

1 **Serotonergic neurons signal reward and punishment on multiple timescales**

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13 **Abstract**

14 Serotonin's function in the brain is unclear. One challenge in testing the numerous hypotheses
15 about serotonin's function has been observing the activity of identified serotonergic neurons in
16 animals engaged in behavioral tasks. We recorded the activity of dorsal raphe neurons while
17 mice experienced a task in which rewards and punishments varied across blocks of trials. We
18 "tagged" serotonergic neurons with the light-sensitive protein channelrhodopsin-2 and identified
19 them based on their responses to light. We found three main features of serotonergic neuron
20 activity: (1) a large fraction of serotonergic neurons modulated their tonic firing rates over the
21 course of minutes during reward versus punishment blocks; (2) most were phasically excited by
22 punishments; and (3) a subset was phasically excited by reward-predicting cues. By contrast,
23 dopaminergic neurons did not show firing rate changes across blocks of trials. These results
24 suggest that serotonergic neurons signal information about reward and punishment on multiple
25 timescales.

26 **Introduction**
27 Reward and punishment play critical roles in shaping animal behavior over short and long
28 timescales (Rolls, 2005; Somerville et al., 2013). On short timescales, moment-to-moment
29 expectations of reward or punishment increase or decrease motivation to perform a specific
30 action associated with the reward or punishment. On long timescales, repeated exposure to
31 reward or punishment can elicit long-lasting positive or negative emotional states (often called
32 “mood”), that can increase or decrease the frequency of performing reward-seeking actions
33 more generally (Niv et al., 2006; Cools et al., 2011; Somerville et al., 2013; Wang et al., 2013).

34 The midbrain raphe nuclei contain the majority of forebrain-projecting serotonergic
35 neurons in mammals (Jacobs and Azmitia, 1992). This small population of neurons
36 (approximately 9000 in the mouse dorsal raphe; Ishimura et al., 1988) projects to almost the
37 entire forebrain (Azmitia and Segal, 1978; Moore et al., 1978; Steinbusch, 1981; O’Hearn and
38 Molliver, 1984; Vertes, 1991; Gagnon and Parent, 2014). The diffuse projection targets of the
39 serotonergic system have led to many theories about its function.

40 Serotonin has been proposed to be involved in processing reward and punishment
41 (Maswood et al., 1998; Daw et al., 2002; Maier and Watkins, 2005; Nakamura et al., 2008;
42 Ranade and Mainen, 2009; Tops et al., 2009; Cools et al., 2011; Amo et al., 2014). One theory
43 proposes that serotonin regulates aversive learning and negative motivation in response to
44 punishments (Soubrié, 1986; Deakin and Graeff, 1991; Daw et al., 2002; Dayan and Huys,
45 2009). According to this theory, serotonin opposes the positive reinforcement and behavioral
46 activation regulated by dopamine. Whereas dopaminergic neurons signal appetitive prediction
47 errors (Schultz et al., 1997), serotonergic neurons could signal punishments, thereby adjusting
48 future behavior to avoid those punishments or inhibiting specific actions that are associated with
49 punishments. This theory has support from lesion, stimulation, tryptophan depletion, and
50 pharmacological studies (Tye et al., 1977; Graeff and Silveira Filho, 1978; Liu and Ikemoto,
51 2007; Crockett et al., 2009; Shin and Ikemoto, 2010), but there is little evidence that

52 serotonergic neurons signal punishments in awake animals (cf. Aghajanian et al., 1978;
53 Montagne-Clavel et al., 1995; Schweimer and Ungless, 2010).

54 A second theory proposes that serotonin signals global reward states, such as tracking
55 average reward (Daw et al., 2002) and modulating mood (Savitz et al., 2009). Here, serotonin is
56 thought to provide its diffuse targets with long-term signals about the value of the environment,
57 which, at the extreme, is correlated with changes in mood. These long-term signals inhibit
58 behavior or increase the vigor of taking actions in a relatively action-general manner. This
59 theory has support from clinical observations (Fava and Kendler, 2000) and genetic studies
60 (Donaldson et al., 2013). However, there is little neurophysiological evidence for such a function.

61 A third theory proposes that serotonin is involved in waiting for reward (Miyazaki et al.,
62 2011a,b; 2014; Fonseca et al., 2015). In this theory, activation of serotonergic neurons
63 promotes patience (Miyazaki et al., 2014) or slows movements that allow an animal to wait for a
64 delayed reward. This theory could explain how waiting for reward could be linked to behavioral
65 inhibition (Soubrié, 1986; Fonseca et al., 2015).

66 Clarifying whether and how serotonin exerts these functions requires understanding how
67 serotonergic neuron firing correlates with both global features of the environment, such as
68 changes in average reward value, and with punishments. These data have been challenging to
69 collect because of the heterogeneity of neurons in and around the raphe nuclei. About two-
70 thirds of dorsal raphe neurons are serotonergic, but others contain GABA, glutamate, dopamine,
71 acetylcholine, or various peptides (Hökfelt et al., 2000; Commons, 2009; Fu et al., 2010; Hioki et
72 al., 2010). Many previous studies have relied on spike waveform characteristics to identify
73 serotonergic neurons in extracellular recordings. This approach may lead to false positives and
74 misses, however (Allers and Sharp, 2003; Kirby et al., 2003; Marinelli et al., 2004; Urbain et al.,
75 2006; Hajós et al., 2007; Schweimer et al., 2011).

76 Given this chemical diversity and the difficulty of identifying neuron types in extracellular
77 recordings, it would be unsurprising to find corresponding physiological diversity. Indeed,

78 previous studies found significant heterogeneity in the activity of dorsal raphe neurons in
79 relation to behavioral tasks (Fornal et al., 1996; Nakamura et al., 2008; Ranade and Mainen,
80 2009; Bromberg-Martin et al., 2010; Miyazaki et al., 2011a; Inaba et al., 2013; Li et al., 2013).
81 One set of experiments demonstrated that dorsal raphe neurons fired in a reward-value-
82 dependent manner during trials of a saccade-to-target task, but that the modulation disappeared
83 before the next trial (Nakamura et al., 2008; Bromberg-Martin et al., 2010). Another set of
84 experiments showed tonic firing rate modulations correlating with waiting for reward from
85 putative dorsal raphe serotonergic neurons (Miyazaki et al., 2011a). These studies suggested
86 that serotonergic neurons signal reward expectation or waiting time (or patience) on the scale of
87 hundreds of milliseconds, but does not determine whether serotonin could be involved in longer-
88 term changes in value.

89 In the present study, we sought to test whether dorsal raphe serotonergic neuron firing
90 correlated with rewards and punishments on different timescales. To address this question, we
91 used a task in which reward value changed on slow and fast timescales. Importantly, we
92 recorded from optogenetically-identified serotonergic neurons. Our results show that
93 serotonergic neurons signal information about reward and punishment across multiple
94 timescales.

95 **Results**

96 **Behavioral task**

97 We classically conditioned head-fixed, thirsty mice with different odor cues that predicted
98 a reward (water), neutral outcome (nothing), or punishment (a puff of air delivered to the
99 animal's face; Cohen et al., 2012). Each behavioral trial began with a conditioned stimulus (CS;
100 an odor, 1 s), followed by a 1-s delay and an unconditioned stimulus (US; the outcome; Figure
101 1a). Mice licked toward the water-delivery tube in the delay period before rewards arrived, but
102 not during neutral or punishment trials, indicating that they had learned the CS-US associations
103 (analysis of variance (ANOVA), t-tests, $P < 0.001$ for each session; Figure 1b). We varied the
104 structure of the task so that animals received blocks of ten reward trials, alternating with ten
105 punishment trials, with a tone indicating transitions between blocks (or, in 35% of sessions,
106 block order was random; Figure 1c; block duration mean \pm S.D., 1.36 ± 0.30 min). Mice licked
107 significantly more during the tone preceding reward versus punishment blocks. Because the
108 tone was the same preceding both block types, this indicates that they attended to the block
109 structure (Wilcoxon signed-rank test, $P < 0.01$; Figure 1-supplement 1). In each block of some
110 sessions, the reward or punishment trial was replaced by a neutral trial with probability 0.1. To
111 ensure that the time of onset of each trial could not be predicted following the end of the
112 previous one, we used an exponentially-distributed (flat hazard rate) inter-trial interval (ITI). The
113 ITIs lasted, on average, longer than twice the duration of trials (ITI, 6.41 ± 1.88 s, mean \pm S.D.).
114 Using this task, we could study neuronal responses on fast (CS and US) and slow (across
115 blocks) timescales.

