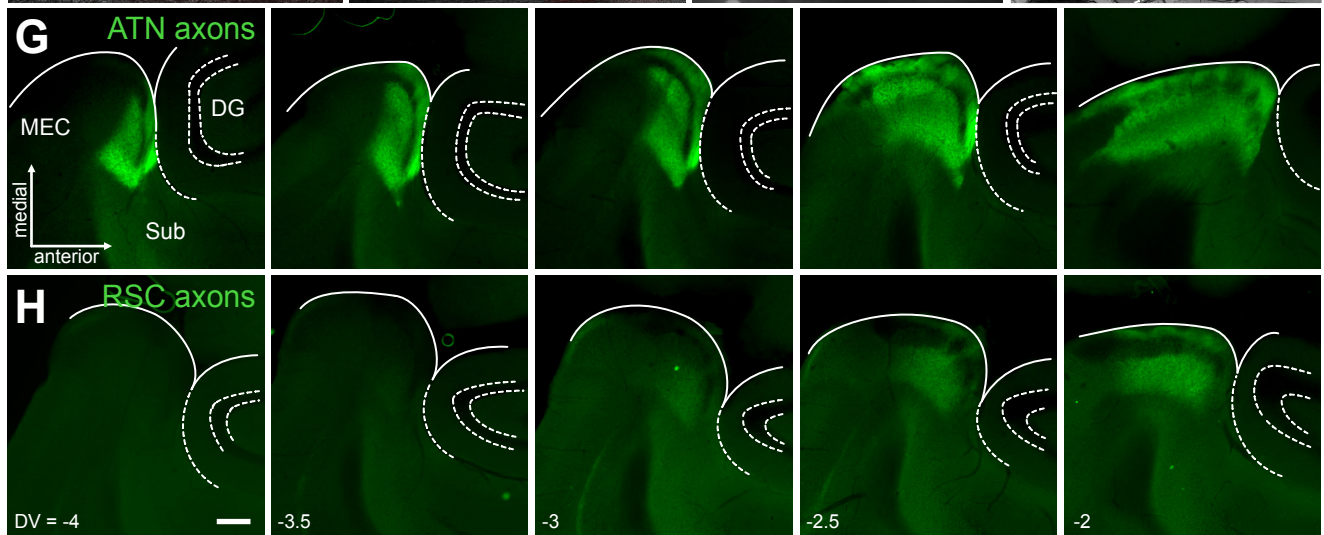
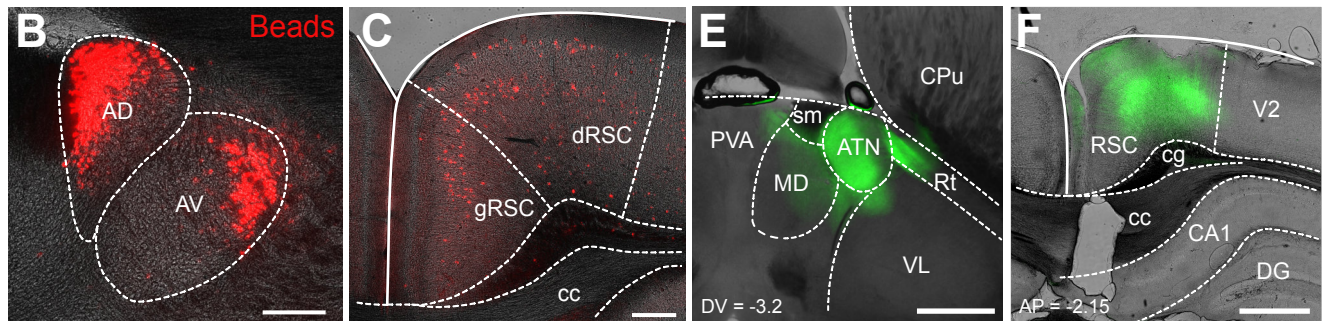
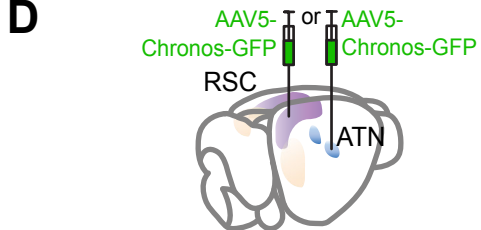
