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Activity of tonically active neurons in the primate striatum reflects interaction between time processing and reward prediction

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Abstract

The striatum and its dopaminergic input participate in temporal processing and numerous studies provide evidence that interactions between dopamine and acetylcholine are critical for striatal functioning. However, the role of local cholinergic innervation of the striatum in behaviors requiring precise timing has not been specifically investigated. Here, we recorded from presumed striatal cholinergic interneurons, identified as tonically active neurons (TANs), in two male rhesus monkeys performing self-initiated movements after specified learned time intervals have elapsed since a visual cue. We found that 38% of all recorded TANs responded to the cue with a pause in firing and the strength of these responses could be modulated according to the duration of the interval being timed and the accuracy of time estimates. By examining the TAN response to the reward itself and by recording from TANs during a Pavlovian procedure in which no action was required, we found evidence that TAN activity modulation may potentially reflect differences in the animal's prediction of reward. Thus, besides their well-known role in predicting and detecting rewarding events, TANs may generate signals related to the processing of time. Our findings suggest a role of the local cholinergic circuitry in the representation of time within the striatum.

1 **Introduction**

2 Convergent evidence indicates that the striatum, the main recipient of afferents to the basal
3 ganglia, is a key component in brain networks involved in temporal processing. Apart from
4 timing deficits observed in striatal-based disorders in humans, such as Parkinson's disease
5 (Pastor et al., 1992; Parker et al., 2013) and attention-deficit hyperactivity disorder (Rubia et
6 al., 2009; Noreika et al., 2013), a large body of data supporting the role of the striatum and its
7 dopaminergic input in the processing of time also comes from brain imaging studies in
8 humans (Harrington et al., 2004; Coull et al., 2011) and lesion/inactivation studies in animals
9 (Meck, 1996; 2006; Paton and Buonomano, 2018). In addition, electrophysiological studies in
10 both rodents and monkeys trained on timing tasks have provided evidence of striatal activity
11 related to the encoding of time at both single-neuron and population levels (Matell et al.,
12 2003; Jin et al., 2009; Chiba et al., 2015; Gouvêa et al., 2015; Mello et al., 2015; Bakhurin et
13 al., 2016; 2017; Wang et al., 2018; Zhou et al., 2020). Until now, hypotheses about the
14 neuronal basis of striatal timing function have focused on the output neurons and the
15 modulating influence of dopamine on temporal processing (Coull et al., 2011; Merchant et al.,
16 2013; Paton and Buonomano, 2018). However, the striatum and its output pathways are
17 strongly regulated by local circuits, including cholinergic interneurons, which exert a
18 powerful influence on striatal network activity (Zhou et al., 2002). How the cholinergic
19 innervation of the striatum, derived from interneurons, contributes to the processing of time
20 remains unclear. On the other hand, there is considerable evidence that dopaminergic
21 transmission interacts closely with the intrinsic striatal cholinergic system (Threlfell and
22 Cragg, 2011; Cachope et al., 2012; Threlfell et al., 2012; Cai and Ford, 2018) and it is
23 therefore possible that cholinergic signaling may complement DA transmission during timing
24 behavior.

25 Although a link has been suggested between cholinergic activity and timing behavior
26 based on lesion and pharmacological studies of cholinergic transmission in animals (Buhusi
27 & Meck 2005; Meck 1996), none of them were specific enough to determine the contribution
28 of the local cholinergic innervation of the striatum. Up to now, studies of behavioral effects of
29 targeted disruption of striatal cholinergic transmission have documented disturbances in the
30 ability to modify behavior in changing environments (Brown et al., 2010; Aoki et al. 2015),
31 without specifically addressing a contribution to timing behavior.

32 Cholinergic interneurons of the striatum, presumed to be tonically active neurons
33 (TANs) extracellularly recorded in behaving animals, are considered as detectors of
34 motivationally salient stimuli whose timing cannot be precisely predicted. Indeed, in previous

35 work, we showed that TANs are particularly sensitive to the expected time of rewarding
36 stimuli (Sardo et al., 2000; Ravel et al., 2001), suggesting that they may have access to
37 temporal prediction information (Apicella et al., 2006). However, no study to date has
38 specifically investigated the contribution of the cholinergic TAN system to timing, under
39 conditions which require precise temporal control of behavior.

40 Another open question is whether reward expectation may bias time-related signals in
41 the striatum. As for dopaminergic neurons, TANs are highly sensitive to reward-predicting
42 stimuli (Aosaki et al., 1994; Apicella et al., 1997) and some of them are involved in the
43 encoding of reward prediction error (Apicella et al., 2009; 2011). The intertwining of reward
44 prediction and temporal processing is the source of an inherent difficulty in disambiguating
45 effects related to timing processes from those related to expectations about reward (Paton and
46 Buonomano, 2018; Fung et al., 2021).

47 In the present study, we analyzed the activity of TANs during a time estimation task
48 which requires monkeys to produce overt estimates of elapsed time. In this task, a visual cue
49 is presented to the animals indicating that the reward is delivered for a self-paced movement
50 executed at a specified time interval since the cue. We found that TANs displayed responses
51 to the timing cue, some of them being scaled according to the duration of the interval to be
52 timed and the accuracy of time estimates. Our results also indicate that the observed
53 modulations in TAN activity may integrate reward prediction, in addition to the processing of
54 time. Based on these findings, we suggest that the local cholinergic circuitry of the striatum
55 could provide a neuronal mechanism that detect and process stimuli relevant for timing
56 behavior and engage the striatal mechanisms more directly involved in the representation of
57 time. A preliminary account of this work previously appeared in a short review (Martel and
58 Apicella, 2021).

59

60 **Results**

61 *Monkey's ability to estimate time intervals*

62 We examined neuronal activity while rhesus monkeys performed a time estimation task
63 (TET) in which they made self-initiated movements based on time estimates (Figure 1A). In
64 this task, animals were presented with a visual spatial cue that indicates reward can be
65 obtained for making reaching movements towards targets after a specified time interval, either
66 *short* (1.0, 1.3s) or *long* (2.0, 2.3s), has elapsed since the onset of the cue. A schematic
67 representation of the sequence of events in the TET is given in Figure 1A.

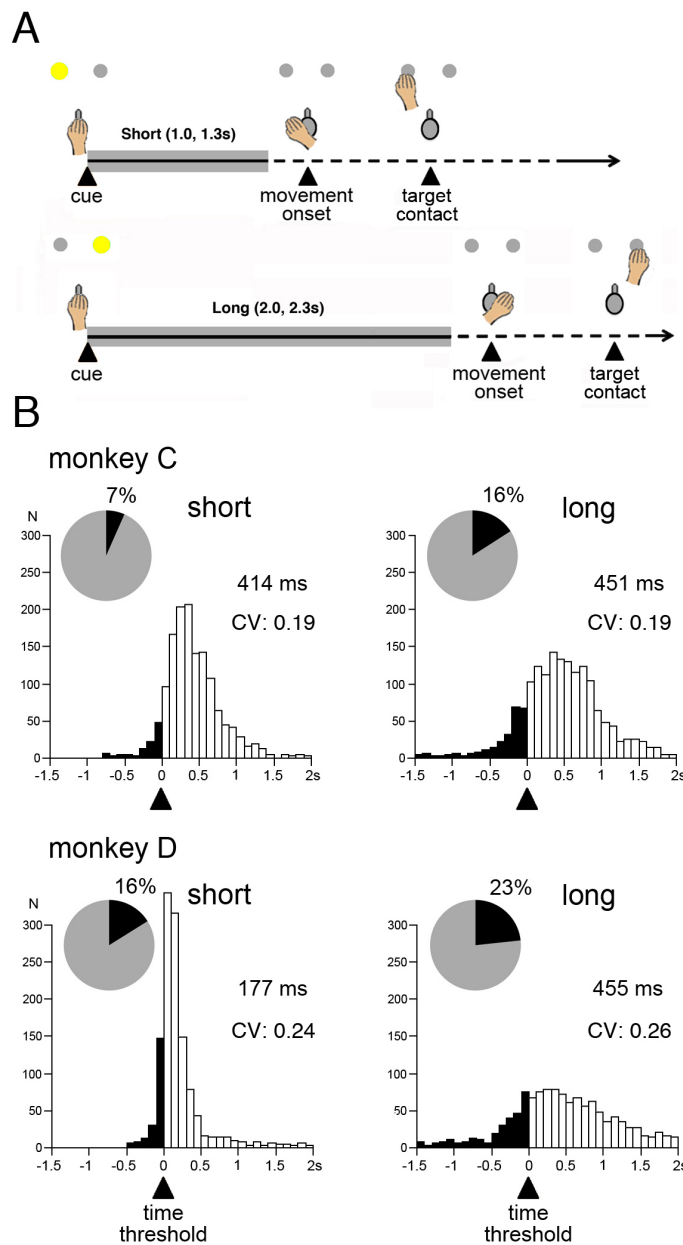


Figure 1. Time estimation task and timing performance. **A.** Temporal sequence of events in the task. Monkeys were required to initiate reaching movement after a specified time interval has elapsed. At the beginning of the trial, a visual spatial cue (yellow light), either on the left or the right side, indicated the duration of the interval (*Short* or *Long*) in the range of seconds, each time interval being associated with a particular location. Gray-shaded horizontal lines indicate the minimal waiting period before movement initiation assigned to each location of the cue (time threshold). Correctly timed movements were reinforced with fruit juice immediately after target contact. **B.** Timing performance. Distribution of movement onset times produced by monkeys in the task. CV, coefficient of variation (i.e., the ratio of the standard deviation to the mean). Values correspond to the mean onset time of movement after the criterion time. Black parts of the histograms correspond to movements which did not reach the criterion time (underestimation) and inset pie charts indicate the proportions of these incorrectly timed movements. Number of trials were 1370 and 1594 for monkey C and 1219 and 1146 for monkey D, for short and long intervals, respectively.