116 To determine whether mice treated air puffs as punishments, we performed a behavioral
117 experiment in which mice were given free choices between a water reward and a water reward
118 delivered simultaneously with an air puff. Mice reliably chose the water reward without the air
119 puff, confirming that air puffs act as a negative reinforcement (Figure 1-supplement 2).

120

121 **Identifying serotonergic neurons**

122 We recorded the activity of 149 dorsal raphe neurons while mice performed the
123 conditioning task (6 mice, 24.8 ± 6.1 neurons per mouse, mean \pm S.E.M.). We expressed
124 channelrhodopsin-2 (ChR2), a light-gated cation channel, in serotonergic neurons by injecting
125 an adeno-associated virus containing FLEX-ChR2 (AAV5-EF1 α -DIO-hChR2(H134R)-EYFP-
126 WPRE-pA) into the dorsal raphe of transgenic mice expressing Cre recombinase under the
127 control of the promoter of the serotonin transporter gene (*Sltc6a4*; *Sert-Cre* mice). Expression
128 was specific (of 260 ChR2-EYFP-positive cells, 6 cells, or 2.3%, were 5-HT-negative) and
129 efficient (of 314 5-HT-positive cells, 254, or 80.9%, were ChR2-EYFP-positive; Figure 1d).

130 For each neuron, we measured the response to light stimulation and the shape of
131 spontaneous spikes (Figure 2a,b). We reasoned that for a neuron to be identified as responding
132 to light stimulation, it must first show a significant response to the stimulus (quantified here as
133 light-evoked energy, defined as the integral of the squared voltage values $\int V^2 dt$). Second, to
134 ensure that the neuron under observation, rather than a population of nearby ChR2-expressing
135 serotonergic neurons, responded to light stimulation, we verified that the response to light
136 stimulation matched the shape of spontaneous spikes. We calculated the distance between the
137 spontaneous spike waveform and light-evoked voltage response and plotted it against the
138 energy of light-evoked response for each recording (Figure 2b). Using an expectation-
139 maximization clustering method, we observed two distinct clusters: one that showed significant
140 responses to light pulses and one that did not. Twenty-nine neurons fell into the former cluster
141 (filled cyan points in Figure 2b). Three points in that cluster were not considered identified
142 serotonergic neurons because they did not reliably respond to light stimulation. Consistent with
143 direct light activation rather than indirect synaptic activation, all 29 neurons showed fast light-
144 evoked spikes (Figure 2c) and followed high-frequency stimulation (Figure 2d,e). These
145 properties indicate that these 29 neurons expressed ChR2 (henceforth called "serotonergic
146 neurons"; 5 mice, 5.8 ± 1.5 neurons per mouse, mean \pm S.E.M.).

147

148 **All serotonergic neurons show task-related activity**

149 The structure of the behavioral task allowed us to ask whether serotonergic neuron firing
150 correlated with short-term (rewards, punishments, and the cues that predicted them within a
151 trial) or long-term (blocks of reward versus punishment trials) changes in the environment.

152 We first asked whether serotonergic neuron firing rates were significantly modulated
153 during the behavioral task. We performed an ANOVA on the trial-by-trial firing rates during the
154 baseline period (1 s before odor onset), CS period (from odor onset to odor offset), delay (from
155 odor offset to outcome onset), and US period (from outcome onset to 500 ms after outcome
156 onset). The factors were task epoch (baseline, CS, delay, or US) and outcome type. All 29
157 serotonergic neurons exhibited task-related modulations in firing rate (ANOVA, all $P < 0.01$).

158

159 **Tonic firing modulation by long-term values**

160 Next, we examined the responses of serotonergic neurons in detail. We observed three
161 main features of the firing patterns of serotonergic neurons. First, a large fraction (41%) of
162 serotonergic neurons fired at a higher or lower rate during the ITIs of reward blocks versus
163 punishment blocks (Figure 3a,b; Figure 3-supplement 1). That is, even before a particular trial
164 began, these neurons fired at a rate that correlated with the value (reward versus punishment)
165 of the block. Remarkably, this response persisted across minutes. To quantify this observation,
166 we calculated the firing rate in the 2 s before the start of each trial during reward and
167 punishment blocks. Twelve of 29 serotonergic neurons showed significantly different pre-trial
168 firing rates between reward and punishment blocks: 7 were more excited during reward blocks,
169 5 were more excited during punishment blocks (Figure 3c,d; Wilcoxon rank sum tests, $P < 0.05$).
170 Interestingly, this tonic signal appeared to build up or down slowly within blocks, rather than
171 sharply increasing or decreasing in response to block transitions (Figure 3-supplement 2). This
172 tonic signal did not depend on the duration of ITIs (Wilcoxon rank sum tests, all $P > 0.3$). In

173 addition, 16 of 29 serotonergic neurons displayed gradually decreasing firing rates over the
174 course of the experiment (Figure 3-supplement 3).

175 To compare this effect to the response of dopaminergic neurons—which have been
176 proposed to be involved in long-term value-related signaling (Niv et al., 2006; Cools et al.,
177 2011)—in the same task, we recorded the activity of 28 ventral tegmental area (VTA) neurons,
178 15 of which were identified as dopaminergic (by ChR2 tagging, as described above), and 13 of
179 which were putatively dopaminergic based on their task-related responses (4 mice, 7.0 ± 2.0
180 neurons per mouse, mean \pm S.E.M.). None of the putative or identified dopaminergic neurons
181 showed significantly different pre-trial firing rates between reward and punishment blocks
182 (Wilcoxon rank sum tests, all $P > 0.5$; Figure 3e-h). Most dopaminergic neurons were excited by
183 predicted rewards to varying degrees (see Figure 3-supplement 4 for a more “canonical”
184 example), similar to prior observations in classical conditioning tasks with trace delays in mice
185 (Cohen et al., 2012) and monkeys (Fiorillo et al., 2008).

186

187 **Serotonergic neurons are phasically excited or inhibited by reward-predicting cues or
188 punishments**

189 The second main feature of serotonergic neuron activity we observed was their
190 response to punishments (Figure 4a-c, Figure 4-supplement 1). Previous studies found
191 identified serotonergic neurons to be excited or inhibited by punishments in anesthetized
192 animals (Aghajanian et al., 1978; Montagne-Clavel et al., 1995; Schweimer and Ungless, 2010).
193 To test whether punishments modulated serotonergic firing, we calculated the area under the
194 receiver operating characteristic (auROC) curve in sliding 100-ms windows for each neuron,
195 comparing each window during the trial to the baseline firing rate (1 s before punishment trials;
196 Figure 4b). The auROC quantifies the discriminability of the two firing rate distributions. Values
197 of 0.5 (black) indicate no change in firing rate relative to baseline. Values greater than 0.5
198 (yellow) indicate increases in firing rate relative to baseline, while values less than 0.5 (blue)

199 indicate decreases in firing rate relative to baseline. Most serotonergic neurons (28 out of 29)
200 responded phasically to punishments: 22 were excited, 6 were inhibited (Figure 4a-c; Wilcoxon
201 rank sum tests in the 500 ms after punishment onset, $P < 0.05$). This response was transient,
202 lasting less than 500 ms for most neurons (315 ± 140 ms, mean \pm S.D.).

