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1 **Dorsal striatum and the temporal expectancy of an aversive event in Pavlovian** 2 **odor fear learning**

3

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16

17 **Abstract**

18 Interval timing, the ability to encode and retrieve the memory of intervals from seconds to minutes,
19 guides fundamental animal behaviors across the phylogenetic tree. In Pavlovian fear conditioning,
20 an initially neutral stimulus (conditioned stimulus, CS) predicts the arrival of an aversive
21 unconditioned stimulus (US, generally a mild foot-shock) at a fixed time interval. Although some
22 studies showed that temporal relations between CS and US events are learned from the outset
23 of conditioning, the question of the memory of time and its underlying neural network in fear
24 conditioning is still poorly understood. The aim of the present study was to investigate the role of
25 the dorsal striatum in timing intervals in odor fear conditioning in male rats. To assess the animal's
26 interval timing ability in this paradigm, we used the respiratory frequency. This enabled us to
27 detect the emergence of temporal patterns related to the odor-shock time interval from the early
28 stage of learning, confirming that rats are able to encode the odor-shock time interval after few
29 training trials. We carried out reversible inactivation of the dorsal striatum before the acquisition
30 session and before a shift in the learned time interval, and measured the effects of this treatment
31 on the temporal pattern of the respiratory rate. In addition, using intracerebral microdialysis, we
32 monitored extracellular dopamine level in the dorsal striatum throughout odor-shock conditioning
33 and in response to a shift of the odor-shock time interval. Contrary to our initial predictions based

34 on the existing literature on interval timing, we found **evidence suggesting** that transient
35 inactivation of the dorsal striatum **may** favor a more precocious buildup of the respiratory
36 frequency's temporal pattern during the odor-shock interval in a manner that reflected the duration
37 of the interval. **Our data** further **suggest** that the conditioning and the learning of a novel time
38 interval were associated with a decrease in dopamine level in the dorsal striatum, but not in the
39 nucleus accumbens. These findings prompt **a reassessment of** the role of the striatum and striatal
40 dopamine in interval timing, at least when considering Pavlovian aversive conditioning.

41

42 **Keywords:** Interval timing, odor fear conditioning, respiration, dorsal striatum, dopamine
43 microdialysis.

44 Introduction

45 Learning time intervals is crucial to survival and goal reaching across the phylogenetic tree. In
46 Pavlovian fear conditioning, an initially neutral stimulus predicts the arrival of an aversive
47 unconditioned stimulus, after a time interval that is encoded, a process pertaining to interval timing
48 (Molet & Miller, 2014; Kirkpatrick & Balsam, 2016). How the brain processes and encodes such
49 information remains poorly understood (Merchant et al., 2013; Tallot & Doyère, 2020). From a
50 neurobiological point of view, there is substantial support for the involvement of the dorsal striatum
51 and its dopaminergic inputs in interval timing (Buhusi & Meck, 2005), both from lesion experiments
52 of the dorsal striatum or intrastriatal infusion of dopaminergic antagonists (De Corte et al., 2019;
53 Meck, 2006), and from lesion or optogenetic manipulation of the substantia nigra pars compacta
54 (SNc), the primary source of dorsostriatal dopamine (Meck, 2006; Soares et al., 2016).
55 Electrophysiological recordings of single neurons in the dorsal striatum of rats also show that
56 striatal neurons firing rate is correlated with the duration of the time interval between signaling
57 cue and reward (Bakhurin et al., 2017; Gouvêa et al., 2015; Matell et al., 2003; Mello et al., 2015),
58 and intrastriatal muscimol infusions produce an impairment in the animals' ability to discriminate
59 durations (Gouvêa et al., 2015). In the context of fear conditioning, using 2-Deoxyglucose (2-DG)
60 metabolic mapping we previously showed that odor-shock pairing in rats was associated with an
61 increase in 2-DG uptake in the dorsal striatum (Boulanger Bertolus et al., 2014). Furthermore,
62 recent work recording oscillatory neural activity in the dorsal striatum in Pavlovian fear
63 conditioning correlated its maximum power in theta and gamma bands with the time at which the
64 rat expected the aversive stimulus (Dallérac et al., 2017). Timing in fear conditioning is also
65 associated with plasticity in the striatum (Dallérac et al., 2017).