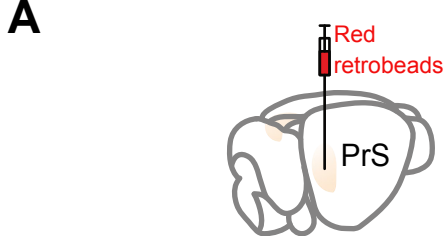
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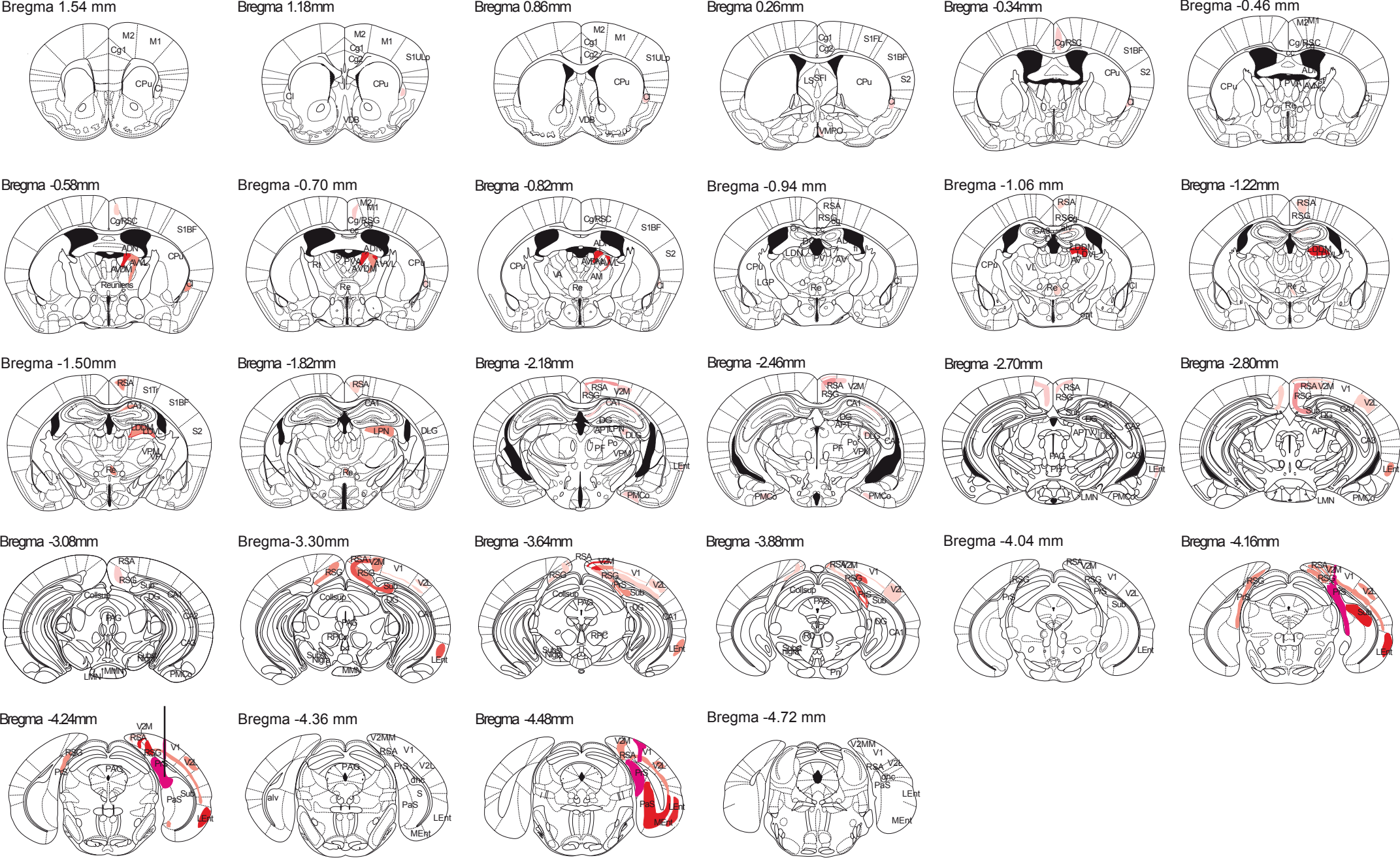