203 The third main feature of serotonergic neuron activity we observed was their response to
204 reward-predicting cues (Figure 4d-f). We calculated the auROC, comparing the baseline (1 s
205 before reward trials) to each 100-ms window during the trial. About half of serotonergic neurons
206 (15 out of 29) were phasically excited by reward-predicting cues (Figure 4d-f; Wilcoxon rank
207 sum tests during the 1 s of odor presentation, $P < 0.05$). Very few neurons (2 out of 29; $P <$
208 0.05) were inhibited by reward-predicting cues. The duration of excitation was brief, lasting less
209 than 500 ms (235 ± 194 ms, mean \pm S.D.). For 9 of these 15 neurons, the response to a
210 reward-predicting CS was significantly greater than the response to a punishment-predicting CS
211 (Wilcoxon rank sum tests, $P < 0.05$). The time of peak response during a reward-predicting CS
212 was significantly shorter for serotonergic than dopaminergic neurons (mean \pm S.E.M., $331 \pm$
213 15.7 ms for serotonergic neurons, 388 ± 12.7 ms for dopaminergic neurons, Wilcoxon rank sum
214 test, $P < 0.05$).

215 Recent work has provided conflicting views on the role of the dorsal raphe in reward
216 behavior (Liu and Ikemoto, 2007; Shin and Ikemoto, 2010; Liu et al., 2014; McDevitt et al., 2014;
217 Miyazaki et al., 2014; Qi et al., 2014; Fonseca et al., 2015). To compare the phasic reward-
218 related responses during the task to responses to unexpected rewards, we examined
219 dopaminergic and serotonergic responses to unexpected reward (delivered at random times
220 prior to the task in 5 *Slc6a4*-Cre mice and 3 *Slc6a3*-Cre mice). Whereas dopaminergic neurons
221 showed a large, phasic excitation in response to unexpected rewards, a subset of serotonergic
222 neurons was weakly and slowly, but significantly, excited (Wilcoxon rank sum tests, $P < 0.05$ for
223 9 of 10 dopaminergic neurons and 11 of 29 serotonergic neurons; Figure 4g,h). We compared
224 neuronal responses to unexpected rewards to responses to expected rewards in the context of

225 the task. Dopaminergic neurons showed larger responses to unexpected versus expected
226 rewards (Figure 4i), similar to previous observations (e.g., Schultz et al., 1997; Cohen et al.,
227 2012). Interestingly, serotonergic neurons showed a weak but significantly larger response to
228 unexpected versus expected rewards (Figure 4i).

229

230 **Correlations between serotonergic response features**

231 Next, we tested whether these three features were correlated within the population of
232 serotonergic neurons. We found that the difference in pre-trial firing rate during reward versus
233 punishment blocks positively correlated with the difference in response to reward- versus
234 punishment-predicting CS; neurons with higher excitation for reward than punishment CS
235 tended to fire at a higher rate before reward than punishment trials (Figure 5a; Pearson's $r =$
236 0.85, $t_{27} = 8.56$, $P < 0.001$). This demonstrates that the same serotonergic neurons can
237 multiplex different signals about reward and punishment on different timescales.

238 The difference in pre-trial firing rate (reward versus punishment) did not significantly
239 correlate with the response to punishment (Figure 5b; Pearson's $r = 0.03$, $t_{27} = -0.17$, $P > 0.8$).
240 The response to reward CS was significantly positively correlated with the response to
241 punishment (Figure 5c; Pearson's $r = 0.60$, $t_{27} = 3.89$, $P < 0.01$).

242

243 **Serotonergic neuron background firing rates signal graded value**

244 To test whether differences between firing rates during reward versus punishment blocks
245 reflected value, as opposed to salience, we compared the firing rates during neutral trials to
246 those during reward and punishment trials. Neurons with responses to neutral stimuli that fall in
247 between those to rewards and punishments are defined as value-coding. Those with responses
248 to rewards and punishments that are either both larger, or both smaller, than responses to
249 neutral stimuli, are defined as salience-coding (Matsumoto and Hikosaka, 2009).

250 We asked whether serotonergic neurons were value-coding across blocks. For 16
251 serotonergic neurons, we modified the task to include blocks of neutral trials randomly
252 interspersed among reward and punishment blocks (with equal probability). Eight of these
253 displayed significantly different pre-trial firing rates during reward versus punishment blocks
254 (Figure 3c). For 7 of these 8 neurons (3 of which displayed higher firing rates during reward
255 blocks, 5 of which displayed higher firing rates during punishment blocks), pre-trial firing rates
256 during neutral blocks did not fall outside of the bounds of those during reward and punishment
257 blocks (Figure 6a; Tukey's Honest Significant Difference tests, $P > 0.05$). Only 1 of the 16
258 neurons had both significantly larger pre-trial firing rates during neutral blocks than during
259 reward and punishment blocks. Thus, 15 of 16 serotonergic neurons were not salience-coding
260 across blocks of trials.

261 So far, we have described differences in background firing rates as being value-related
262 due to their differences between reward (water), neutral (no stimulus), and punishment (air puff)
263 conditions. We asked whether responses across blocks were truly value-related, or, rather, due
264 to differences in responses to the different sensory modalities for reward and punishment
265 (gustatory versus somatosensory). We performed two experiments to address this. We
266 recorded from 13 additional identified serotonergic neurons in which blocks contained trials with
267 one of three reward sizes: zero, small (1 μ l), or big (4 μ l; Figure 6-supplement 1). If pre-trial,
268 tonic responses are value-related, they should vary monotonically with reward size. Indeed, for
269 each of the 13 neurons, firing rates were either strictly increasing (5 neurons) or strictly
270 decreasing (8 neurons) with reward size (Figure 6b; Tukey's Honest Significant Difference tests,
271 $P > 0.05$). Next, we recorded from 21 additional identified serotonergic neurons (4 mice, $5.3 \pm$
272 1.0 neurons per mouse, mean \pm S.E.M.) in a similar task with four types of blocks: water or
273 chocolate milk reward, neutral, air-puff punishment, and quinine punishment. Quinine, a bitter-
274 tasting solution, is a punishment of the same sensory modality as a water reward. We found that
275 8 of these 21 neurons were tonically more (6 neurons) or less (2 neurons) active in water versus

276 quinine blocks, during ITIs (Figure 6c,d). These neurons also showed a positive correlation in
277 their response to the two different punishments, albeit with a weaker response to quinine
278 (Figure 6-supplement 2). This could be due to the longer timecourse or smaller magnitude of the
279 aversiveness of quinine compared to air puffs. Across both experiments, 20 of 50 identified
280 serotonergic neurons showed tonic differences in firing for reward versus punishment blocks of
281 trials. Thus, background firing rate in serotonergic neurons signal value across different
282 magnitudes of reward and different types of punishment.

283

284 **Serotonergic neuron CS responses signal value**

285 Next, we asked whether serotonergic neurons were value-coding within trials. We
286 compared the firing rates during the CS, rather than US, for two reasons. First, there was no US
287 during neutral trials. Second, outcomes were predicted by the CS, therefore neuronal responses
288 to the US could be confounded by expectation. For each of the 23 serotonergic neurons with
289 sufficient data for this analysis, the CS-induced firing rate as a function of value was monotonic
290 (Figure 7a,b). As a population, the CS firing rate was larger for reward than punishment (paired
291 *t*-test, $t_{22} = 2.6$, $P < 0.05$), larger for reward than neutral ($t_{22} = 2.9$, $P < 0.05$), and larger for
292 neutral than punishment ($t_{22} = 2.2$, $P < 0.05$; Figure 7c-e). This suggests that firing rate
293 differences between reward and punishment trials reflected the value, not the salience, of those
294 stimuli.

295

296 **Unidentified neuron responses**

297 As with identified serotonergic neurons, we observed many unidentified neurons with
298 firing-rate fluctuations from block to block (Figure 8a,b). We note that this population of
299 unidentified neurons likely contains serotonergic as well as non-serotonergic neurons because
300 of incomplete ChR2 expression or our strict criteria for identification.

301 We calculated the firing rate in the 2 s before the start of each trial during reward blocks
302 and punishment blocks. Twenty-nine of 120 unidentified neurons showed significantly different
303 pre-trial firing rates between reward and punishment blocks: 10 were more excited during
304 reward blocks, 19 were more excited during punishment blocks (Figure 8c,d, Wilcoxon rank sum
305 tests, $P < 0.05$).