68

69

70 Distributions of movement onset times (MOTs) for each interval, indicate that the monkeys’
71 timing accuracy was lowered in both animals when the duration of the interval was longer, as
72 reflected by a broader distribution around the time threshold for movement onset (Figure 1B).
73 The relative constancy of the coefficients of variation in the range of intervals we used
74 indicates that the extent of the MOT distribution was proportional to the length of the interval
75 being timed, consistent with the scalar property of interval timing which refers to the
76 tendency for response time variability to increase with interval duration (Gibbon, 1977). This
77 supports the idea that both monkeys have learned to adjust the timing of their movements
78 according to the location of the cue. We also quantified the timing accuracy of movements by
79 calculating the percentage of trials in which animals correctly awaited the end of the

80 minimum waiting period. Both monkeys responded after the criterion time in more than 80%
81 of the trials, regardless of interval duration, reflecting their ability to adjust time estimates
82 according to temporal information from cue. There was a mean 11.6% and 19.7%
83 underestimation errors in monkeys C and D, respectively (Fig. 1B, insets), this difference
84 being statistically significant ($\chi^2 = 66.29$, $df = 1$, $p < 0.0001$). In monkey D, the timing of
85 movements was remarkably accurate for the short interval, the mean MOT being 177 ms after
86 the time threshold, compared to 414 ms in monkey C. On the other hand, MOTs were
87 relatively similar between the two animals for the long interval (451 ms and 455 ms in
88 monkeys C and D, respectively). These interindividual differences may reflect different
89 timing strategies. Namely monkey C could efficiently use the cue associated with each
90 interval duration, leading to similar levels of timing accuracy in self-timed movements,
91 whereas monkey D apparently used a strategy to optimize the estimated onset time of its
92 movement specifically for the short interval.

93

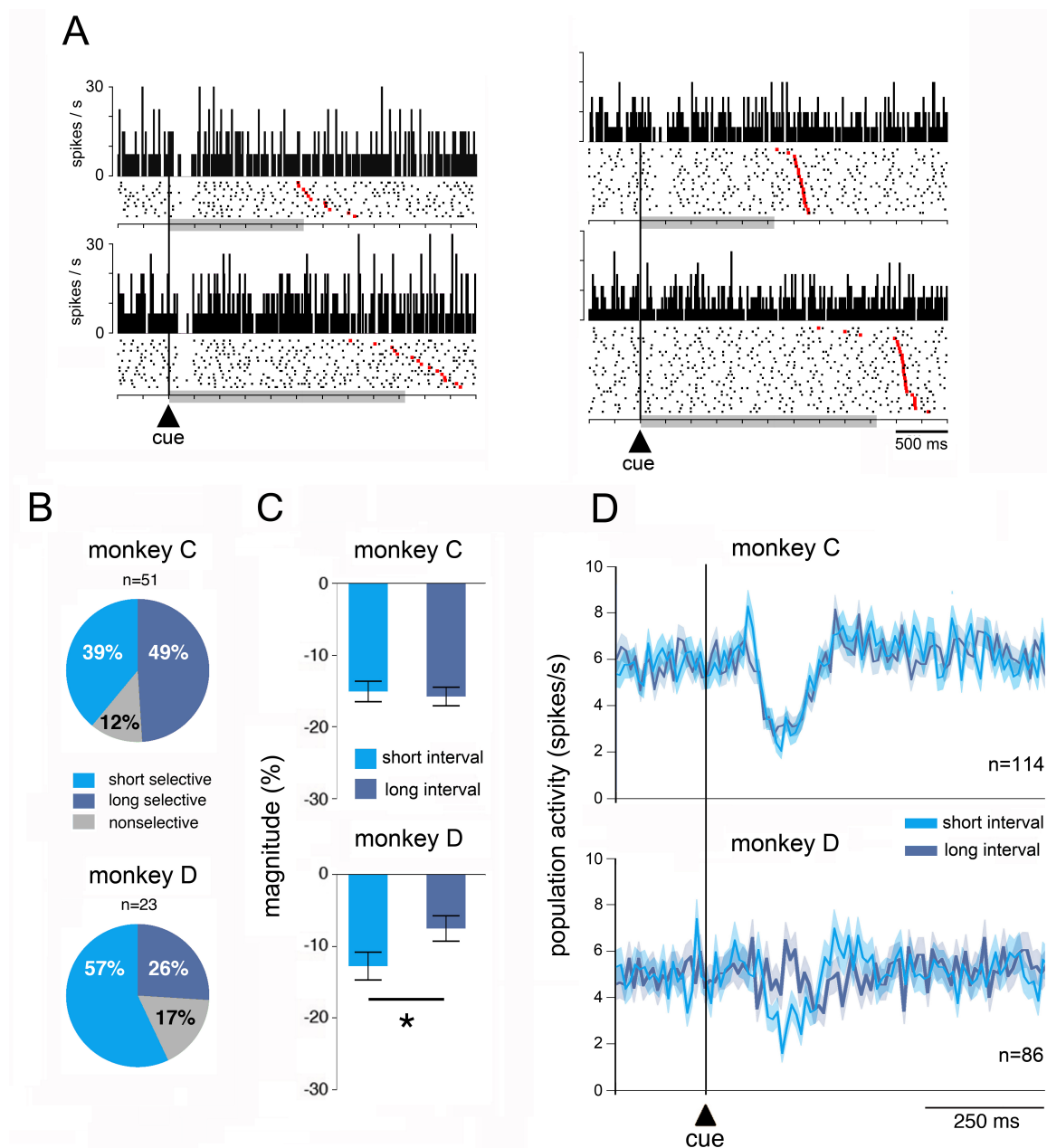
94 *Sensitivity of TANs to the timing cue*

95 We recorded the activity of 200 neurons (114 in monkey C, 86 in monkey D)
96 electrophysiologically identified as tonically active neurons (TANs). Previous work in
97 behaving monkeys has shown that TANs constitute a group of neurons that share
98 characteristic spiking features (Figure 2-figure supplement 1) and display relatively
99 homogeneous changes in activity related to the detection of motivationally salient stimuli
100 (Apicella, 2017). In the present study, the predominant task-related modulation of TAN
101 activity consisted in a short-lasting depression in activity (*pause*) after the presentation of the
102 timing cue. This pause could be followed by a transient increase in activity (*rebound*) and, in
103 some rare instances, preceded by a brief excitatory phase. The histological verification of
104 recording sites in both monkeys showed that most of the recorded TANs were located in the
105 dorsal putamen, at precommissural and postcommissural levels, as will be reported in detail
106 later (Figure 7).

107 For each recorded TAN, we analyzed the responsiveness to the timing cue separately
108 for short and long intervals. Over 38% of all TANs displayed statistically significant
109 decreases in activity in response to the onset of the cue associated with the short and/or long
110 interval (monkey C: 51/114, 45%; monkey D: 23/86, 27%), monkey C showing a larger
111 fraction of responsive neurons than monkey D ($\chi^2=6.80$, $df=1$, $p=0.009$). Example responsive
112 neurons are shown in Figure 2A. The first neuron (Fig. 2A, left) displayed a pause response to
113 the cue for the short- and long-interval trials. In the second neuron (Fig. 2A, right), a pause

114 response to the cue was detected for the short-interval trials, whereas a weak nonsignificant
 115 change occurred for the long-interval trials.

116



117

118

Figure 2. Sensitivity of TANs to the timing cue. **A.** Two example neurons responding to the cue. Left, nonselective response. Right, selective response. Each trial is displayed as a row of spikes (dots) aligned at cue onset, with perievent time histogram above each raster plot. Data are separated by interval duration and sorted by MOT. Red markers indicate the times of movement onset. Gray-shaded bars indicate the minimum waiting period before movement onset. **B.** Percentages of different response types evoked by the cue. n, number of responsive neurons. **C.** Boxplot representation of magnitudes of changes in TAN activity after the cue for short and long intervals. Data are indicated as decreases in percentage below baseline activity and expressed as means \pm SEM (* $p < 0.05$, Wilcoxon rank-sum test). **D.** Population activity of TANs aligned on the cue onset marked by the vertical line. The analysis included rewarded trials (i.e., correctly timed movements) and unrewarded trials (i.e., incorrectly timed movements). Colored curves represent mean activity of TANs separately for short and long interval trials calculated in nonoverlapping time bins of 10 ms. Shading indicates SEM. n, number of neurons included for population curves.

119

120 Of the 74 neurons responding to the cue, 10 (14%) responded regardless of the interval and 64
121 (86%) responded with only one interval, indicating that most TANs were able to discriminate
122 between short- or long-interval cues. As illustrated in Figure 2B, in monkey C, there were no
123 significant differences in the fraction of TANs showing selectivity for one interval or the
124 other (short-preferring, $n=20/51$, 39%; long-preferring, $n=25/51$, 49%, $\chi^2=0.99$, $df=1$,
125 $p=0.318$), whereas in monkey D the percentage of selective responses was significantly
126 higher for the short interval than for the long interval (short-preferring, $n=13/23$, 57%; long-
127 preferring, $n=6/23$, 26%, $\chi^2=4.16$, $df=1$, $p=0.041$). It therefore appears, in both animals, that
128 most TANs showed selective responses to the cue depending on the interval duration, with
129 monkey D showing an increased responsiveness of TANs to the cue associated with the short
130 interval, possibly reflecting the higher accuracy of time estimates with this interval.

131 To analyze quantitatively whether the sensitivity of TANs to the timing cue differed
132 according to interval duration, we rated the magnitude of changes in TAN activity during
133 specified time windows which we selected on the basis of our analysis of latency and duration
134 of individual neuronal responses for each monkey and each time interval (see Materials and
135 Methods). We did this for each neuron of the entire sample of TANs tested in the TET. The
136 results of this analysis are given in Fig. 2C. The magnitude of pauses in TAN activity varied
137 insignificantly between short and long intervals in monkey C (Wilcoxon rank-sum test,
138 $z=0.415$, $p=0.677$), whereas it was significantly higher for the short interval than for the long
139 one in monkey D (Wilcoxon rank-sum test, $z=2.241$, $p=0.025$). We also examined the average
140 activity from all neurons separately for the short and long intervals. As shown in Figure 2D, a
141 clear population response after the cue onset was obvious for both intervals in monkey C,
142 whereas a response occurred for the short interval in monkey D, with comparatively little sign
143 of activity modulation for the long interval. In this animal, a preference for the short interval
144 was therefore evident at the level of both the population and the individual neurons, reflecting
145 the monkey's tendency to be more efficient in using temporal information from short-interval
146 cues. On the other hand, in monkey C, the magnitudes of the TAN response were not
147 dependent of the interval duration, suggesting that this animal made use of temporal
148 information to initiate actions in a more homogeneous way. This indicates that the different
149 responsiveness of TANs in the two monkeys may potentially reflect differences in strategies
150 for performing the timing task.