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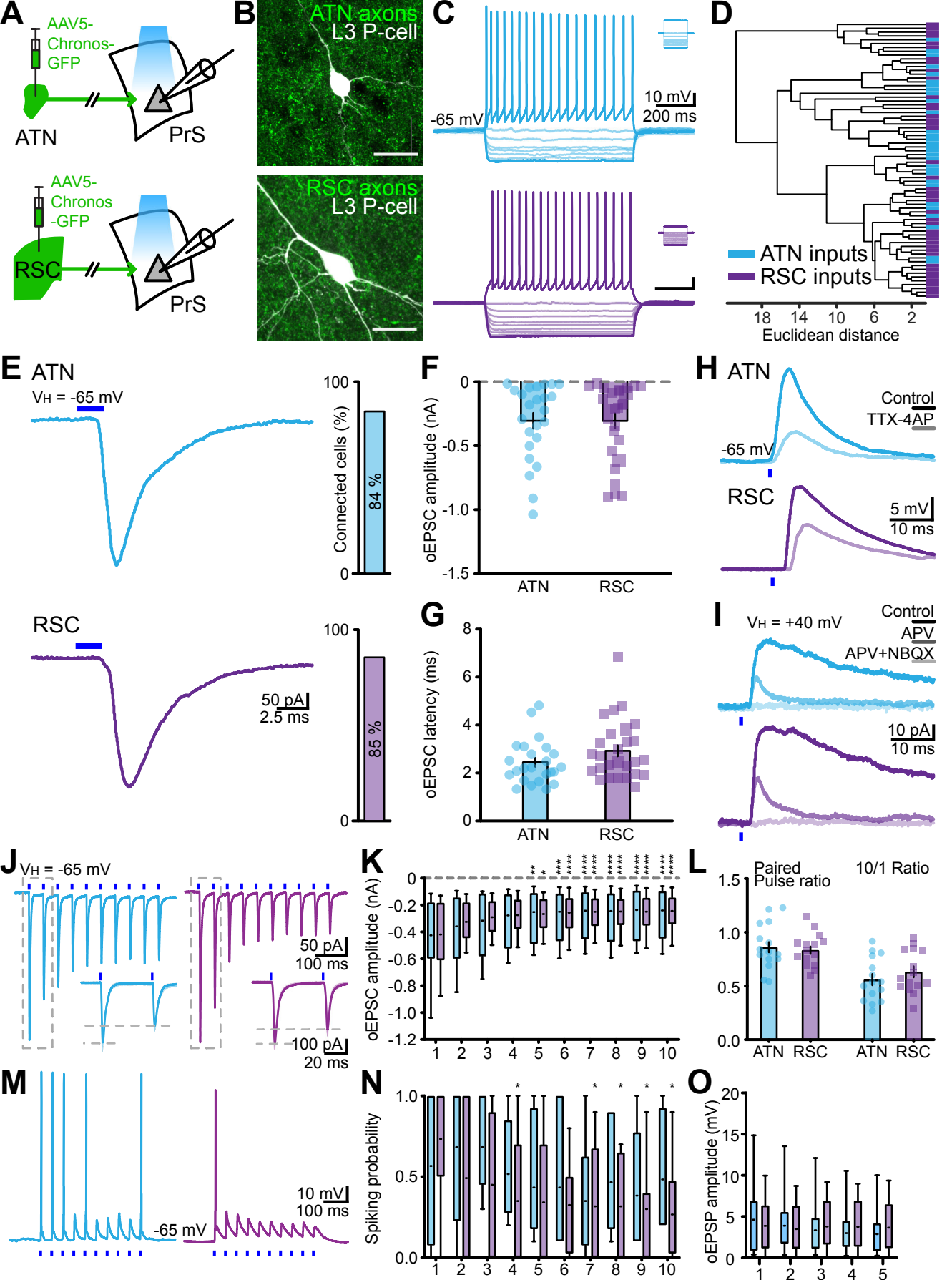
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