306 In addition to these slow firing-rate fluctuations across minutes, 92 of 120 unidentified
307 neurons showed task-related responses during the trial (ANOVA, all $P < 0.01$; Figure 8-
308 supplement 1). These neurons were either excited or inhibited by rewards, punishments,
309 reward-predicting cues, and punishment-predicting cues to varying degrees and durations.
310 Although it is likely that there were false negatives (serotonergic neurons contained in the
311 populations of unidentified neurons; Figure 8-supplement 1), there were significant differences
312 between serotonergic and unidentified neurons. For instance, the duration of the significant
313 response (excitation or inhibition) was significantly longer for unidentified neurons than for
314 serotonergic neurons. That is, serotonergic neuron responses during the trial epoch (from CS to
315 US) tended to be more phasic than those of the 92 task-responsive unidentified neurons
316 (Fisher's exact test on the proportion of 100-ms bins significantly different from baseline, odds
317 ratio = 0.643, $P < 0.01$). Finally, as has been observed previously in anesthetized cats and rats,
318 we found both serotonergic and unidentified neurons that displayed "clock-" and "non-clock-like"
319 firing patterns (Figure 8-supplement 2b; Nakahama et al., 1981; Schweimer and Ungless, 2010).
320

321 **Spike waveform properties of serotonergic neurons**

322 Serotonergic neurons are typically identified extracellularly based on their wide spike
323 shapes and low firing rates (Bramwell, 1974; Aghajanian et al., 1978; Baraban et al., 1978;
324 Baraban and Aghajanian, 1980; Aghajanian and Vandermaelen, 1982; Gallager, 1982; Heym et
325 al., 1982; Wang and Aghajanian, 1982; Chiang and Pan, 1985; Fornal et al., 1985, 1987;
326 Cunningham and Lakoski, 1988; Levine and Jacobs, 1992; Ceci et al., 1994; Guzmán-Marín et

327 al., 2000; Celada et al., 2001; Sakai and Crochet, 2001; Waterhouse et al., 2004; Miyazaki et al.,
328 2011). These criteria have recently been called into question, however (Park, 1987; Allers and
329 Sharp, 2003; Kirby et al., 2003; Marinelli et al., 2004; Kocsis et al., 2006; Urbain et al., 2006;
330 Hajós et al., 2007; Ranade and Mainen, 2009; Bromberg-Martin et al., 2010; Schweimer and
331 Ungless, 2010; Schweimer et al., 2011; Gocho et al., 2013; Li et al., 2013). We asked whether
332 we could have identified serotonergic neurons in our data set in this way. Serotonergic neurons
333 had significantly longer spike duration than unidentified neurons (Wilcoxon rank sum test, $P <$
334 0.05), although there was significant overlap in the distributions (Figure 8-supplement 2a).
335 There was no significant difference between the mean firing rate (Figure 8-supplement 2a;
336 Wilcoxon rank sum test, $P > 0.6$) or coefficient of variation of the inter-spike interval distributions
337 (Wilcoxon rank sum test, $P > 0.1$) between serotonergic and unidentified neurons. Neither an
338 expectation-maximization nor a k-means clustering algorithm could classify serotonergic
339 neurons based on spike duration, firing rate, or shape of the inter-spike interval distribution.

340 **Discussion**

341 By recording from identified serotonergic neurons, we showed that (1) a large fraction of
342 serotonergic neurons showed tonic firing modulation depending on state value on long
343 timescales (tens of seconds to minutes); (2) serotonergic neurons phasically responded (mostly
344 by excitation) to punishments; and (3) a subset of serotonergic neurons was phasically excited
345 by reward-predicting cues. These observations showed that serotonergic neurons signal reward
346 and punishment on multiple timescales.

347 The dorsal raphe nucleus contains diverse types of neurons (Marinelli et al., 2004;
348 Commons, 2009; Fu et al., 2010; Hioki et al., 2010). Although previous recording studies have
349 used indirect methods based on spike shape or firing rate measures (Fornal et al., 1996;
350 Miyazaki et al., 2011a), it has been difficult to separate the responses of serotonergic from non-
351 serotonergic raphe neurons during behavioral tasks. Indeed, our data set of identified
352 serotonergic neurons contained diverse waveforms and firing properties, making it critical to use
353 a more direct method to identify serotonergic neurons. We observed some diversity in the
354 responses of identified serotonergic neurons (cf. Ranade and Mainen, 2009) but, to describe the
355 full diversity of serotonergic neurons, a larger sample size is required. We thus focused on
356 salient features that were shared by a large fraction of identified serotonergic neurons, which we
357 observed in our sample size of 50 identified serotonergic neurons. In the following, we discuss
358 potential implications of our findings in the context of theories of behavioral regulation and
359 serotonin signaling.

360

361 **Tonic firing of serotonergic neurons tracks state values**

362 Reward and punishment can exert their effects on behavior on multiple timescales. It
363 has been proposed that the average reward rate on relatively long timescales (or state value)
364 regulates the vigor of behavioral responding in a manner relatively non-specific to actions (Niv
365 et al., 2006; Wang et al., 2013). However, the neural mechanisms that regulate this process

366 remain unclear. Much attention has been paid to a potential involvement of tonic dopaminergic
367 firing in this process (Niv et al., 2006; Cools et al., 2011). In contrast to this proposal, we did not
368 find that dopaminergic neurons changed their baseline firing according to state values. Our data
369 showed, instead, that 40% of serotonergic neurons changed their baseline firing depending on
370 the state value of the environment. We observed this effect using rewards and punishments of
371 the same sensory modality, and using brief punishments (air puffs), in which the acute
372 aversiveness likely did not persist into the ITI. These firing rate changes were relatively small in
373 magnitude (around 1-2 spikes s⁻¹), but given the low baseline firing rates of serotonergic
374 neurons, these changes corresponded to around 20-100% increases in firing rates, which could
375 have led to substantially higher serotonin release.

376 Our data also showed that serotonergic neurons exhibited transient activations
377 associated with various task events (Nakamura et al., 2008; Ranade and Mainen, 2009).
378 Serotonergic neuron responses to reward- or punishment-predictive cues as well as reward or
379 punishment were transient, typically lasting less than 500 ms, and relatively small in magnitude
380 (5-10 spikes s⁻¹), in agreement with previous studies (Nakamura et al., 2008; Ranade and
381 Mainen, 2009; Bromberg-Martin et al., 2010; Miyazaki et al., 2011a; Inaba et al., 2013), though
382 substantially smaller than a recent one (Li et al., 2013). Previous studies observed that dorsal
383 raphe neurons exhibited sustained activities that appeared to track moment-to-moment changes
384 in value triggered by sensory cues and outcomes, within a trial (Nakamura et al., 2008;
385 Bromberg-Martin et al., 2010). Although these activities lasted for several hundreds of
386 milliseconds to seconds, our identified serotonergic neurons showed relatively transient
387 activities within trials. In contrast, our data showed that many unidentified neurons showed
388 sustained activities within trials, suggesting that sustained activities may be more common in
389 non-serotonergic neurons. Another recent set of studies found that putative serotonergic
390 neurons showed firing modulations lasting up to several seconds, during a task in which rats
391 waited for a reward (Miyazaki et al., 2011a), and that manipulations of serotonergic signaling

392 altered waiting behavior (Miyazaki et al., 2014; Fonseca et al., 2015). This raises the interesting
393 possibility that both time and reward value modulate the firing of serotonergic neurons,
394 increasing the flexibility of the serotonergic signal. Indeed, the tonic signal we observed could
395 be the subjective value of waiting (i.e., waiting for punishments elicits a low-value state,
396 whereas waiting for rewards elicits a high-value state). A third study found that dorsal raphe
397 neurons, on average, fired at a higher rate before cues that predicted rewards relative to cues
398 that predicted no reward (Li et al., 2013). In this work, trials were also delivered in a block-wise
399 fashion, though it was not possible to identify serotonergic neurons, nor was there an analysis of
400 the slow modulations in firing rate in individual neurons.

401 The sign of value-dependent changes in tonic firing varied across serotonergic neurons:
402 some increased and others decreased during periods of high state values although, on average,
403 high-value blocks were associated with higher tonic firing rates. Nevertheless, the topography of
404 projections from the raphe (Imai et al., 1986; Vertes et al., 1991; Lowry et al., 2000; Chandler et
405 al., 2013) and the physiological topography within the raphe (Lowry et al., 2000; Crawford et al.,
406 2010) suggest the potential for a specific mapping between subsets of serotonergic neurons
407 and their functions. Our results cannot distinguish these subpopulations of serotonergic neurons.
408 It remains to be examined whether different firing patterns of serotonergic neurons correspond
409 to specific subpopulations.