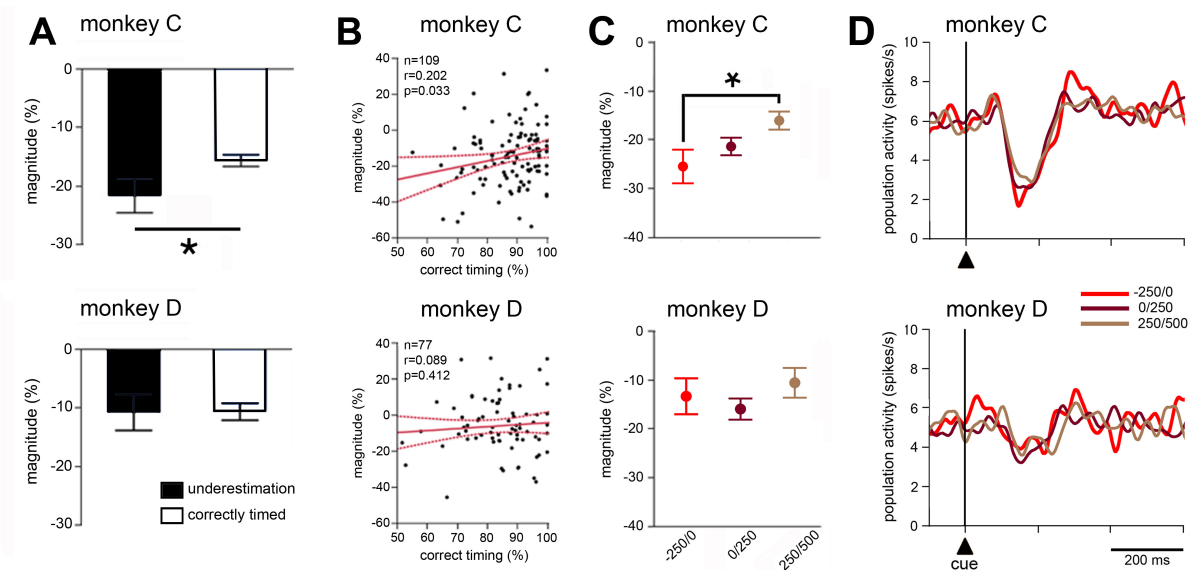
151

152 *Influence of timing accuracy on TAN responsiveness*

153 We next examined whether TAN activity after cue onset may be related to the monkeys'
154 timing accuracy. To this end, we first compared movements initiated before reaching the time
155 threshold (i.e., movements with negative MOTs) and movements that meet the time threshold
156 (i.e., movements with positive MOTs), combining data from both intervals. As shown in
157 Figure 3A, in monkey C, the magnitude of TAN pauses was stronger for incorrectly timed
158 movements, compared with correctly timed movements (Wilcoxon rank-sum test, $z=2.239$,
159 $p=0.025$), whereas differences in activity between correctly and incorrectly timed movements
160 were not statistically different in monkey D (Wilcoxon rank-sum test, $z=0.177$, $p=0.859$) (Fig.
161 3A).

162 To further assess a possible link between the TAN activity and timing performance,
163 we performed a correlation analysis across neurons between the magnitude of TAN responses
164 to the cue and the percentage of trials in which monkeys correctly reached the time threshold.
165 As shown in Figure 3B, in monkey C, we found a statistically significant linear relationship
166 between the magnitude of TAN modulations and percentage of correctly timed movements
167 ($r=0.202$, $p=0.033$), indicating that trials in which the magnitude of the TAN response to the
168 cue was higher were followed by shorter production time intervals. In contrast, we did not
169 found any significant correlation between the strength of the TAN response to the cue and
170 timing accuracy in monkey D ($r=0.089$, $p=0.412$).

171



172

Figure 3. Sensitivity of TANs to timing accuracy. **A.** Comparison of magnitudes of TAN responses to the cue between correctly and incorrectly timed movements. Same conventions as in Fig. 2C. **B.** Correlations between magnitudes of TAN responses to the cue and levels of timing performance, for each block of trials in which a TAN was recorded. The solid lines (in red) indicate the fit of a linear regression and dashed lines indicate the 95% confidence interval from the regression. *n*, number of neurons. *r*, Pearson correlation coefficient. **C.** Influence of the proximity to the time threshold for movement onset. Magnitudes of TAN responses to the cue were computed separately from trials within different ranges of MOT (three consecutive 250 ms periods starting from 250 ms before to 500 ms after the time threshold), * $P < 0.05$. **D.** Population activity of TANs for trials in different MOT ranges. Same conventions as in Fig. 2D, except that curves are smoothed with a Gaussian filter ($\alpha=0.04$).

173

174 We then asked whether the strength of TAN modulation is dependent on proximity to the time
 175 threshold. To examine this, we sorted the trials by their MOT value and computed the
 176 magnitude of the pause response to the cue in three ranges of values around the time criterion
 177 (Fig. 3C). We found that the pause was significantly higher for the trials with the lower
 178 MOTs than for the trials with the higher MOTs in monkey C. This indicates that TAN
 179 response magnitude to the cue was influenced by the ability of monkey C at estimating the
 180 elapsed time before movement onset, with TAN modulations being stronger for movements
 181 initiated just before the time threshold (i.e., underestimation errors). Such a relationship
 182 between TAN response and timing accuracy was not observed for monkey D. However, when
 183 comparing across different MOT ranges in monkey C, there was no visible difference in the
 184 response of the population of neurons (Fig. 3D), indicating that individual TANs can
 185 discriminate better between the short- and long-interval cues than the average activity from
 186 the whole sample of neurons.

187 In summary, we showed, in one of our monkeys, that the strength of TAN response to
 188 the cue seemed to reflect the animal's intention to move at a particular time, suggesting that

189 TANs are involved in signaling motor initiation in a time-dependent manner. The
190 interindividual difference in the sensitivity of TANs to timing performance emphasizes a
191 possible link with the monkey's strategy to determine the onset time of movements.

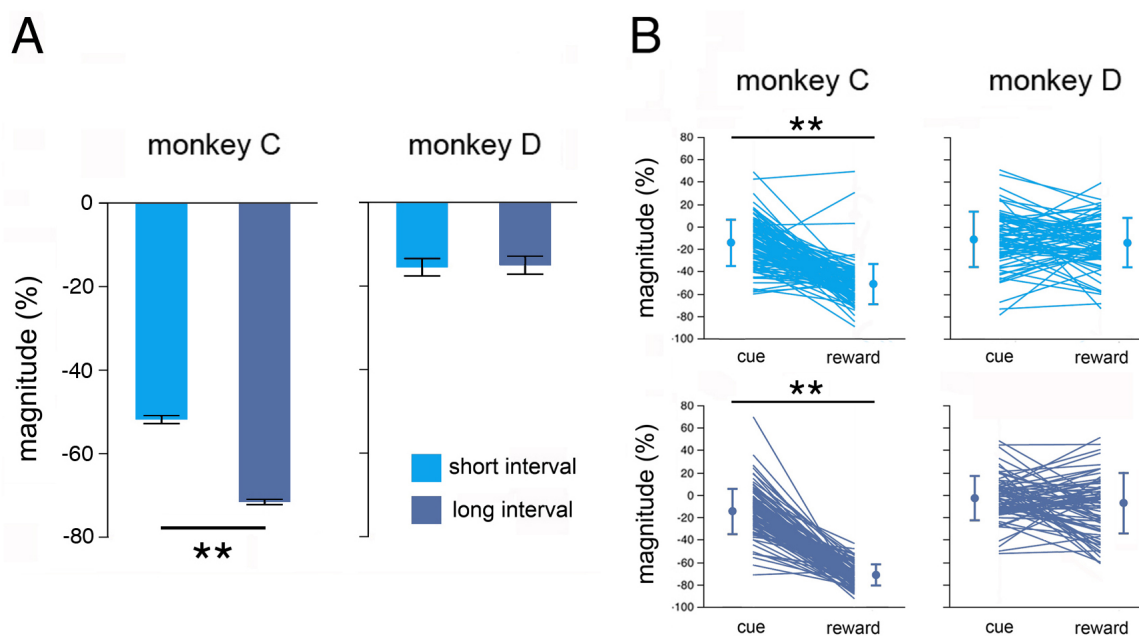
192

193 *Influence of reward prediction on TAN responsiveness*

194 In the task employed here, the cue not only contains temporal information, but also predicts
195 reward after a correctly timed movement. Because of the role TANs play in the detection of
196 reward-predictive events, we should expect that prediction of the upcoming reward
197 contributes to TAN sensitivity to the cue. Our behavioral data analysis showed that the
198 proportion of correctly timed movements was higher in monkey C (88.4%) than in monkey D
199 (80.3%), and this was paralleled by an increased TAN responsiveness to the cue in monkey C,
200 suggesting that the increased ability of this animal at producing correctly timed movements
201 favored predictable rewards and then enhanced the TAN responsiveness to the cue. To test
202 this suggestion indirectly, we examined the effect of the reward itself on TAN activity. It was
203 reasoned that a higher reward predictive value of the cue would lead to a decreased TAN
204 responsiveness to reward, consistent with the inverse relationship between reward prediction
205 and TAN responses to reward we have reported in previous studies (i.e., the more predictable
206 the reward, the less strongly TANs respond to it). We then turned to the effects of reward on
207 TAN activity using a similar time window analysis to that done for assessing the
208 responsiveness of TANs to the cue. We found statistically significant pause responses to
209 reward in 28 of 114 neurons (25%) and 31 of 86 neurons (36%) in monkeys C and D,
210 respectively, the TANs in monkey C showing a tendency to lower, albeit not significant,
211 responsiveness to reward than did TANs in monkey D ($\chi^2=3.10$, $df=1$, $p=0.07$). This
212 indicates that reward prediction could potentially be incorporated in TAN responses to the
213 timing cue in monkey C.