66 Notably, a majority of studies investigating the neurobiological substrate of interval timing in
67 rodent models rely on operant conditioning. Such protocol relies heavily on the motor response
68 of the subject, which could bias our understanding of the neural substrate of interval timing *per*
69 *se*. Indeed, the striatum and cortico-striatal inputs are also a neural substrate for motor and
70 procedural learning (Barnes et al., 2005; Koralek et al., 2013; Martiros et al., 2018), and action
71 selection when the task involves temporal discrimination (Howard et al., 2017). To avoid these
72 possibly confounding factors, we used a **non-striatum-dependent** behavioral measure, the
73 respiratory frequency, to assess the animal's interval timing ability in a Pavlovian fear conditioning
74 associating an odor to a mild footshock. Indeed, the pattern of respiratory frequency has been
75 shown to be a good index of the animal's temporal expectation of the shock arrival (Boulanger
76 Bertolus et al., 2014; Dupin et al., 2020; Shionoya et al., 2013). This allowed us to look more

77 closely at the role of dorsal striatum and its dopamine level in the initial acquisition of an interval
78 duration, as well as when a change in this duration is applied. Based on the existing literature, we
79 made two hypotheses 1) inactivating the dorsal striatum should impair timing behavior and its
80 adaptation to a new interval duration and 2) dopamine level in the dorsal striatum should increase
81 when a new interval duration is introduced. We report that, while a transient inactivation of the
82 striatum did alter the expression of the temporal pattern of respiration, and dopamine level in the
83 striatum was indeed modulated during the learning of a new duration, the directions of the effects
84 were the opposite of those hypothesized. These findings prompt a reconsideration of the role of
85 the striatum and striatal dopamine in interval timing, at least when considering Pavlovian aversive
86 conditioning.

87

88 **Methods**

89 **Animals**

90 Twenty-five pair-housed and fifteen single-housed male Long Evans rats (Janvier, France)
91 contributed data for experiment 1 and 2 respectively. They weighed 250-300 g at the start of the
92 experimentation, were housed at 23°C under a 12h light–dark cycle, and food and water were
93 available *ad libitum*. All experiments and surgical procedures were conducted in strict accordance
94 with the 2010/63/EU Council Directive Decree and the French National Committee (87/848) for
95 care and use of laboratory animals. The experiments were carried out under the approval of
96 Direction of Veterinary Service (#69000692), and care was taken at all stages to minimize stress
97 and discomfort to the animals.

98 **Surgery**

99 Details of the procedures can be found in the supplementary methods. Briefly, animals were
100 anesthetized, received subcutaneous local analgesia and were placed in a stereotaxic frame. For
101 experiment 1, rats were implanted bilaterally in the dorsal striatum with stainless steel guide
102 cannulae. For experiment 2, rats were implanted unilaterally (left side) with a guide cannula
103 targeting the dorsal striatum. To assess the specific involvement of the dopaminergic system
104 innervating the dorsal striatum in this task, control rats were implanted in the nucleus accumbens
105 which receives abundant dopaminergic inputs, albeit from another source, the ventral tegmental
106 area (VTA). All animals were allowed two weeks of post-surgical recovery. **During this period, the
107 animals' behavioral state was daily monitored and scored, and if any sign of suffering was
108 detected, the animal was injected with 2-4mg/kg carprofen i.p.**

109 **Experimental apparatus and paradigm**

110 The experimental cages (described in previous studies (Hegoburu et al., 2009, 2011) and
 111 detailed in the supplementary methods) consist of a whole-body plethysmograph for Experiment
 112 1 and Plexiglass cylinder for Experiment 2, both customized with tubing in the ceiling connected
 113 to a programmable custom olfactometer, and a shock floor. In this setup, rats underwent odor
 114 fear conditioning.