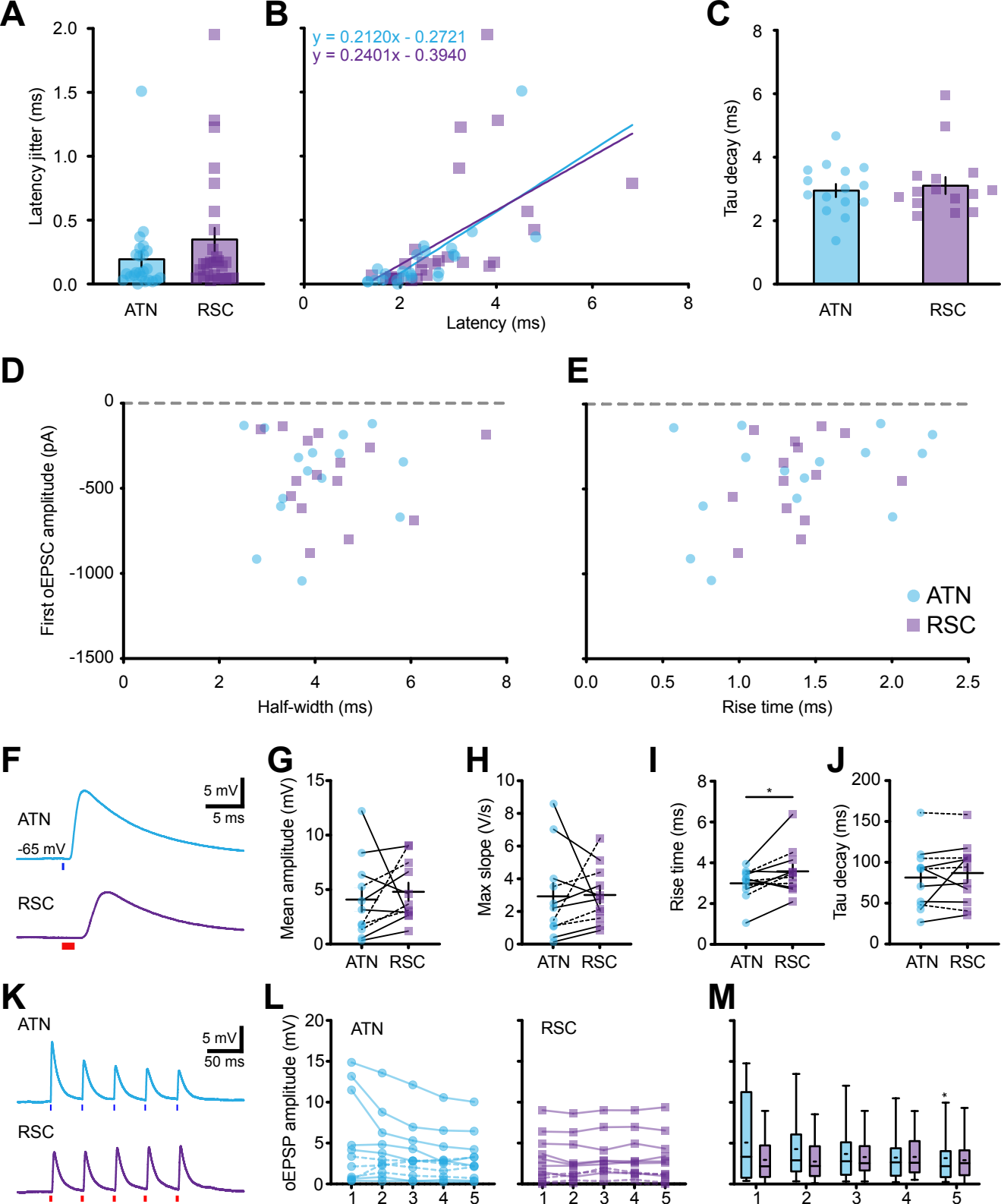
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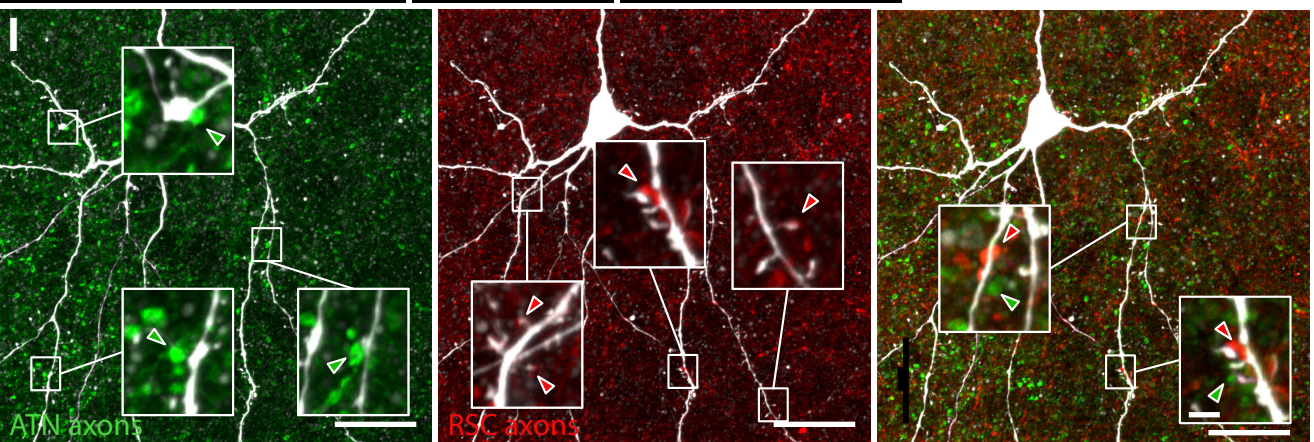