214 To further investigate sensitivity to reward for all TANs, we examined the magnitude
215 of changes in activity during specified time windows adjusted to latency and duration of
216 individual TAN responses to reward. As shown in Figure 4A, in monkey C, we found that
217 TAN responses to reward were stronger for the long interval, compared to the short interval
218 (Wilcoxon rank-sum test, $z=16.30$, $p < 0.0001$). This is in contrast with what we observed in
219 monkey D, in which the effect of the delivery of reward on TAN activity was relatively mild
220 and (did not differ) in magnitude between intervals (Wilcoxon rank-sum test, $z=0.21$,
221 $p=0.833$). In addition, when comparing across neurons the magnitudes of activity changes
222 following the cue and reward, we found an inverse relationship between cue and reward

223 responses in monkey C (i.e., TANs that responded weakly to the cue responded strongly to
224 reward) irrespective of interval duration (Fig. 4B) (Wilcoxon rank-sum test, $z=0.69$, $p=0.487$),
225 providing support to the idea that differences in the responsiveness of TANs to the timing cue
226 may partly result from differences in reward prediction. This effect was not present in
227 monkey D (Wilcoxon rank-sum test, $z=0.33$, $p=0.534$). The above results suggested that
228 changes in reward prediction, in at least one animal, could account for TAN sensitivity to the
229 timing cue we observed.
230



231

Figure 4. Sensitivity of TANs to reward. **A.** Boxplot representation of magnitude of changes in TAN activity after the delivery of reward separately for short and long intervals. Conventions are the same as in Fig. 2C, except that only rewarded trials were included. ** $P < 0.01$ (Wilcoxon signed-rank test). **B.** Changes in TAN activity after cue and reward separately for short and long intervals for each neuron recorded. Each line indicates the data of one neuron. Thick dots and error bars represent mean and SEM respectively. * $P < 0.05$, ** $P < 0.01$ (Wilcoxon signed-rank test).

232

233 In the TET, the movement was triggered by an internal decision process based on the
234 estimation of elapsed time from the cue onset and there may be a possibility, in the absence of
235 an external movement-triggering stimulus, that the movement itself serves as an event that
236 predicts reward. We then focused on the influence of the movement onset on TAN activity.
237 By analyzing TAN activity aligned on the initiation of movements in both monkeys, we
238 showed that TANs, as a population, did not display activity modulation around the onset of
239 movement, regardless of the interval duration (Figure 4-figure supplement 1).

240

241 *Influence of interval duration in the Pavlovian protocol*

242 To assess more specifically the possible contribution of reward prediction in modulating TAN
243 activity, we examined TAN responses when monkeys were engaged in a Pavlovian
244 conditioning task (PCT) in which visual cues solely conveyed differences in the time elapsed
245 until reward delivery, with no requirement for action. Such a condition allowed us to examine
246 the influence of elapsed time while reward was invariably delivered on every trial. By using
247 two time intervals in the same range as those in the TET and same relationships between cue
248 location and interval duration, we found that both monkeys showed anticipatory licking
249 movements prior to the delivery of reward indicating that the cue served as a predictor of
250 reward (Fig. 5A).

251 Among 90 TANs tested in the PCT, responses to the cue and reward were detected in
252 30 neurons (monkey C: 14/52, 27%; monkey D: 16/38, 42%) and 52 neurons (monkey C:
253 32/52, 62%; monkey D: 20/38, 53%), respectively. In contrast with what we observed in the
254 TET, the proportion of TANs responding to the cue did not vary significantly between the
255 two monkeys ($\chi^2 = 2.27$, $df = 1$, $p = 0.131$). Fig. 5B shows an example of a neuron sensitive
256 to the interval duration in the PCT. In a first block of trials (*usual cue-interval mapping*), the
257 neuron displayed a response to the cue which was stronger for long interval trials than for
258 short interval trials. Then, the same neuron was tested in another block of trials (*inverted cue-*
259 *interval mapping*) in which we reversed the assignments of the spatial cues for the short and
260 long interval. As can be seen, the response selectivity was maintained, suggesting that the
261 observed modulations in TAN activity did not appear to be related to the location of the cue.
262 This neuron also responded selectively to reward delivered at the end of the long interval in
263 both trial blocks. We examined the sensitivity to cue location in seven TANs by using
264 standard and inverted cue-interval mappings and found that magnitudes of response to the cue
265 did not depend on the spatial location of the cue (Figure 5-figure supplement 1).

266 As a next step, we undertook in the PCT a similar analysis to that done in the TET to
267 assess quantitatively the effects of interval duration on changes in TAN activity within time
268 windows determined for the cue and reward. As shown in Fig. 5C, the magnitude of TAN
269 response to the cue failed to show any significant difference between time intervals, whereas
270 a clear time-dependent modulation of reward response emerged in both monkeys, with
271 stronger responses to long intervals compared to short intervals. This finding is consistent
272 with results from our previous studies showing that a lack of prediction about the time of
273 reward delivery has an enhancing effect on the TAN response to this event (Sardo et al., 2000;
274 Ravel et al., 2001), which has been interpreted as reflecting a lowered monkey's time-keeping

275 ability with increased duration of the cue-reward interval. Contrary to what we observed in
 276 TET in monkey C, we did not find a clear relationship between cue and reward responses in
 277 PCT (Fig. 5D), with the exception of a weak, albeit significant, effect for the long interval
 278 (Wilcoxon rank-sum test, $z=2.29$, $p=0.021$). It therefore appears that interval duration exerts a
 279 more homogeneous effect on the activity of TANs in the PCT with the presence of a time-
 280 dependent effect on the reward response and the absence of relationship between cue and
 281 reward responses.
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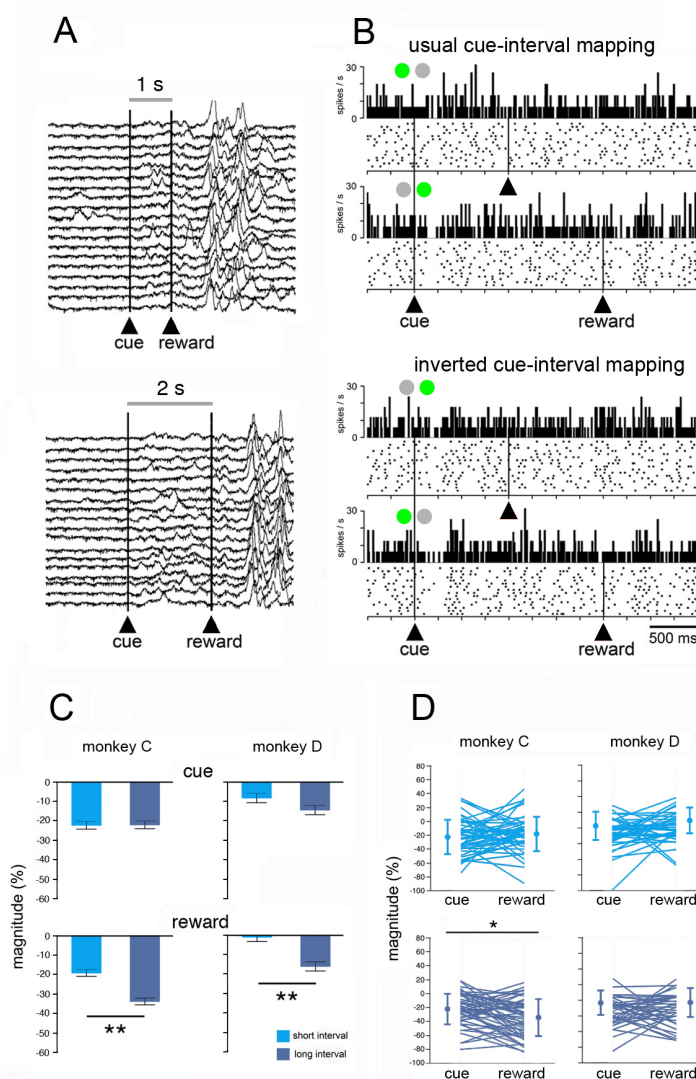


Figure 5. Effects of interval duration on TAN activity in the Pavlovian conditioning task (PCT). **A.** Licking movements after the onset of the reward predictive cue. For each interval duration are shown traces of lick records aligned on the cue onset. Licking started immediately after the presentation of the cue, indicating that animals have learned the reward predictive value of this signal. **B.** Raster plots of the activity of a single TAN recorded in the PCT. On this example, two blocks of trials were administered, corresponding to two cue-interval configurations. Rasters are separated by interval duration, with the upper two panels showing the TAN activity in the usual cue-interval configuration, and the lower two when the configuration has been inverted. The two circles at the top of each histogram indicate the left or right location of the cue (in green). Same conventions as in Fig. 2A. **C.** Magnitude of changes in TAN activity after cue and reward separately for short and long intervals. Same conventions as in Fig. 2C. **D.** Changes in TAN activity after cue and reward separately for short and long intervals for each neuron recorded. Same conventions as in Fig. 4B.