410 Together, these results suggest that background serotonin could serve as an explicit
411 signal of state values. It is important to note that our data demonstrate a relative value code
412 during the task (blocks of trials of different values), but do not speak to the possibility that
413 serotonergic neurons signal an absolute state value (cf. Figure 3-supplement 3). As discussed
414 above, reward and punishment affect behavior on multiple timescales. Our finding that tonic
415 serotonergic firing tracks values over long timescales raises the possibility that tonic serotonin
416 regulates long-lasting affective states.

417

418 **Dopamine and serotonin do not signal opposite information**

419 In addition to the changes in tonic firing, our data showed that a large fraction of
420 serotonergic neurons were excited by reward-predictive cues and unpredicted reward on shorter
421 timescales (i.e., within trials). Although there was substantial diversity in serotonergic neuron
422 firing patterns, during reward trials, on average, their response to reward-predicting cues
423 resembled those of dopaminergic neurons signaling reward prediction error (Schultz et al.,
424 1997; Cohen et al., 2012). It is important to note, however, that the magnitude of increases was
425 smaller, and did not appear to signal prediction errors for most neurons (Figures 3d,h, 4i). In
426 addition, we found that many serotonergic neurons were excited by punishments. It is important
427 to disentangle whether the phasic responses to air puffs we observed are due to aversiveness
428 or other factors such as saliency or relief from punishment (Heym et al., 1982; Waterhouse et al.,
429 2004; Dayan and Huys, 2009; Cools et al., 2011). The short response latency suggests that it is
430 unlikely to be relief from punishment, but the nature of phasic air puff responses remains to be
431 clarified. Of course, all of these signals could be expressed by the population of serotonergic
432 neurons. In addition, it will be important to understand why the phasic response to air puffs was
433 stronger than the phasic response to air-puff-predicting cues.

434 It has been proposed that serotonergic and dopaminergic neurons work largely in an
435 opponent manner (Daw et al., 2002). This idea was supported by pharmacology and intracranial
436 self-stimulation (Redgrave et al., 1978), as well as a few recording studies that suggested
437 phasic activations of serotonergic neurons by aversive stimuli (Aghajanian et al., 1978;
438 Montagne-Clavel et al., 1995; Schweimer and Ungless, 2010). However, in these studies,
439 responses were measured in anesthetized animals or the effect of reward was not examined,
440 leaving unanswered whether phasic serotonin signals purely aversive information. Our data
441 showed that many single serotonin neurons were activated by both reward-predictive cues and
442 punishment (Figure 5c). Although it remains unclear whether these phasic firing patterns can be
443 unified to encode a particular variable, or whether the response to punishment was due to its

444 sensory nature or the relief arriving at the end of the punishment, these firing patterns appear to
445 be inconsistent with the idea that serotonergic neurons send an opponent signal compared to
446 dopaminergic neurons, though median raphe serotonergic neurons could provide such a signal
447 (Daw et al., 2002). This lack of pure opponency is consistent with recent studies measuring
448 putative serotonergic firing and serotonin concentration in a waiting task (Miyazaki et al.,
449 2011a,b).

450

451 **Multiplexing of signals on multiple timescales**

452 Our results suggest that serotonergic neurons multiplex information about reward and
453 punishment on multiple timescales. We propose that slow, value-related firing could represent
454 state value, whereas phasic responses to CS or US could encode a different variable. This slow
455 signal appears to require some time to change (Figure 3-supplement 2). Future studies should
456 clarify how downstream neurons read out tonic versus phasic serotonin signals. It is possible
457 that tonic serotonin has very different effects at target neurons and on behavior than phasic
458 serotonin due to receptors with different affinities or other mechanisms (Daw et al., 2002). In
459 addition, serotonergic neurons are known to contain other transmitters (Varga et al., 2009; Liu et
460 al., 2014), suggesting that the slow and fast timescales could correspond to the action of
461 different transmitters on target neurons, or to downstream circuit effects with differing durations.
462 The function of serotonin could be target-dependent (Deakin and Graeff, 1991), timing-
463 dependent (Daw et al., 2002; Dayan and Huys, 2009), or dependent on co-release of other
464 transmitters (Dayan and Huys, 2009; Varga et al., 2009; Liu et al., 2014; McDevitt et al., 2014).
465 Another possibility is that serotonin combines with other circuits to form logical combinations
466 postsynaptically (for example, serotonin AND dopamine codes for reward, whereas serotonin
467 AND NOT dopamine codes for punishment).

468 How do serotonergic neurons compute the signals over short (hundreds of milliseconds)
469 and long (minutes) timescales? Serotonin release is controlled by many types of afferents. The

470 densest include frontal cortex, basal forebrain areas (bed nucleus of the stria terminalis,
471 substantia innominata, ventral pallidum), hypothalamic nuclei (preoptic nucleus, lateral and
472 posterior nuclei), lateral habenula, and several midbrain and brainstem structures (Peyron et al.,
473 1998; Ogawa et al., 2014; Pollak Dorocic et al., 2014; Weissbourd et al., 2014). Neurons in the
474 VTA and substantia nigra pars compacta (SNC), some dopaminergic, provide input to raphe
475 serotonergic neurons (Beckstead et al., 1979; Ogawa et al., 2014; Pollak Dorocic et al., 2014;
476 Weissbourd et al., 2014). These neurons are phasically excited by rewards or reward-predicting
477 cues (Schultz et al., 1997; Cohen et al., 2012), but their activity appears not to correlate with
478 longer-term changes in value (Figure 3 above; Matsumoto and Hikosaka, 2009). They may
479 excite serotonergic neurons via D2 receptors (Haj-Dahmane, 2001; Aman et al., 2007; but see
480 Martín-Ruiz et al., 2001). Interestingly, dopaminergic stimulation of putative serotonergic
481 neurons *in vitro* caused excitation that persisted beyond the application of dopamine (Haj-
482 Dahmane, 2001). It is possible that serotonergic neurons could accumulate information about
483 rewards via short pulses of input from dopaminergic neurons, although our observation of
484 shorter reward-predicting CS response latencies in serotonergic versus dopaminergic neurons
485 suggests the opposite.

486

487 **Spike waveform properties of serotonergic neurons**

488 There are several possible explanations for why we could not use spike width to clearly
489 identify serotonergic neurons. First, unidentified neurons likely contained serotonergic neurons
490 that we could not identify using our stringent criteria. Second, the shape of extracellularly-
491 recorded spikes depends on the position of the electrode tip to the soma and dendrites of the
492 recorded neuron (Schultz, 1986; Harris et al., 2000; Henze et al., 2000; Buzsáki et al., 2012).
493 Given that the geometry of dendritic and axonal processes in the dorsal raphe is diverse, with
494 fusiform, multipolar, and ovoid somata (Diaz-Cintra et al., 1981), it is potentially difficult to
495 optimize the location of electrode tip relative to cell bodies across the population (cf. Schultz,

496 1986). Indeed, we did not attempt to optimize this position, once we found an identified
497 serotonergic neuron. Third, our nichrome wires could have introduced recording bias away from
498 small cells or certain cell shapes, potentially less of an issue for glass pipette recordings (Towe
499 and Harding, 1970; Shoham et al., 2006; O'Connor et al., 2010). Indeed, in our previous study
500 in the ventral tegmental area, using the same type of electrode, we did not find the oft-reported
501 difference between spike width for dopaminergic versus non-dopaminergic neurons (Cohen et
502 al., 2012). Careful simultaneous intra- and extracellular measurements (Harris et al., 2000;
503 Henze et al., 2000) in the dorsal raphe are needed to resolve this.