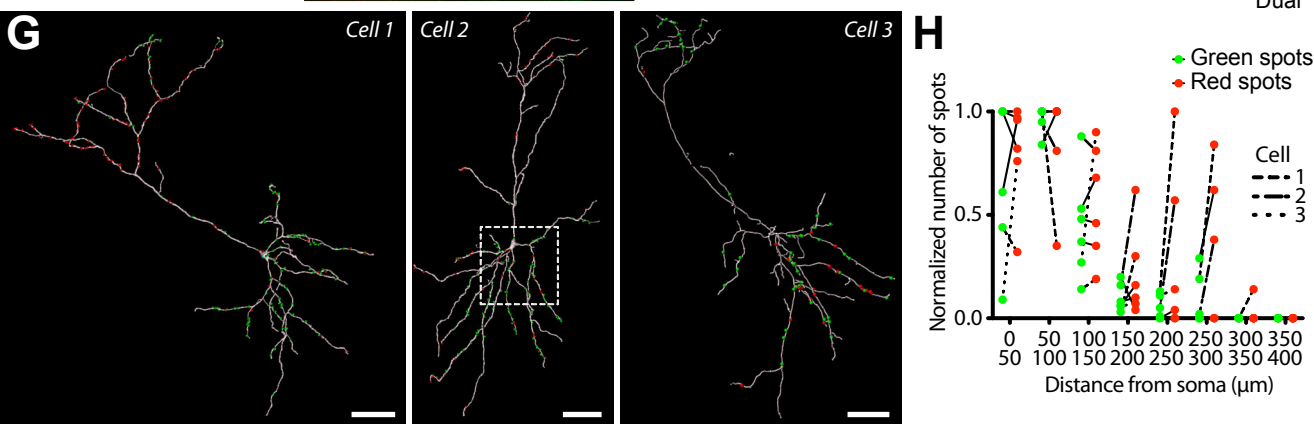
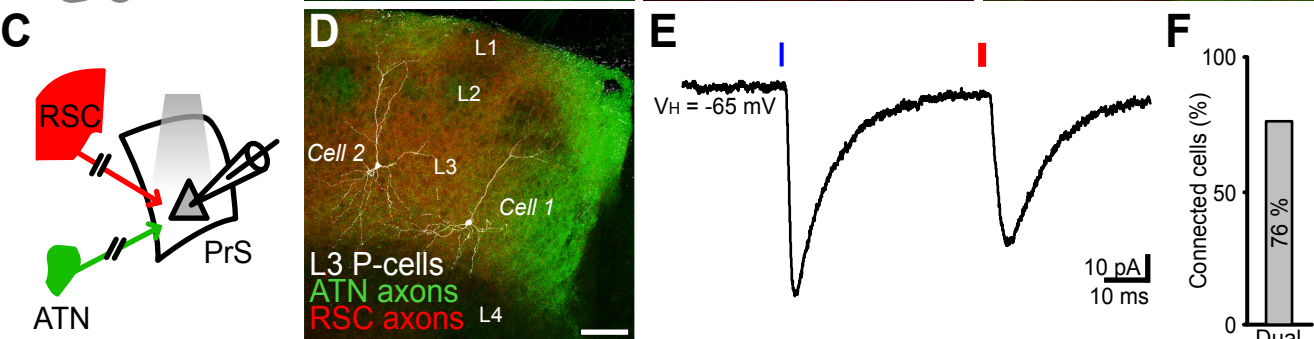
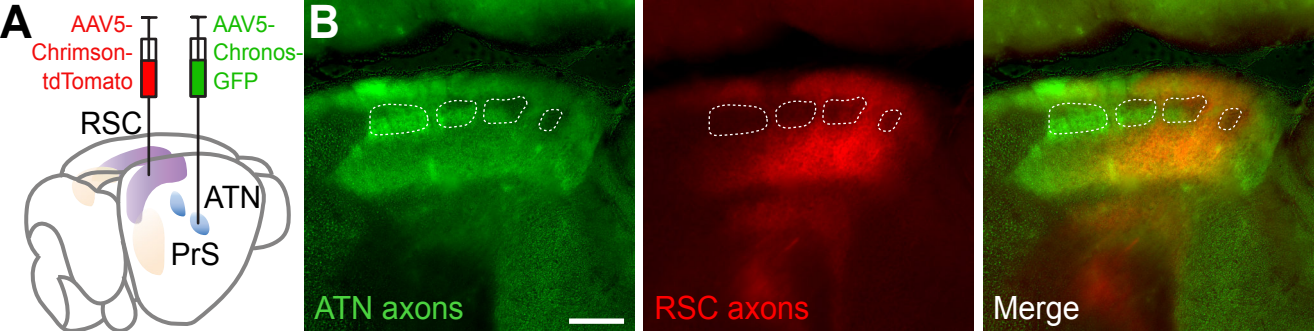