283

284

285

286 Lastly, we compared activity changes in a subset of 31 TANs recorded in both the TET and
 287 PCT. This comparison allowed us to differentiate the sensitivity of TANs to the timing cue
 288 when a similar underlying temporal representation in the seconds range was used either to

289 produce an overt estimate of elapsed time (TET) or to simply predict reward after a time
290 interval has elapsed (PCT). For some neurons, the strength of TAN modulation after the cue
291 onset was different when passing from the TET to the PCT, while for other neurons cue
292 responses remained constant in the two conditions. Examples of neurons responding to the
293 cue are shown in Figure 6. The first neuron (Fig. 6, left) showed a weak response to the cue in
294 the TET, while the cue response was more pronounced when the animal was shifted to the
295 PCT, indicating that the response may vary with respect to changes in the learning context. As
296 is apparent in the figure, the same neuron also responded to reward regardless of the
297 condition, this response being greatest for the long interval. The second neuron (Fig. 6, right)
298 showed a response to the cue that was clearly invariant to the condition. Although there was
299 no modulation of activity following reward delivery in the TET, a response to reward was
300 present only for the long-interval trials in the PCT. These findings indicate that some TANs
301 showed different degrees of modulation of their activity upon variation of the task context in
302 which timing cues were presented.
303

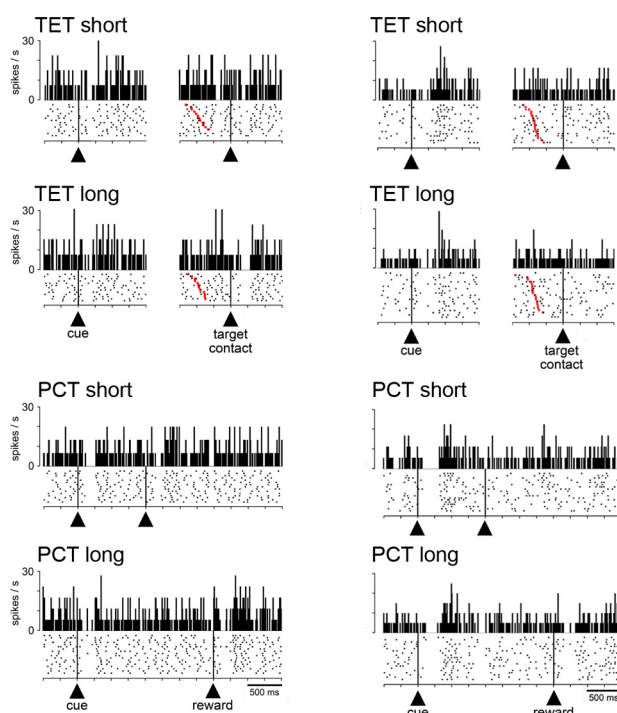


Figure 6. Influence of task condition on TAN responses to the cue and reward. Two example neurons tested in the two conditions. Rasters are separated by interval duration and task condition. Same conventions as in Fig. 2A, except that activity of each neuron is aligned separately to the cue onset and target contact in the TET. A few unrewarded trials are visible at the bottom of each raster aligned on reward delivery (absence of red marker) corresponding to monkey's responses before the criterion time. TET, time estimation task; PCT, pavlovian conditioning task.

304

305

306 *Sensitivity of TANs to the timing cue among striatal regions*

307 In primates, the posterior putamen is related to sensorimotor functions while dorsal and
308 ventral parts of the anterior striatum are involved in associative and limbic functions
309 respectively. In order to look for region-specific signals in TANs, the location of the recorded

310 neurons was identified histologically in both monkeys. As illustrated in Figure 7A, TANs
311 were mostly sampled from the dorsal parts of the precommissural and postcommissural
312 putamen and were distributed across the same regions of the nucleus explored in the two
313 animals. Only a small number of neurons (15 and 3 neurons in monkeys C and D,
314 respectively) were recorded in the ventral striatum (i.e., the ventral part of the precommissural
315 caudate nucleus and putamen). They were not assessed in detail because the sample size was
316 too small for statistical analysis. We then compared the sensitivity of TANs to the timing cue
317 in the two parts of the dorsal striatum (i.e., the motor and associative striatum). Responses to
318 the cue were found in 37 neurons of the associative striatum (monkey C: 23/53, 43%; monkey
319 D: 14/49, 29%) and 33 neurons of the motor striatum (monkey C: 22/46, 48%; monkey D:
320 11/34, 32%). Frequencies of cue responses did not vary significantly between the two regions
321 (monkey C: $\chi^2 = 0.195$, $df = 1$, $p = 0.658$; monkey D: $\chi^2 = 0.136$, $df = 1$, $p = 0.711$),
322 suggesting that response modulations related to the timing cue were not significantly different
323 for the two regions of the striatum. Data from the two monkeys were also analyzed separately
324 at the level of the population of TANs sampled in each dorsal striatal region, regardless of the
325 responsiveness of individual neurons (Fig. 7B). The population responses to the timing cue in
326 the motor and associative striatum were relatively weak in monkey D, compared with monkey
327 C, and they did not appear to differentiate short- and long-interval trials.
328

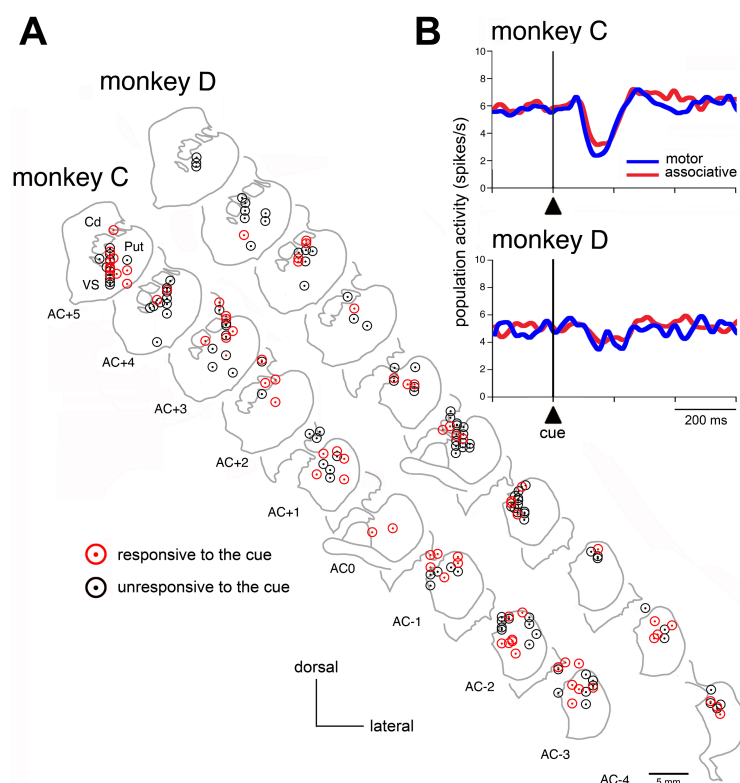


Figure 7. Sensitivity of TANs to the timing cue among striatal regions. **A.** Recording sites of TANs in the striatum of both monkeys. The location of all recorded TANs is plotted in the rostrocaudal direction on coronal sections from 5 mm anterior to 3-4 mm posterior to the anterior commissure (AC), with 1 mm intervals. Cd, caudate nucleus, Put, putamen, VS, ventral striatum. **B.** Comparison of population activities of TANs aligned on cue onset, grouped for short and long intervals, between motor and associative striatum. Same conventions as in Fig. 3D.

330 **Discussion**

331 Previous experimental studies and theoretical models assessing the role of the striatum in the
332 processing of temporal information have focused on the output neurons and did not outline
333 any contribution of local circuit neurons which are critical regulators of the striatal network
334 activity (Buhusi and Meck, 2005; Merchant et al., 2013). In previous work, we have
335 documented that TANs, presumed cholinergic interneurons in the striatum, display strongest
336 activity changes when rewarding stimuli are presented at unexpected times (Sardo et al.,
337 2000; Ravel et al., 2001), suggesting that the integration of temporal information interacts
338 with TAN signaling of reward-related events (Apicella et al., 2006). Here, we specifically
339 addressed the role of TANs in temporal processing by analyzing their activity in monkeys
340 performing self-paced movements based on time estimates in the range of seconds. Our
341 results indicate that the responsiveness of TANs to the timing cue was influenced not only by
342 temporal variables, but also by predictable rewards. This study provides the first account in
343 behaving monkeys of modulation of TAN activity related to the animal's ability to react in a
344 temporally controlled manner, suggesting that the intrinsic striatal cholinergic system may
345 contribute to timing function.

346

347 *TAN activity in relation to time estimation*

348 To perform the TET, our monkeys were required to retrieve temporal information from visual
349 cues, wait for a specified time interval, and then initiate a self-paced movement. Over 38 % of
350 the TANs we recorded were responsive to the cue that marked the beginning of the time
351 interval prior to movement initiation. Behavioral data confirm that the onset time of self-
352 paced movements became more variable as interval duration increased, reflecting the
353 increased variability in time estimates with the duration being timed, termed the scalar
354 property of interval timing (Gibbon, 1977). Although both monkeys were trained extensively
355 prior to recordings, our results revealed a difference in the degree of timing accuracy between
356 the two animals, suggesting that they did not perform the task in the same way. On average,
357 monkey D made more premature responses than monkey C and demonstrated an optimized
358 timing performance for short-interval trials in terms of proximity to the time threshold. On the
359 other hand, the timing strategy of monkey C did not change according to the interval duration,
360 achieving approximately similar levels of timing accuracy for both intervals. Interestingly,
361 these interindividual differences in timing performance were associated with differential
362 responsiveness of TANs to the timing cue. The neurons in monkey C showed higher
363 responsiveness to the timing cue (45%) than did neurons in monkey D (27%), suggesting that

364 the timing cue was better taken into account, regardless of interval duration, for the former
365 animal. Also, in monkey C, the strength of TAN modulation was similar for both short- and
366 long-interval trials, whereas, in monkey D, TAN activity showed selectivity for the short
367 interval compared to the long one, in terms of fraction of responsive neurons and the
368 magnitude of responses. This may reflect, in this latter animal, the development of a spatial
369 bias favoring the short-interval cue over the other. An intriguing result in monkey C was that
370 TAN responses to the timing cue were stronger in trials with movements initiated before
371 reaching the time threshold than in trials with correctly timed movements. We also found a
372 relationship between the overall magnitude of TAN responses to the cue and the accuracy of
373 timing (i.e., the stronger the neuronal modulation, the sooner was the self-timed movement).
374 It therefore appears, at least for this animal, that the TAN response to the cue was specifically
375 coupled to the onset time of the movement, providing support for the idea that TANs might
376 participate in the adjustment of timed movements.