504

505 **Summary**

506 The present study revealed that serotonergic neurons can use tonic as well as phasic
507 firing to convey reward information. This raises questions as to whether these different modes of
508 firing convey distinct information, whether they have different impacts on target neurons (notably,
509 on dopaminergic neurons [Fibiger and Miller, 1977; Watabe-Uchida et al., 2012; Ogawa et al.,
510 2014]), and how they are calculated. Although our data likely do not reveal the full diversity of
511 serotonergic neuron firing dynamics, our results suggest distinguishing these distinct modes is
512 crucial to teasing apart the seemingly complex functions of serotonin in various brain functions
513 and disorders.

514 **Materials and methods**

515 **Animals**

516 For dorsal raphe recordings, we used nine adult male mice, backcrossed with C57BL/6J
517 mice, heterozygous for Cre recombinase under the control of the serotonin transporter gene
518 (*Slc6a4*^{tm1(cre)Xz}; Zhuang et al., 2005). For VTA recordings, all of which came from the task in
519 Figure 1, we used four adult male mice backcrossed with C57BL/6J mice, heterozygous for Cre
520 recombinase under the control of either the dopamine transporter (3 mice) or tyrosine
521 hydroxylase gene (1 mouse) (*Slc6a3*^{tm1.1(cre)Bkmn}/J and B6.Cg-Tg(Th-cre)^{1tmd}/J, respectively, The
522 Jackson Laboratory; Savitt et al., 2005; Bäckman et al., 2006). We did not observe any
523 differences between the different genotypes during the behavioral task. For cell counting, we
524 used three additional adult male *Sert-Cre* mice. Animals were housed on a 12h dark/12h light
525 cycle (dark from 06:00-18:00) and each performed the conditioning task at the same time of day,
526 between 07:00 and 19:00. All surgical and experimental procedures were in accordance with
527 the National Institutes of Health Guide for the Care and Use of Laboratory Animals and
528 approved by the Harvard or Johns Hopkins Institutional Animal Care and Use Committees.

529

530 **Surgery and viral injections**

531 Mice were surgically implanted with a head plate and a microdrive containing electrodes
532 and an optical fiber. During a prior surgery, we injected 200-500 nl adeno-associated virus
533 (AAV), serotype 2/5, using the EF1 α promoter, carrying an inverted ChR2 (H134R)-EYFP
534 flanked by double loxP sites (Nagel et al., 2003; Boyden et al., 2005; Atasoy et al., 2008) into
535 the dorsal raphe stereotactically (from bregma: 4.4-4.7 mm posterior, 0.1-0.2 mm lateral, 1.9-2.3
536 mm ventral), or into VTA as previously described (Cohen et al., 2012).

537 All surgery was performed under aseptic conditions with animals under
538 ketamine/meperidine (60 and 0.5 mg/kg, I.P., respectively) or isoflurane (1-2% at 0.5-1.0

539 L/min) anesthesia. Analgesia (ketoprofen, 5 mg/kg, I.P.; buprenorphine, 0.1 mg/kg, I.P.) was
540 administered postoperatively.

541

542 **Behavioral task**

543 After at least 1 week of recovery, mice were water-deprived in their home cage. Weight
544 was maintained within 90% of their full body weight. Animals were head-restrained using a
545 custom-made metal plate and habituated for 1-2 d while head-restrained before training on the
546 task. Odors were delivered with a custom-made olfactometer (Uchida and Mainen, 2003). Each
547 odor was dissolved in paraffin oil at 1/10 dilution. 30 μ l of diluted odor was placed inside a filter-
548 paper housing. Odors were isoamyl acetate, 1-butanol, N-citral, eugenol, (+) limonene, (-)
549 carvone, (+) carvone, allyl tiglate, eucalyptol, acetophenone,
550 hydroxymethylpentylcyclohexenecarboxaldehyde, 3-hexanone, pentyl acetate, 1-hexanol, p-
551 cymene, and ethyl butyrate, and differed for different animals. Odorized air was further diluted
552 with filtered air by 1:10 to produce a 500 ml/min total flow rate. Licks were detected by breaks of
553 an infrared beam placed in front of the water tube.

554 We delivered one of three odors, selected pseudorandomly, for 1 s, followed by a delay
555 of 1 s and an outcome. Each odor predicted a different outcome: a drop of water (4 μ l), no
556 outcome, or an air puff delivered to the animal's face. ITIs were drawn from an exponential
557 distribution with a rate parameter of 10 (i.e., $P(ITU) = 1/10 \exp(-x/10)$). This resulted in a flat ITI
558 hazard function, ensuring that expectation about the start time of the next trial did not change
559 over time. A 15 kHz tone lasting 1 s signaled block changes, ending 1 s before the start of the
560 next trial. Data were obtained from 141 sessions (19-28 sessions per animal). For 17 identified
561 serotonergic neurons, we omitted rewards during 10% of big-reward trials. Animals performed
562 between 400 and 700 trials per day (533 ± 120 trials, mean \pm SD). Free rewards were delivered
563 before the start of the conditioning task. We did not observe differences in lick rate (Figure 1) or
564 sniff rate (counted manually during 1-s intervals of 20 reward trials and 20 punishment trials for

565 two mice; t-tests, $t_{38} = 1.28$, $P > 0.20$, $t_{38} = 1.05$, $P > 0.29$ for each mouse) during ITIs of
566 different block types.

567 For 16 of 29 serotonergic neurons, block type varied randomly, whereas for 13 of 29
568 serotonergic neurons, and all dopaminergic neurons, block type alternated between reward and
569 punishment. For 16 of 29 serotonergic neurons, neutral blocks were included, in which case an
570 additional odor was used as a CS. For 2 of 29 serotonergic neurons, block size was 20 trials.
571 We trained each animal on the task with randomly-interleaved trials for five days before
572 beginning the blocked structure.

573 For the task including quinine as a US, we used water (4 μ l) or chocolate milk (1%, Hood,
574 Lynnfield, Massachusetts, 4 μ l) as a reward US and quinine HCl (0.5-1.0 mM, 4 μ l) as a
575 punishment US. To ensure that animals ingested the quinine, which is known to be aversive
576 (Schoenbaum et al., 1998; Berridge et al., 2000; Peciña and Berridge, 2005; Roitman et al.,
577 2005), we placed the delivery tube at the entry to their mouths. Other task parameters were the
578 same as above.

579 For the freely-moving behavioral task, we trained five adult male C57BL/6J mice to
580 perform a two-alternative forced choice task as follows (Figure 1-supplement 1). An odor cue (1-
581 hexanol) delivered at a central port was a start cue that signaled the mouse to choose between
582 one peripheral port that contained water (4 μ l) and a second that contained water (4 μ l) and an
583 air puff. After an ITI (same distribution as for the tasks described above), the next trial began.
584 Mice performed 300 trials of this task for five days, after six days of shaping and training.

585

586 **Electrophysiology**

587 We recorded extracellularly from multiple neurons simultaneously using custom-built
588 200- μ m-fiber-optic-coupled screw-driven microdrives with eight implanted tetrodes (four
589 nichrome wires wound together, Sandvik, Palm Coast, Florida). Tetrodes were glued to fiber
590 optics with epoxy or cyanoacrylate. The ends of tetrodes were 400-600 μ m from the ends of

591 fiber optics. Neural signals and time stamps for behavior were recorded using DigiLynx
592 recording systems (Neuralynx, Bozeman, Montana). Broadband signals from each wire were
593 filtered between 0.1 and 9,000 Hz and recorded at 32 kHz. To extract the timing of spikes,
594 signals were bandpass-filtered between 300 and 6,000 Hz and sorted online and offline.

595 To verify that our recordings targeted serotonergic or dopaminergic neurons, we used
596 ChR2 to observe stimulation-locked spikes (Cardin et al., 2009; Lima et al., 2009; Cohen et al.,
597 2012). The optical fiber was coupled to a diode-pumped solid-state laser with analog amplitude
598 modulation (Laserglow Technologies, Toronto, Canada). For each neuron, we delivered trains
599 of 10 light pulses, each 5 ms long, at 1, 5, 10, 20, and 50 Hz at 473 nm at 5-20 mW/mm², before
600 and after the experimental session. Higher intensities typically resulted in light-evoked spike
601 waveforms that did not match spontaneous ones. Therefore, we adjusted the light intensity after
602 observing the responses at the beginning of experiments. The increasing latency of light-evoked
603 spiking as a function of stimulation frequency indicates that the response was not due to
604 photochemical artifact (Figure 2e). Spike shape was measured using a broadband signal (0.1-
605 9,000 Hz) sampled at 32 kHz.