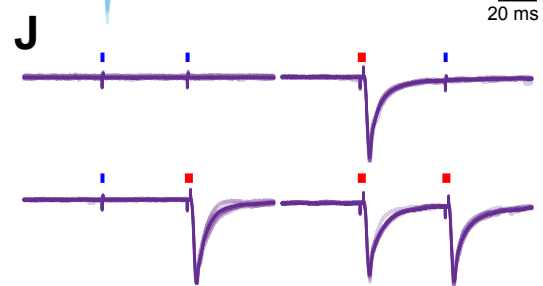
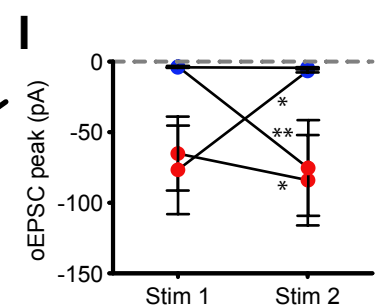
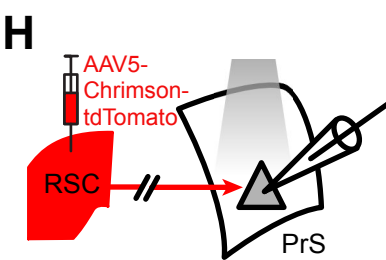
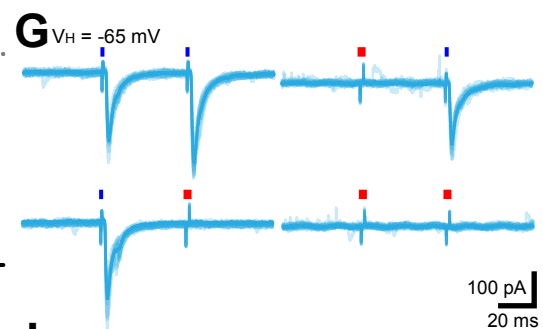
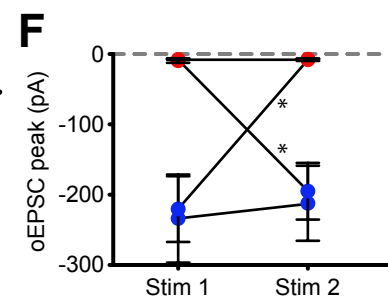
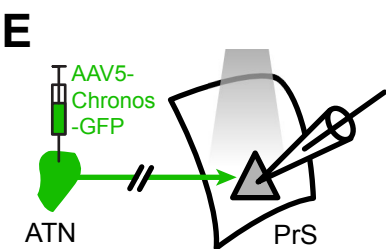
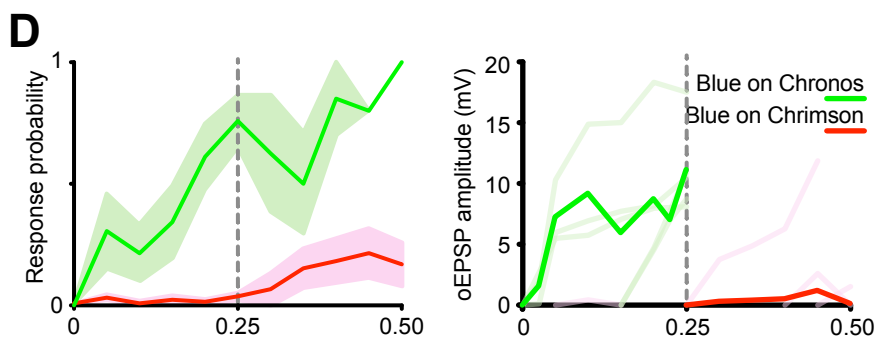
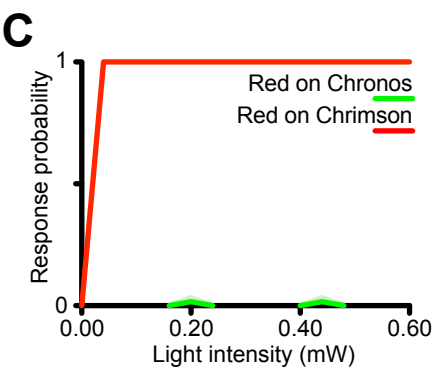
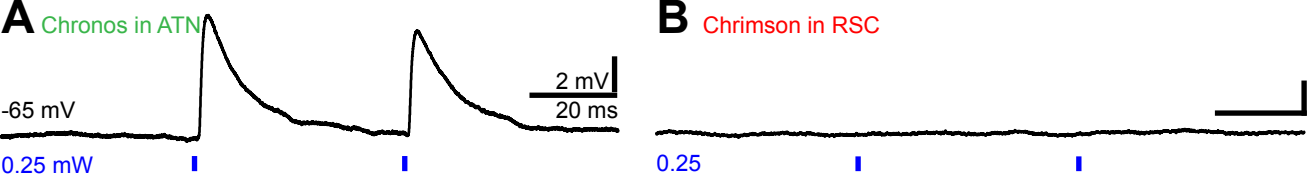