377

378 *Contribution of TANs to the encoding of time*

379 The finding that TANs were responsive to the timing cue suggests a role for these presumed
380 interneurons in initiating the timing process at the beginning of the interval to be timed,
381 possibly through the shaping of sustained and/or sequential activity of striatal output neurons.
382 Indeed, several recording studies in behaving animals have reported changes in the activity of
383 output neurons of the striatum, at both single-neuron and population levels, that could convey
384 information about how much time has elapsed (Matell et al., 2003; Jin et al., 2009; Chiba et
385 al., 2015; Gouvêa et al., 2015; Mello et al., 2015; Bakhurin et al., 2016; 2017; Wang et al.,
386 2018; Zhou et al., 2020). Our work supports the notion that the TAN response to the cue
387 signals the detection of a stimulus associated with a learned time interval. This role for TANs
388 may be quite similar to that proposed for midbrain dopaminergic neurons in a theoretical
389 model of timing control, called the striatal beat frequency model, in which the dopaminergic
390 response is considered as crucial for initiating the timing process (Matell and Meck, 2000;
391 2004; Buhusi and Meck, 2005; Meck, 2006). It is possible, based on known interactions
392 between dopaminergic and cholinergic transmissions within the striatum (Threlfell and Cragg,
393 2011; Cachope et al., 2012; Threlfell et al., 2012; Cai and Ford, 2018), that signals arising
394 from these two neuromodulatory systems provide striatal circuitry with a starting signal to
395 keep track of elapsed time.

396 Apart from the characteristic brief firing rate changes observed at the level of
397 individual neurons, TANs are also known to exhibit synchronous activity during behavior

398 (Raz et al., 1996; Kimura et al., 2003; Morris et al., 2004) and one cannot exclude that
399 synchronized firing among TANs that was not detected by our analyses may also participate
400 in the encoding of time. Recent work using two-photon calcium imaging and population
401 recording with cell-type-specific labeling in behaving mice has reported modulations of the
402 cholinergic tone during spontaneous behavior which may impact striatal output dynamics
403 (Howe et al., 2019). It is therefore conceivable that other encoding mechanisms across the
404 cholinergic population may represent temporal information.

405 Our findings show that TANs responsive to the timing cue were distributed across the
406 anterior caudate and putamen, a region assumed to serve cognitive-related functions, and the
407 posterior putamen, which is important for motor control. Previous electrophysiological
408 studies have reported that output neurons of the striatum, which make up the vast majority of
409 neurons in this structure, convey temporal information both in motor and non-motor striatal
410 regions in rodents (Matell et al. 2003; Mello et al., 2015; Gouvêa et al. 2015; Bakhurin et al.,
411 2016; 2017; Zhou et al. 2020) and monkeys (Jin et al., 2009; Chiba et al., 2015; Wang et al.,
412 2018). Although these studies have focused on dorsal striatal activity, at least one of them has
413 reported that output neurons located in the ventral striatum, a region related to affective and
414 motivational states, are involved in encoding time (Bakhurin et al., 2017). In our study, the
415 responsiveness of TANs in the most ventral parts of the anterior striatum have not been
416 assessed in detail, because the data set was small, and further recordings from this region are
417 needed to draw conclusions about the potential contribution of ventral striatal TANs to
418 temporal processing.

419

420 *Reward prediction and timing processes*

421 In our task, the cue marks the beginning of the interval to be timed and indicates that reward
422 is delivered for responding after a specified duration has elapsed. It therefore remains unclear
423 whether TAN responses to the cue reflect time processing, as distinct from expectations about
424 reward. It is increasingly recognized that timing and motivation processes are concurrently
425 active during tasks used to elicit timing behavior in animals and humans (Fung et al., 2021).
426 For example, a link between time estimation and reward expectation has been demonstrated
427 for midbrain dopaminergic neurons in mice performing a temporal discrimination task
428 (Soares et al., 2016). In the present study, we have assessed the sensitivity of TANs to reward
429 prediction via TAN responses to the reward itself, since it is well established that fully
430 predicted reward elicits little or no TAN response relative to reward delivered in an
431 unpredictable way (Sardo et al., 2000; Ravel et al., 2001). As we found in monkey C, the

432 magnitude of the cue response was negatively correlated with the magnitude of reward
433 response across TANs, a stronger response to the cue being paralleled by a weaker response
434 to reward irrespective of interval duration. This supports the notion, at least in this animal,
435 that reward prediction could potentially be incorporated in TAN responses to the timing cue.
436 In contrast, such a correlation was not observed in monkey D indicating little or no
437 contribution of reward-predictive value of the cue, likely because this animal was less
438 successful in obtaining rewards in the timing task, compared to monkey C. It is also
439 noticeable that the overall magnitude of TAN responses to reward in monkey D was relatively
440 weak, perhaps reflecting a lower level of motivation to perform the task.

441 In an attempt to assess more specifically the influence of reward prediction on the
442 sensitivity of TANs to the timing cue, we used the PCT in which timing relies on expectation
443 of when reward will be delivered. In this condition, differences in the magnitude of the TAN
444 response to reward between short- and long-interval trials were consistent in both monkeys,
445 response magnitude being higher when the timing of reward became less precise with longer
446 intervals. Based on these findings, we suggest that TANs show a clear reward prediction
447 effect in the PCT. On the other hand, the magnitude of the TAN response to the cue did not
448 show a consistent relationship with the magnitude of the reward response, indicating that the
449 modulation of the response of TANs to the reward-predicting stimulus was less marked than
450 that following reward delivery, as previously observed in a study manipulating reward
451 probability during a Pavlovian task (Apicella et al., 2009).

452 Although a clear-cut dissociation between temporal and motivational variables is
453 difficult to make in the TET, our analysis of TAN responses to reward indicates that reward
454 prediction should be considered as a possible confounding effect. This is in line with studies
455 showing that the motivational state may affect time processing abilities (Gable and Poole,
456 2012; Avlar et al., 2015; Balci, 2014; Kirkpatrick, 2014; Daniels and Sanabria, 2017). The
457 few neuroimaging studies in humans that have attempted to dissociate reward and timing
458 processes, have reported activations in striatal and prefrontal cortical areas that may reflect
459 the integration of time perception and reward expectation (Tomasi et al., 2015; Apaydin et al.,
460 2018). In addition, theoretical models incorporating time as a crucial aspect of reward
461 processing also emphasize the importance of motivational variables on timing processes (Daw
462 et al., 2006; Galtress et al., 2012; Gershman et al., 2014; Petter et al., 2018; Mikhael and
463 Gershman, 2019). Further work using behavioral procedures that will allow independent
464 variation of temporal and reward parameters is required to clarify whether TANs participate
465 in time processing independently of reward prediction.

466 *Context detection and timing processes*

467 By focusing on a subset of TANs recorded in the TET and PCT, we sought to directly
468 compare the efficiency of specific behavioral contexts for eliciting TAN responses to a cue
469 which serves to either estimate a duration or simply predict the expected time of reward using
470 short and long intervals in the same range. Results suggest that some TANs can adapt their
471 responsiveness by taking into account the condition in which the timing cue is presented. This
472 differential sensitivity to the timing cue indicates that the encoded information is likely to
473 change with the particular learning context in which the cue was experienced, suggesting that
474 TANs do participate in temporal processing in a context-dependent manner. This is in
475 agreement with the notion that the local cholinergic circuitry of the striatum could play a role
476 in the recognition of a learned context for stimulus detection and action generation (Apicella,
477 2007; Stocco, 2012; Bradfield et al., 2013). Single neuron recordings in behaving animals
478 have reported in many circumstances an influence of context on TAN responses to salient
479 events (Shimo and Hikosaka, 2001; Yamada et al., 2004; Lee et al., 2006; Apicella et al.,
480 2011; Stalnaker et al., 2016).

481 Although dopaminergic neurons and cholinergic TANs are able to transmit signals that
482 convey temporal information (Martel and Apicella, 2021), their contribution to the processing
483 of time may not be equivalent. From an anatomical viewpoint, dopaminergic neurons send
484 information to different brain regions, including the striatum and frontal cortex, thus exerting
485 a widespread influence on multiple cortical and subcortical targets involved in the production
486 of reward-guided behaviors, whereas the influence of the TAN signal is confined within the
487 striatal circuitry. One can speculate that this local signaling system relates to contextual
488 information that may impact behavior. The fact that the TAN response is modulated by
489 multiple factors, including possible individual strategies for initiating self-timed movements,
490 is consistent with the notion that the information encoding capacity of the TAN system might
491 specify the context upon which timing is applied.

492

493 *Limitations of the study*

494 In the present study, monkeys produced either a short or a long time interval by making
495 spatially directed movements. An obvious limitation of this design is that the cue not only
496 provided a temporal reference for self-initiated movements, but also contained information
497 about which action to select, each time interval being associated with a movement to a right
498 or left target. Therefore, we cannot rule out an influence of the visuospatial nature of the
499 timing task on the TAN responses to the cue we observed. Although previous studies have

500 shown that TANs may display changes in activity in response to visuospatial aspects of task
501 performance (Kimura, 1992; Shimo and Hikosaka, 2001; Ravel et al., 2006), it is generally
502 acknowledged that TAN activity does not have a consistent relationship with the production
503 of a specific action (but see Lee et al., 2006 for the observation of TAN modulations related
504 to self-paced movements). In addition, TANs have been proposed to drive attentional shifts
505 required for stimulus detection (Matsumoto et al., 2001; Ding et al., 2010; Schulz and
506 Reynolds, 2013), suggesting that differences in the monkey's allocation of attention across
507 the two cue locations may bring about differences in the TAN response. However, in the
508 Pavlovian protocol, we reversed the relationship between the location of the timing cue and
509 the interval duration and did not find compelling evidence that a spatial effect may account
510 for observed changes in TAN activity. Additional work is needed to disambiguate possible
511 effects related to time processing from those related to visuospatial attentional demands.