606 We used two criteria to include a neuron in our data set. First, the neuron must have
607 been recorded within 500 µm of an identified serotonergic neuron, to ensure that all neurons
608 came from the dorsal raphe (except for dopaminergic neurons, which all came from VTA).
609 Second, the neuron must have been well isolated. To measure isolation quality, we calculated
610 the L-ratio (Schmitzer-Torbert and Redish, 2004), which approximates the fraction of
611 "contaminated" spikes. Smaller L-ratios indicate better isolation. All neurons in the data set had
612 L-ratios < 0.05 and signal-to-noise ratios of > 5 dB. Identified serotonergic neurons, of which
613 there were 50 across experiments, came from all nine mice (a range of 1-10 per mouse).

614

615

616

617 **Data analysis**

618 To measure firing rates, peristimulus time histograms (PSTHs) were constructed using
619 10-ms bins. To calculate spike density functions, PSTHs were convolved with a function
620 resembling a postsynaptic potential, $(1-\exp(-t)) \exp(-t/200)$, for time t . For display (but not
621 analysis), we smoothed spike density functions with a smoothing spline with 30 degrees of
622 freedom (Kass et al., 2005). To determine whether a neuron showed a significant task-related
623 response, we calculated an ANOVA on the trial-by-trial firing rates during the baseline period (1
624 s before odor onset), CS period (from odor onset to odor offset), delay (from odor offset to
625 outcome onset), and US period (from outcome onset to 500 ms after outcome onset). The
626 factors were task epoch (baseline, CS, delay, or US) and outcome type. Normality was tested
627 by Kolmogorov-Smirnov tests and quantile-quantile plots. All two-group comparisons were two-
628 sided. Effect sizes for each experiment were determined post-hoc using Cohen's U_3 (cf.
629 Hentschke and Stütgen, 2011).

630 Light-evoked spikes were detected during the 10 ms after light onset. If less than 20% of
631 light pulses evoked a spike (defined as a waveform that matched that of the isolated unit) during
632 the 10 ms after light onset (upper left points in Figure 2b), the maximum absolute voltage during
633 that interval was used as the light-evoked "response." Euclidean distances between
634 spontaneous and light-evoked spike waveforms were calculated by aligning the larger of the
635 positive or negative peak of each waveform, averaging separately, and aligning the peaks of the
636 averages. The distance was calculated using the full duration of the spontaneous spike (spike
637 duration was measured as the first time until the last time at which the voltage was significantly
638 different from baseline using Wilcoxon rank sum tests). The energy of the light-evoked
639 waveform is defined as the integral of the squared voltage values ($\int V^2 dt$).

640 ROC curves were calculated by comparing the distribution of firing rates (spike density
641 functions) across trials in 100-ms bins to the distribution of baseline firing rates (1 s before odor
642 onset, using 100-ms bins). The duration of significant responses were calculated using

643 Wilcoxon rank sum tests comparing the baseline firing rate to the firing rate in the response
644 window of interest, bin by bin. The number of bins in which $P < 0.05$ was taken as the duration
645 of the significant response (after Bonferroni corrections).

646 Expectation-maximization clustering was performed using hierarchical clustering for
647 parameterized Gaussian mixture models, setting the number of clusters to 2 (one cluster of
648 "identified serotonergic neurons" and one of "unidentified neurons"), with model selection by
649 Bayesian Information Criterion.

650 All statistical tests were done with Bonferroni corrections for multiple comparisons.
651 Analyses were done with R (<http://www.r-project.org/>).

652

653 **Immunohistochemistry**

654 After recording, which lasted between 19 and 28 days, mice were given an overdose of
655 ketamine/medetomidine, exsanguinated with saline, perfused with 4% paraformaldehyde, and
656 brains were cut in 50-100 μm coronal sections. Sections were immunostained overnight with a
657 primary antibody to 5-HT (1:200; S5545, Sigma-Aldrich, St. Louis, Missouri; Steinbusch et al.,
658 1978; Bras et al., 1986; Dai et al., 2008), then incubated with an Alexa594-coupled secondary
659 antibody for 2 hr (1:200; Invitrogen, Carlsbad, California). Sections were further counterstained
660 with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Recording sites were identified
661 and verified to be amid EYFP expression and 5-HT staining in the dorsal raphe.

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670

671 **Author contributions**

672 J.Y.C. and M.W.A. collected and analyzed data. J.Y.C. and N.U. designed experiments and
673 wrote the paper.

674 **Figure 1** Behavioral task. (a) Structure of individual trials. (b) Average lick rate for all animals
675 during each trial type. (c) Representative sequence of trials from one experiment. Each point
676 represents an odor cue. Shaded regions indicate reward blocks. (d) Schematic of midbrain
677 indicating recording sites (shaded), with low and high magnification of 5-HT labeling (cyan),
678 ChR2-EYFP (magenta), nuclei (DAPI), and their overlay. Scale bars are 100 μ m and 10 μ m for
679 low and high magnification, respectively.

680 **Figure 1-figure supplement 1** Histogram of lick rate during tones indicating block transitions,
681 for experiments in which reward blocks alternated with punishment blocks.

682 **Figure 1-figure supplement 2** Mice treat air puffs as punishments. (a) Mice performed a two-
683 alternative forced choice task, in which they chose between a water reward and a water reward
684 together with an air puff, indicating their response by moving to the associated response port.
685 (b), Task timing, in which an odor cue in a central port signals a choice, followed by an outcome.
686 (c), Median (horizontal line), interquartile range (box), and 1.5 times interquartile range
687 (whiskers) of proportion of water choices for each mouse, across five experiments of 300 trials
688 each. Each mouse chose water over water together with air puffs significantly more than chance
689 (Tukey's Honest Significant Difference tests, $P < 0.001$).
690

691 **Figure 2** Identifying serotonergic neurons. (a) Example voltage trace from 10 pulses of 10-Hz
692 light stimulation (cyan bars; light duration, 5 ms). Each light-triggered spike is shown below. The
693 lower right is the first two principal components of all waveforms from one tetrode wire, showing
694 the neuron's ("unit") isolation quality, with 100 randomly-chosen light-evoked spikes in cyan. (b)
695 Quantification of light-evoked responses to identify serotonergic neurons (filled points).
696 Abscissa: energy (integral of the squared voltage values) of the light-evoked response from
697 each neuron. Ordinate: Euclidean distance between the mean spontaneous spike and the light-
698 evoked response. Example neurons are shown to the right (black, spontaneous spikes; cyan,
699 light-evoked voltages; SD of spike waveforms are smaller than line thicknesses). Note that three

700 unfilled points in the lower-right cluster are not considered identified serotonergic neurons
701 because of low probability of firing in response to light stimulation. (c) Mean and SD of the light-
702 evoked spike latency for identified serotonergic neurons. Probability (d) and latency (e) of light-
703 evoked spikes from serotonergic neurons as a function of stimulation frequency (filled points are
704 means across neurons).

705

706 **Figure 3** A population of serotonergic neurons is more or less active during blocks of reward
707 trials than punishment trials. (a) Average firing rates of four example serotonergic neurons
708 during reward trials (black) and punishment trials (orange). Shaded regions denote S.E.M. Note
709 the higher pre-trial firing rate during reward trials than punishment trials in the top two neurons
710 and the higher firing rate during punishment versus reward blocks in the third neuron. (b) Firing
711 rate of the same four neurons across the timecourse of the experiment. Note the slow (across
712 minutes) fluctuations in firing rate in the top three neurons that correlated with block type
713 (reward: black, shaded regions; punishment: orange). (c) Mean \pm 95% confidence intervals
714 around firing rates during the baseline epoch for punishment versus reward blocks for each
715 serotonergic neuron (significant data points are filled). Examples from (a) are labeled. (d)
716 Average firing rates of serotonergic neurons with significantly higher baseline firing rates during
717 reward (top) or punishment (bottom) blocks. (e) Average firing rate of an example identified
718 dopaminergic neuron during reward and punishment trials. (f) Firing rate across the timecourse
719 of the experiment for the dopaminergic neuron in (e). (g) Mean \pm 95% confidence intervals
720 around firing rates during the baseline epoch for punishment versus reward blocks for each
721 dopaminergic neuron (identified in cyan, putative in white). (h) Average firing rate of
722 dopaminergic (identified and putative) neurons during reward and punishment trials.
723 **Figure 3-figure supplement 1** Raster plots showing spike times during 40 trials for the four
724 example neurons in Figure 3a.