	ATN			RSC			<i>p</i> -value
	Mean	SEM	n	Mean	SEM	n	Mann-Whitney
Resting membrane potential (mV)	-71.37	1.85	27	-73.50	1.52	38	<i>ns</i>
Neuronal input resistance (MΩ)	414.5	31.5	27	349.8	23.4	38	<i>ns</i>
Tau 1 (ms)	26.46	1.80	27	22.25	1.19	38	<i>0.0481</i>
Sag ratio at -100 mV	1.07	0.01	27	1.10	0.01	38	<i>ns</i>
Rheobase current (pA)	51.88	5.25	27	63.00	3.67	38	<i>0.0188</i>
Firing rate at 200 pA (Hz)	39.21	3.87	27	45.10	3.02	38	<i>ns</i>
Maximum firing frequency (Hz)	48.19	4.56	27	64.84	5.02	38	<i>0.0035</i>
Input-output slope (Hz/nA)	357.5	30.0	27	376.7	24.6	38	<i>ns</i>
AP threshold (mV)	-34.13	0.86	27	-33.48	0.91	38	<i>ns</i>
AP width (ms)	0.77	0.06	27	0.68	0.04	38	<i>ns</i>
AP AHP (mV)	-20.25	0.64	27	-18.77	0.47	38	<i>ns</i>
AP rise amplitude (mV)	77.84	1.67	27	79.77	1.26	38	<i>ns</i>
AP maximum depolarization rate (V/s)	323.7	11.0	27	350.7	9.2	38	<i>0.0326</i>
AP maximum repolarization rate (V/s)	-107.9	6.1	27	-121.7	5.9	38	<i>ns</i>
Onset latency at rheobase (ms)	181.2	24.3	27	139.4	17.6	38	<i>ns</i>