512 To conclude, our results support a view that TANs appear to act like detectors of
513 stimuli relevant for initiating a timing process in the seconds range, providing evidence that
514 the information encoding capacity of TANs extends to cognitive functions, including the
515 representation of time. Although identifying the involvement of TANs in timing behavior
516 remains challenging due to the difficulty to distinguish signals specific to temporal processing
517 from those related to other confounding processes, changes in TAN activity we observed
518 likely reflect a combination of time perception and reward prediction. An important question
519 for future studies will be to clarify how the coordination between the TAN system and striatal
520 output pathways, under the control of dopamine, participates in striatal timing function. Until
521 now, there is no experimental evidence from inactivation studies in animals that a selective
522 alteration in intrinsic striatal cholinergic innervation disrupts timing behavior. However,
523 human studies have pointed to an impaired temporal control of motor behavior in patients
524 with Tourette syndrome (Vicario et al., 2010; Graziola et al., 2020; Schuller et al., 2021) in
525 which a loss of striatal cholinergic interneurons has been reported (Kataoka et al., 2010).
526 There is also strong evidence that striatal dopamine-depleted states are associated with
527 disturbances in timing behavior in both animals and humans (Buhusi and Meck, 2005;
528 Merchant et al., 2013), and it is conceivable that impaired local interactions between
529 dopaminergic and cholinergic transmissions in the striatum are central to the expression of
530 time processing abnormalities. Future work will be necessary to fully understand the
531 integration of cholinergic TANs and dopaminergic input into striatal circuits underlying the
532 encoding of time.

533 **Materials and methods**

534 *Animals and recording procedures*

535 Experiments were conducted on two adult male rhesus monkeys (*Macaca mulatta*), C and D,
536 weighing 11 and 7 kg, respectively, kept in pairs in their home cage. This study was
537 performed in accordance with the principles of the European Union Directive 2010/63/EU on
538 the protection of animals used for scientific purposes and all procedures were approved by the
539 Ethics Committee of the Institut de Neurosciences de la Timone (protocol #3057-
540 2015120809435586). Prior to the experiments, each monkey was implanted with a head-
541 holding device and recording chamber (25×35 mm) under general gas anesthesia (sevoflurane
542 2.5%) and aseptic conditions. The center of the chamber was stereotaxically directed to the
543 anterior commissure (AC) based on the atlas of Paxinos et al. (1988). The animals received
544 antibiotics and analgesics for a period of 5 days after the surgery.

545 Recordings were obtained with glass-coated tungsten electrodes (impedance: 2–3 MΩ)
546 passed inside a guide tube (0.6 mm outer diameter) and lowered to the striatum with a manual
547 hydraulic microdrive (MO-95; Narishige, Tokyo, Japan). Electrode tracks were made
548 vertically into regions of the striatum anterior and posterior to the AC, mostly in the putamen.
549 Few penetrations were continued through the precommissural caudate nucleus and putamen to
550 reach the ventral striatum.

551 Neuronal activity was amplified (x5000), bandpass-filtered (0.3–1.5 kHz), and spike
552 sorting was performed on-line using a window discriminator (NeuroLog; Digitimer,
553 Hertfordshire, UK). Continuous monitoring of the spike waveform on a digital oscilloscope
554 allowed us to check the isolation quality of the recorded neurons. Recordings took place in
555 the left striatum and all reach movements were made with the arm contralateral to the
556 recorded hemisphere. In line with many previous single-neuron recording studies in the
557 striatum of behaving monkeys, TANs were electrophysiologically identified by waveform
558 shape, firing rate, and response to motivationally salient stimuli consisting of a transient
559 decrease in activity (*pause*). A computer controlled the behavioral task and data acquisition
560 using a custom-made software developed by E. Legallet under LabVIEW (National
561 Instruments).

562

563 *Behavioral task*

564 Training and recording sessions took place in a setup similar to that described previously
565 (Marche and Apicella, 2017). Monkeys were seated in a restraining box facing a vertical
566 panel containing two metal knobs (diameter, 10 mm) serving as movement targets, positioned

567 18 cm apart at the animal's eye level, and two light-emitting diodes (LEDs), one above each
568 target. A resting bar was mounted in the lower part of the panel and a tube was positioned
569 directly in front of the monkey's mouth for dispensing drops of fruit juice (0.3 ml) as a
570 reward. Animals were trained in a *time estimation task* (TET) in which one of the two LEDs
571 was lit as a cue indicating both the target of movement and the minimum waiting period
572 before initiating a reaching movement, the left and right locations being associated with *short*
573 and *long* intervals, respectively. A schematic representation of the sequence of events in the
574 TET is given in Figure 1A. The trial began when the monkey kept its hand on the bar. After 1
575 s, a LED was illuminated for 0.5 s. This cue marked the beginning of the to-be-timed interval,
576 referred to as the time threshold, and its left or right location indicated that the minimum
577 waiting period before movement onset was short or long, respectively (monkey D: 1.0 and 2.0
578 s; monkey C: 1.3 and 2.3 s from cue onset). The onset of movement after the minimum
579 waiting period has elapsed was limited to 2 s. The cue location was pseudorandomly selected
580 from trial to trial. Trials lasted 6 s, so that the overall temporal structure of the task remained
581 the same. Because of this fixed trial duration, the monkey had no possibility to predict the
582 timing of the cue accurately while keeping the hand on the bar.

583 Only monkeys' reaching movements made after the specified time interval had
584 elapsed were rewarded. If the monkey released the bar before reaching the time criterion or
585 touched the target in more than 1 s after bar release, it was not rewarded. In these cases, the
586 trial continued until the end of its total duration, and the same trial was repeated until a
587 rewarded movement was successfully completed. Trials on which animals did not react after
588 cue onset or did not touch a target after bar release were excluded from the analyses. Both
589 monkeys received training on the TET until they reached a stable level of performance
590 corresponding to a criterion of at least 80% correctly performed trials.

591 We also tested the effects of intervals in the seconds range in a Pavlovian conditioning
592 task (PCT) that excludes possible confounding factors that were linked to target reaching. In
593 this condition, access to the working panel was prevented by closing the sliding door at the
594 front of the restraining box and monkeys remained motionless with their arms relaxed in a
595 natural position. Animals were then exposed to a visual stimulus (red light, 0.5 s duration),
596 presented either on the left or right side, whose onset was initiated by the experimenter. This
597 cue was followed by the delivery of reward after a fixed interval of 1.0 or 2.0 s, depending
598 upon stimulus location. The PCT matched the TET in terms of location-interval combinations
599 (left/short and right/long) and total trial duration (6 s). During PCT sessions, the licking
600 movements of the monkeys were monitored using force transducers (strain gauges) attached

601 to the tube delivering liquid. Signals from the strain gauge device were digitized at 500 Hz
602 and stored into an analog file. In addition, we sometimes reversed the cue-interval
603 associations of the PCT. By comparing TAN activity before and after reversal, we assessed
604 whether activity changes following the cue onset were related to the ensuing interval duration
605 independent of the cue location.

606 *Data analysis*

607 Performance in the TET was assessed by measuring the time between the onset of the cue and
608 bar release (movement onset time, MOT), for each time-interval combination. We examined
609 MOT distributions to determine whether the spread of these distributions is proportional to
610 the length of the interval being estimated, as reflected by the ratio between the dispersion and
611 the mean of MOT (coefficient of variation, CV).

612 Neurons were determined to be task-responsive according to methods similar to those
613 described previously (Marche et al., 2017). Briefly, a “sliding window” analysis was used to
614 quantify the time course of changes in TAN activity after a specific task event. A test window
615 of 100 ms duration was advanced in 10 ms increments across the trial, starting at cue onset,
616 movement onset, or target contact. The average spike counts in the test window was
617 compared, at each step, with that calculated during the 500 ms immediately preceding the
618 onset of the cue (control period). The onset of a modulation was taken to be the beginning of
619 the first of at least five consecutive steps showing a significant difference (Dunn's test for
620 multiple nonparametric comparisons, $p < 0.01$) as against the activity in the control period.

621 Quantitative analysis of spike rates was also performed for windows with fixed
622 durations that were specifically determined for each monkey on the basis of the analysis of
623 latency and duration of statistically significant decreases in activity (i.e., pauses) detected
624 with the sliding window procedure. We set these time windows such that they included most
625 of the pause onset and offset times. According to this analysis, time windows after the cue
626 onset were 93-309 ms for both short- and long-interval trials, for monkey C, and 127-313 ms
627 and 211-418 ms for short- and long-interval trials respectively, for monkey D. The windows
628 determined by the average onset and offset times of pause responses to reward were 139-369
629 ms and 194–391 ms for both short- and long-interval trials, for monkey C and D, respectively.
630 We then rated the magnitude of activity changes in each time window for each neuron and
631 each time interval, irrespective of whether neurons were individually responsive or not. We
632 set quantification windows in the same way for assessing the magnitude of TAN activity after
633 the stimulus and reward in the PCT. The percentages of TANs showing pause responses in

634 relation to the total number of neurons tested were calculated for each condition and
635 differences in proportions of responding neurons among conditions were statistically assessed
636 with the χ^2 test. Differences in the magnitude of changes in activity were assessed with a
637 Wilcoxon rank-sum test.

638 *Recording sites*

639 The location of individual recorded neurons was confirmed histologically in both monkeys.
640 Near the end of the experiments, small electrolytic lesions (20 μ A for 30 s, cathodal current)
641 were made at several points along selected electrode tracks. The monkey was deeply
642 anesthetized with pentobarbital and perfused through the heart with 0.9% saline followed by
643 4% paraformaldehyde (pH 7.4 phosphate buffer). Frozen sections (40- μ m thick) were made in
644 the frontal plane and stained with cresyl violet. We then reconstructed the location of each
645 recorded neuron according to the depth and coordinates of electrode penetrations based on the
646 retrieved sites of marking lesions. In line with previous work, we used the AC as a structural
647 boundary separating the striatum into motor and associative parts. Based on the reconstruction
648 of recording sites, TANs were sampled between 5 mm anterior and 4 mm posterior to the AC,
649 mainly over the medio-lateral extent of the putamen. Only a few penetrations were made in
650 the most ventral part of the precommissural striatum.

651

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654 MATLAB programming, and Dr. M. Esclapez for help with histology. We also thank animal
655 care personnel of the Mediterranean Primate Research Center.