725 **Figure 3-figure supplement 2** (a) Normalized mean \pm S.E.M. firing rate within reward and
726 punishment blocks for the positive-coding serotonergic neurons. Note the build-up and build-
727 down activity within blocks. (b) Raw firing rates for reward and punishment blocks for six
728 example serotonergic neurons with tonic firing rate differences across blocks. (c) Mean \pm S.E.M.
729 lick rate during the delay between CS and US as a function of trial within block. Note the lack of
730 resemblance to the firing rates in (a).

731 **Figure 3-figure supplement 3** (a) Trial-by-trial scatter plot of lick rate against spike rate for an
732 example serotonergic neuron. (b) Pearson correlation coefficient of trial-by-trial lick rate and
733 spike rate across serotonergic neurons. The two neurons with significant correlations are
734 indicated in gray. (c) Firing rate across reward trials for two example serotonergic neurons, with
735 linear fits in dashed gray. The top neuron had a significantly decreasing firing rate across trials,
736 whereas the bottom neuron did not. (d) Slope of firing rate across reward trials for all
737 serotonergic neurons, with slopes significantly different from zero indicated in gray. (e-h) Slope
738 of firing rate across reward (e and f) and punishment (g and h) trials for all serotonergic neurons,
739 during CS (odor onset to odor offset; e and g) and US (US onset to 500 ms after US onset; f
740 and h), with slopes significantly different from zero indicated in gray. (i) Difference between
741 background firing rates during reward blocks and pre- (top panel) or post-session (top panel)
742 activity, for serotonergic neurons. (j) Difference between background firing rates during
743 punishment blocks and pre- (top panel) or post-session (top panel) activity, for serotonergic
744 neurons.

745 **Figure 3-figure supplement 4** Example activity of a dopaminergic neuron with a smaller
746 response to predicted reward than unpredicted reward-predicting cue.

747

748 **Figure 4** Serotonergic neurons are briefly excited or inhibited by punishments or reward-
749 predicting cues. (a) Average firing rates of two example serotonergic neurons during
750 punishment trials. CS and US analysis windows are shaded in gray. (b) Area under the ROC

751 curve for punishment trials for all serotonergic neurons, sorted by the sum of the auROC for
752 reward trials in (e). Yellow indicates excitation, blue indicates inhibition, and black indicates no
753 change relative to baseline. (c) Histogram of changes in firing rate relative to baseline during the
754 CS and US epochs of punishment trials. (d) Average firing rates of two example serotonergic
755 neurons during reward trials. (e) Area under the ROC curve for reward trials for all serotonergic
756 neurons, sorted by their sum. (f) Histogram of changes in firing rate relative to baseline during
757 the CS and US epochs of reward trials. (g) Area under the ROC curve for free reward for
758 dopaminergic and serotonergic neurons. (h) Average firing rate of dopaminergic and
759 serotonergic neurons around free reward (shaded curves show S.E.M.). (i) Histograms of
760 average differences between mean firing rates during expected versus unexpected rewards for
761 serotonergic and dopaminergic neurons.

762 **Figure 4-figure supplement 1** (a) Area under the ROC curve for reward (water) versus
763 punishment (air puff) trials for serotonergic neurons, sorted by the sum of the auROC. Yellow
764 indicates excitation, blue indicates inhibition, and black indicates no change relative to baseline.
765 (b) Histogram of changes in firing rate during the CS and US epochs of reward versus
766 punishment trials.

767

768 **Figure 5** Correlations between serotonergic neuron response features. (a) Difference in firing
769 rate during the 2-s pre-trial epoch versus the difference in firing rate during the CS (reward -
770 punishment), corrected for baseline differences. (b) Area under the ROC curve for the 2-s pre-
771 trial epoch during reward versus punishment trials plotted against area under the ROC curve
772 during the punishment US epoch. (c) Area under the ROC curve for during the reward CS
773 versus punishment US epochs.

774

775 **Figure 6** Serotonergic neuron background firing rates signal graded value. (a) Median
776 (horizontal line), interquartile range (box), and 1.5 times interquartile range (whiskers) of pre-trial

777 firing rates during reward (black), neutral (gray), and punishment (orange) blocks. Brackets
778 indicate significant differences. (b) Mean \pm 95% confidence intervals pre-trial response during
779 blocks of three reward sizes for 13 serotonergic neurons with strictly increasing (blue) or
780 decreasing (red) firing rates as a function of reward size. (c) Mean \pm S.E.M. firing rate of two
781 example serotonergic neurons during reward (water or chocolate milk) and punishment (air puff,
782 orange; quinine, green) trials. (d) Mean \pm 95% confidence intervals around firing rates during
783 the baseline epoch for punishment (quinine) versus reward (water or chocolate milk) blocks for
784 each serotonergic neuron (significant data points are filled).

785 **Figure 6-figure supplement 1** Behavioral task. (a) Structure of individual trials. (b) Average lick
786 rate for all animals during each trial type.

787 **Figure 6-figure supplement 2** (a) Area under the ROC curve for reward, air-puff punishment,
788 and quinine punishment trials for 21 serotonergic neurons. (b) Area under the ROC curve for
789 the punishment period for quinine versus air-puff punishments. Pearson correlation with
790 significance test is shown. (c) Mean \pm S.E.M. firing rate of serotonergic neurons in the task that
791 included different reward sizes.

792

793 **Figure 7** Serotonergic neurons signal value, not salience, in response to the CS. (a) Mean \pm
794 S.E.M. firing rate of serotonergic neurons during all three trial conditions. (b) Mean \pm S.E.M.
795 firing rate during the CS epoch (region bounded by dashed lines in a) for each trial condition.
796 (c)-(e) Mean \pm 95% confidence intervals around firing rates during the CS epoch for neutral
797 versus reward (c), punishment versus reward (d), and punishment versus neutral (e).

798

799 **Figure 8** A population of unidentified neurons is more or less active during blocks of reward
800 trials than punishment trials. (a) Mean \pm S.E.M. firing rates of three example neurons during
801 reward trials (black) and punishment trials (orange). Note the higher pre-trial firing rate during
802 reward trials than punishment trials in the bottom two neurons. (b) Firing rate of the same three

803 neurons across the timecourse of the experiment. Note the slow (across minutes) fluctuations in
804 firing rate in the bottom two neurons that correlated with block type (reward: black, shaded
805 regions; punishment: orange). **(c)** Mean \pm 95% confidence intervals around firing rates during
806 the baseline epoch for punishment versus reward blocks for each unidentified neuron
807 (significant data points are filled). **(d)** Mean \pm S.E.M. firing rates of unidentified neurons with
808 significantly higher baseline firing rates during reward (top) or punishment (bottom) blocks.

809 **Figure 8-figure supplement 1** Response profiles across all neurons and all trial types. The
810 figure shows the heterogeneity of unidentified neuron responses relative to serotonergic
811 neurons. Area under the ROC curve for reward (left) and punishment trials (right) for each
812 neuron. Neurons are sorted by the sum of the auROC values from reward trials. Serotonergic
813 neurons are marked with arrows to the left of the plot.

814 **Figure 8-figure supplement 2** Serotonergic neurons cannot be identified based on firing
815 properties in this data set. **(a)** Firing rate versus spike duration with marginal density histograms.
816 Serotonergic neurons had significantly longer spike duration than unidentified neurons
817 (Wilcoxon rank sum test, $P < 0.05$). **(b)** Example inter-spike interval (ISI) density histograms for
818 three serotonergic neurons and three unidentified neurons. **(c)** Mean spontaneous spikes
819 (black) and light-evoked voltages (cyan) from all serotonergic and unidentified neurons.
820 Asterisks indicate the three unidentified neurons from the lower-right cluster in Figure 2b.

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