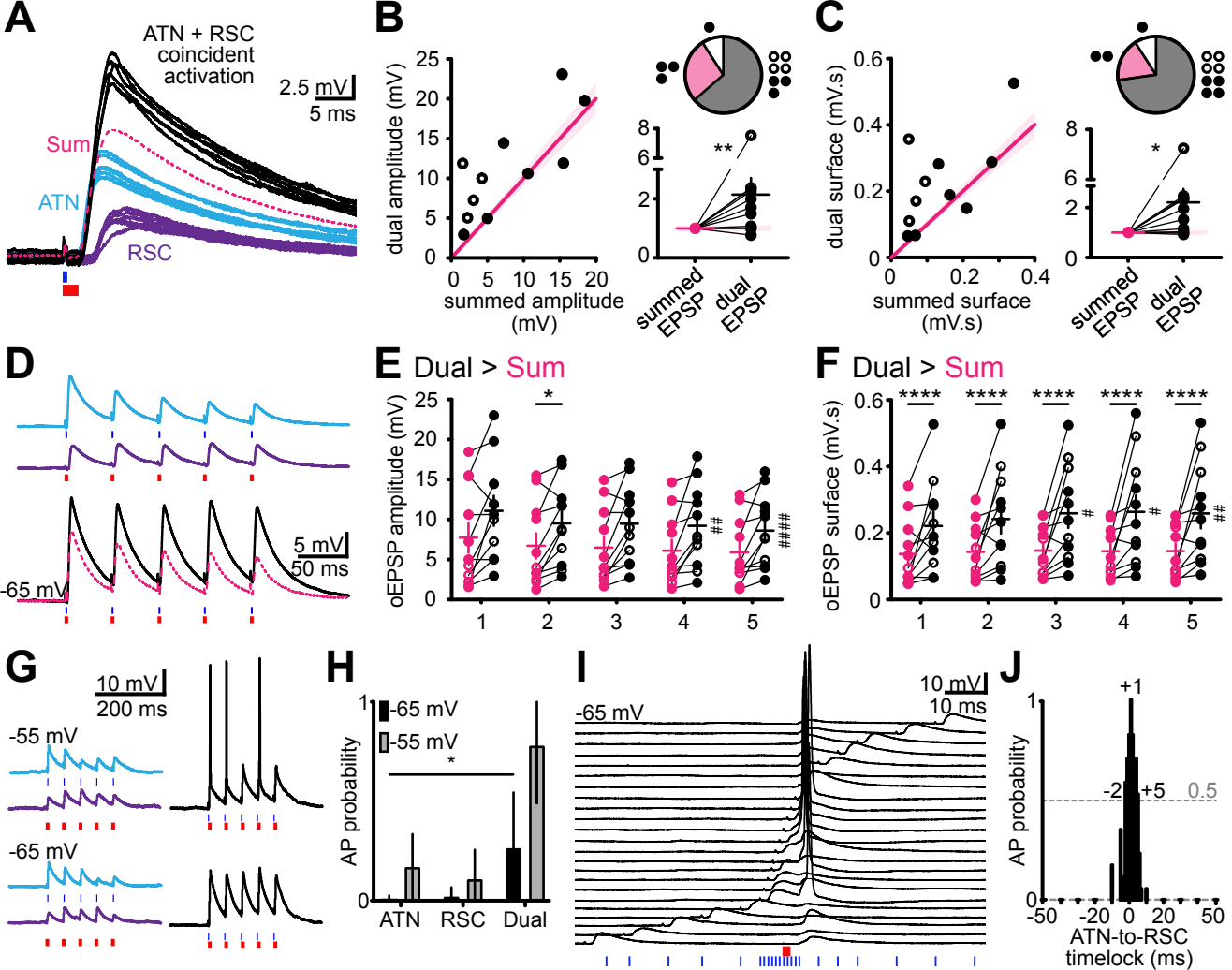






K

		Stim 1		Stim 2		n	Wilcoxon test
		Mean amplitude (pA)	SEM	Mean amplitude (pA)	SEM		
Chronos in ATN	Blue - Blue	-233.8	62.7	-212.1	53.3	6	ns
	Blue - Red	-220.1	46.7	-7.0	0.7	6	0.0312
	Red - Red	-9.5	3.2	-8.2	1.7	6	ns
	Red - Blue	-6.8	0.4	-194.4	40.1	6	0.0312
Chrimson in RSC	Blue - Blue	-4.1	0.3	-4.4	0.5	8	ns
	Blue - Red	-3.7	0.6	-75.3	33.8	8	0.0156
	Red - Red	-65.1	26.2	-84.0	32.0	8	0.0234
	Red - Blue	-76.7	31.4	-6.6	1.0	8	0.0078



A

CsGluc/QX-314

ATN

-65 mV

10 mV
200 ms

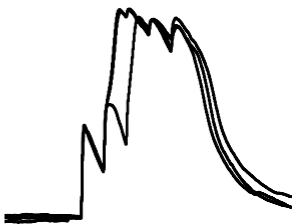
RSC

-65 mV

-65 mV

B

+ Gabazine

**C**

+ Gabazine + APV