656

657 *Data availability statement*

658 All datasets generated for this study will be made available upon publication. To make our
659 data openly accessible, we will consider sharing source data files and codes by using a public
660 open repository.

661

662 *Competing interests*

663 The authors declare no competing interests.

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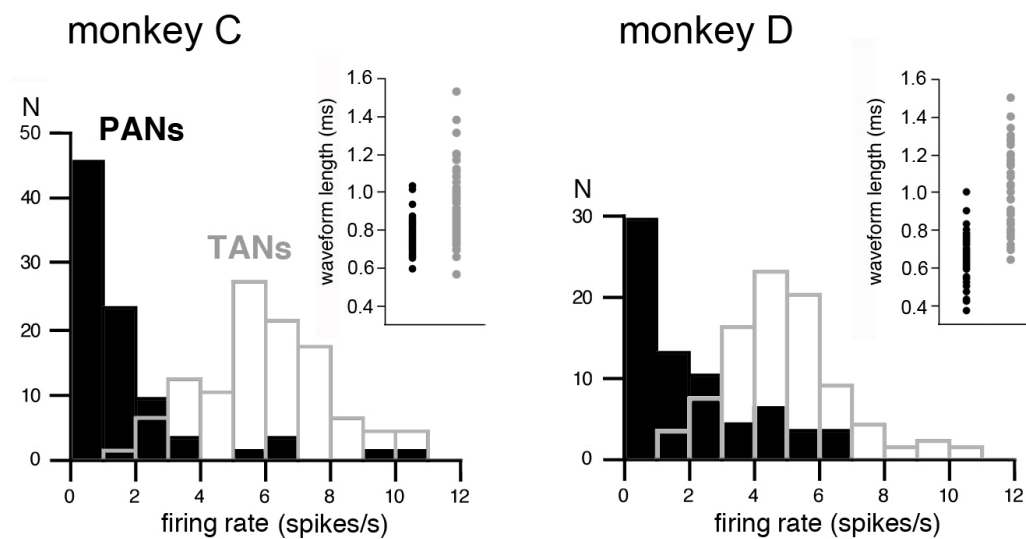
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972 **Figure 2-figure supplement 1**
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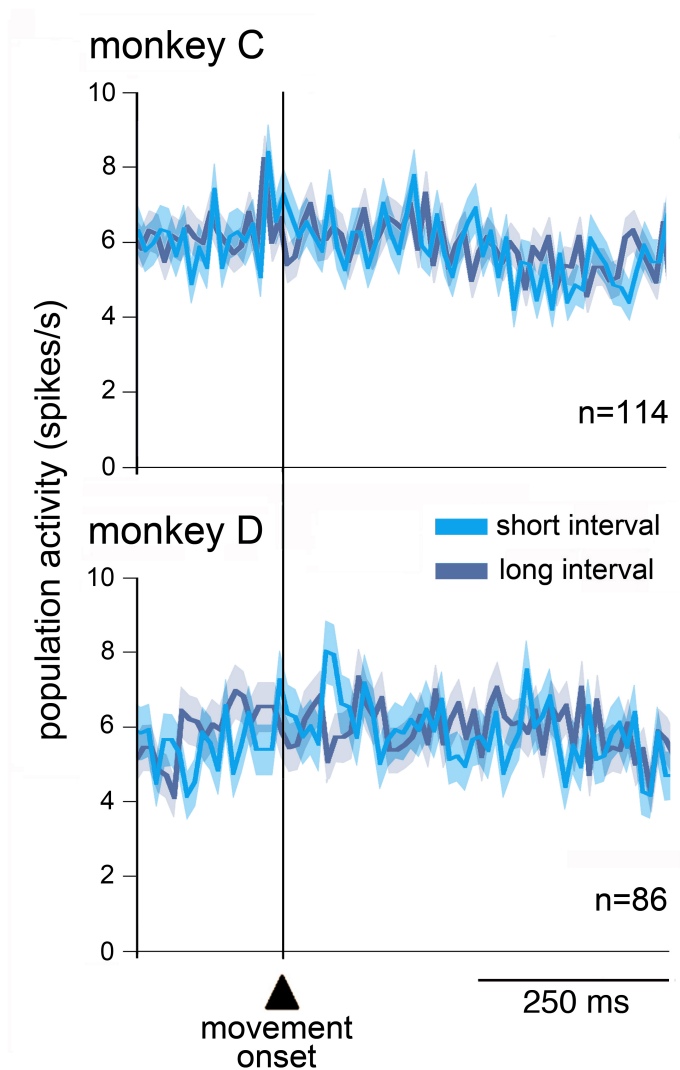
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976 **Electrophysiological characterization of TANs.**

977 Tonically active neurons (TANs), presumed cholinergic interneurons in the striatum, were
978 identified based on previously established electrophysiological criteria, i.e., spontaneous
979 firing rate and spike waveform width. Consistent with previous studies, the TANs had a mean
980 baseline activity which is higher than that of the phasically active neurons (PANs), which
981 correspond to striatal output neurons (Monkey C: TANs: n=103, 5.94 ± 1.94 spikes/s; PANs:
982 n=86, 1.47 ± 1.91 spikes/s; Monkey D: TANs: n=86, 4.82 ± 1.72 spikes/s; PANs: n=68,
983 1.83 ± 1.92 spikes/s). Also, the mean spike waveform width of TANs was larger than that of the
984 PANs (Monkey C: TANs: n=109, 0.90 ± 0.17 ms; PANs: n=104, 0.76 ± 0.08 ms; Monkey D:
985 TANs: n=85, 1.08 ± 0.22 ms; PANs: n=68, 0.65 ± 0.11 ms). In addition, the stereotyped
986 *pause* in activity in response to motivationally salient stimuli is a reliable indicator of TAN
987 identity which clearly distinguishes them from PANs. We excluded from analysis a relatively
988 small sample of the so-called fast spiking interneurons, presumed striatal GABAergic
989 interneurons, characterized by having a higher firing frequency than TANs and a narrower
990 spike waveform than PANs. Spontaneous activity was measured as the mean firing rate
991 during the 500 ms before the onset of the timing cue. N indicates the number of neurons.
992 Insets: distributions of average spike lengths. Each dot represents data from an individual
993 neuron. The spike length of each neuron was defined as the time interval between the first
994 negative and second positive peaks of the waveform.

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1007 **Figure 4-figure supplement 1**
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1011 **Lack of changes in TAN activity around self-timed movements.**

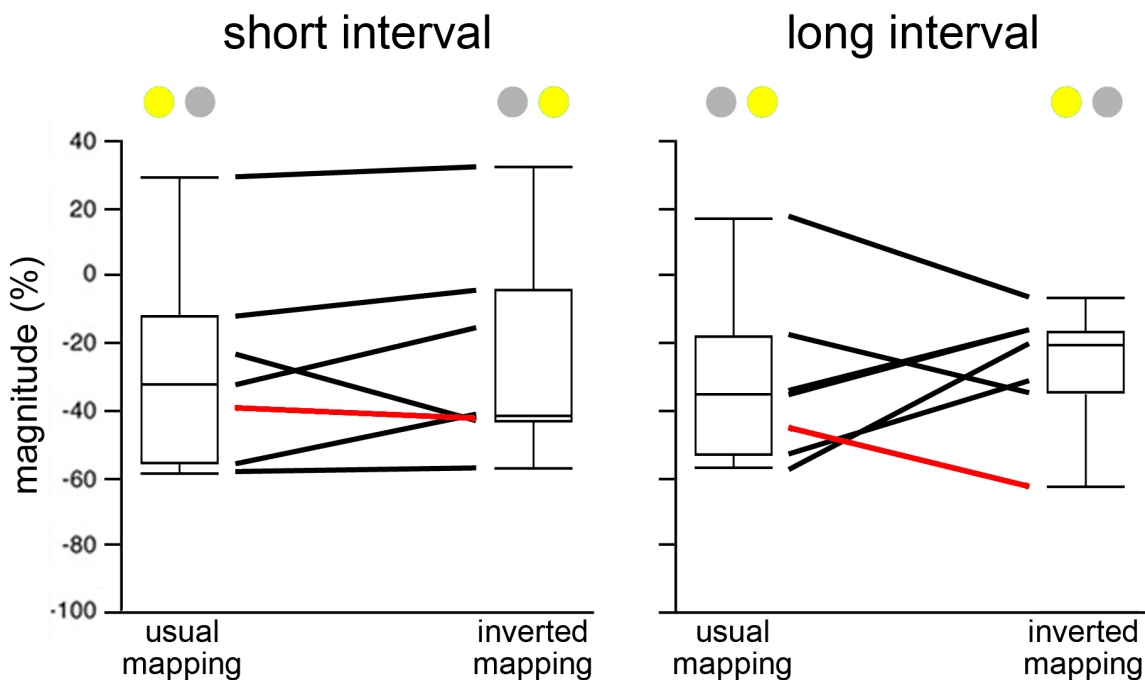
1012 Population activity of TANs aligned on the onset of movements marked by the vertical line.
1013 At a population level, TANs did not exhibit clear activity modulation around the movement
1014 onset, regardless of the time interval. Same conventions as in Fig. 2D.

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1029 **Figure 5-figure supplement 1**

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1034 **Influence of changing the cue-interval mapping in the Pavlovian protocol.**

1035 Box plots of the magnitude of changes in TAN activity after the onset of the cue, separately
1036 for short and long intervals. Boxes indicate 25–75 percentile ranges of the distributions, lines
1037 through boxes indicate medians and vertical lines indicate tails of distributions. Red lines
1038 correspond to the example TAN illustrated in Fig. 5B. We recorded a sample of seven TANs
1039 in the PCT to test whether TAN responses to the cue are attributable to the time interval or the
1040 location of the cue. After carrying out a block of trials with the usual assignment of cues for
1041 the two time intervals (usual mapping), we reversed the assignments of the spatial cues for the
1042 short and long interval (inverted mapping). The activity across this sample of TANs was not
1043 altered when the cue-interval mapping was reversed, suggesting that it was not influenced by
1044 the location of the cue.

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