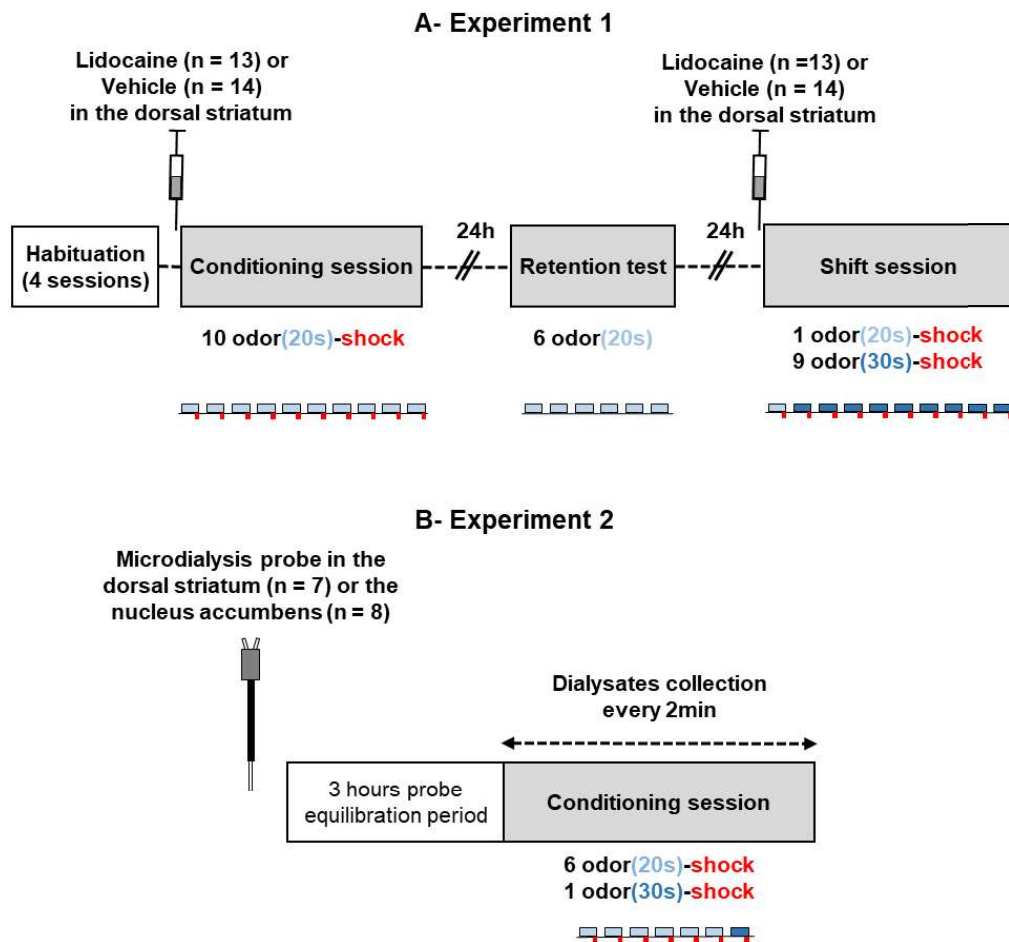


Figure 1: A) Training protocol for Experiment 1. After a period of habituation to the experimental setup for four 20-min sessions, animals were injected with either lidocaine or vehicle in the dorsal striatum, and immediately trained with 10 Odor-Shock pairings using a 20-s odor-shock interval (Conditioning session). Twenty-four hours later, they received 6 presentations of the odor alone to assess their learned fear to the odor (Retention test). Forty-eight hours after conditioning, they were re-injected with the same drug they received previously, and trained in a Shift session where they received 1 odor-shock pairing with a 20-s odor-shock interval followed by 9 odor-shock pairings with a 30-s interval. B) Training protocol for Experiment 2. The microdialysis probe was inserted in the left dorsal striatum or left nucleus accumbens and the animals were placed in the experimental cage for a 3h probe equilibration period after which they were trained with 6 Odor-Shock pairings with a 20-s interval, followed with a single Odor-Shock pairing with a 30-s interval.

116 For Experiment 1 (Figure 1A), after familiarization to the conditioning cage, the animals
117 received a Conditioning session (ten odor-shock pairings with a 20s odor-shock interval), a
118 Retention test (six odor presentations) and a Shift session (one 20-s odor-shock pairing, followed
119 by nine 30-s odor-shock pairings) at 24h intervals. They were injected with 0.5 μ L of either
120 lidocaine (2%, Sigma-Aldrich France, in sterile saline 0.9%, Lidocaine group, n = 13) or saline
121 (Control group, n = 14) just before the Conditioning and Shift sessions. During each session, the
122 animal's behavior and respiratory rate were continuously recorded for offline analysis.

123 For Experiment 2 (Figure 1B), after a 3-hour probe equilibration period, rats received a
124 conditioning session including six 20-s odor-shock pairings, followed by one 30-s odor-shock
125 pairing. Dialysates from the dorsal striatum (n = 7) and the nucleus accumbens (n = 8) were
126 collected every 2 min.

127 At the end of the experiment, the animals were sacrificed with a lethal dose of pentobarbital
128 for histological verification of the canulae tips localization (Figure S1 and S2 for Experiment 1
129 and 2 respectively).

130 **Data acquisition, pre-processing and analysis**

131 In Experiment 1, the respiratory signal and behavior were analyzed as described before
132 (Boulanger Bertolus et al., 2014). We assessed the effects of treatment on the temporal dynamics
133 of the respiratory frequency from the odor onset to shock arrival. For this analysis, the time course
134 of the respiratory frequency, in 1-s time bins, during the 19 seconds of the odor-shock interval
135 was compared using a two-way ANOVA with Group (Lidocaine or Control) as an independent
136 factor, and Time (1 to 19 seconds) as a repeated measure factor. During the Retention test, the
137 freezing rate was analyzed using a two-way ANOVA with Group as an independent factor, and
138 Period (Pre-Odor vs Odor) as a repeated measure factor. For the Shift session, a three-way
139 ANOVA was performed with Group as an independent factor, and Time (1 to 19 seconds) and
140 Interval (20s vs 30s) as repeated measures factors.

141 In Experiment 2, microdialysis data were acquired as previously described to accurately
142 correlate the neurochemical data with the behavioral events (Parrot et al., 2004; Hegoburu et al.,
143 2009; Hegoburu, Denoroy, et al., 2014), using homemade concentric microdialysis probes (see
144 supplementary methods) continuously infused with artificial cerebrospinal fluid (aCSF). Dialysates
145 were collected in PCR tubes rinsed with an acidic preservative medium, and stored at -30°C until
146 analysis using ultra-high-performance liquid chromatography (see supplementary methods).

147 Data were expressed as percentage of the baseline obtained by averaging the dopamine
148 concentrations measured in the seven samples collected before the start of the conditioning.
149 Changes in dopamine concentration were then analyzed using a two-way ANOVA with Structure

150 (dorsal striatum or nucleus accumbens) as an independent factor, and Time as a repeated
151 measure factor.

152 All ANOVA results are reported in the legend of the corresponding figures. For all statistical
153 comparisons performed, post-hoc pairwise comparisons were carried out when allowed by the
154 ANOVA results, the significance level being set at 0.05.

155

156 Results

157 **Experiment 1: Reversible inactivation of the dorsal striatum during the shift** 158 **session hastens the adaptation of the respiratory temporal curve to the new** 159 **interval duration**

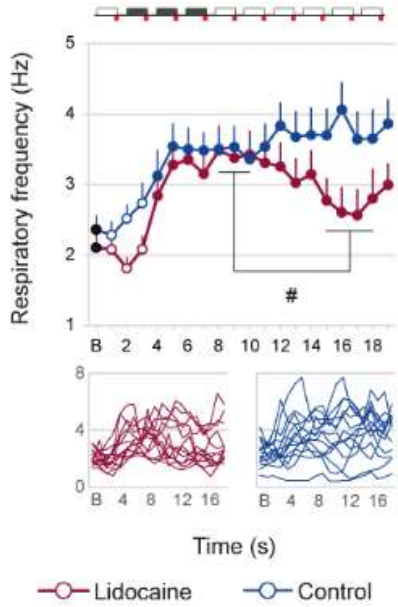
160 We previously showed that adult rats submitted to an odor fear conditioning exhibit a typical
161 temporal respiratory frequency pattern (using 1-s time bins) during the odor-shock interval,
162 consisting in a rapid respiratory frequency increase upon odor delivery and a U-shaped decrease
163 just before shock arrival (Boulanger Bertolus et al., 2014; Shionoya et al., 2013). We assessed
164 the effects of dorsal striatum inactivation on this respiratory temporal pattern.

165 During the Conditioning session, rats with lidocaine-inactivated dorsal striatum exhibited the
166 typical temporal respiratory frequency pattern as early as within the first 3 trials following the first
167 odor-shock presentation (Figure 2A). Within-group comparisons showed that the respiratory rate
168 increased in response to odor arrival in both groups ($p < 0.05$ from second 4 on), but contrary to
169 the Control group, the Lidocaine group showed a U-shaped decrease preceding shock arrival
170 (significant difference between seconds 8-10 and seconds 15-18). Importantly, the temporal
171 patterns of respiration were similar in both groups by the end of the session, as well as in
172 subsequent odor presentations (Figure S3). Furthermore, during the Retention test, 24h later,
173 both the experimental and control groups showed a significant increase of freezing to the odor
174 compared to the pre-odor baseline (Figure 2B), suggesting a similarly strong odor-shock
175 association in the two groups.

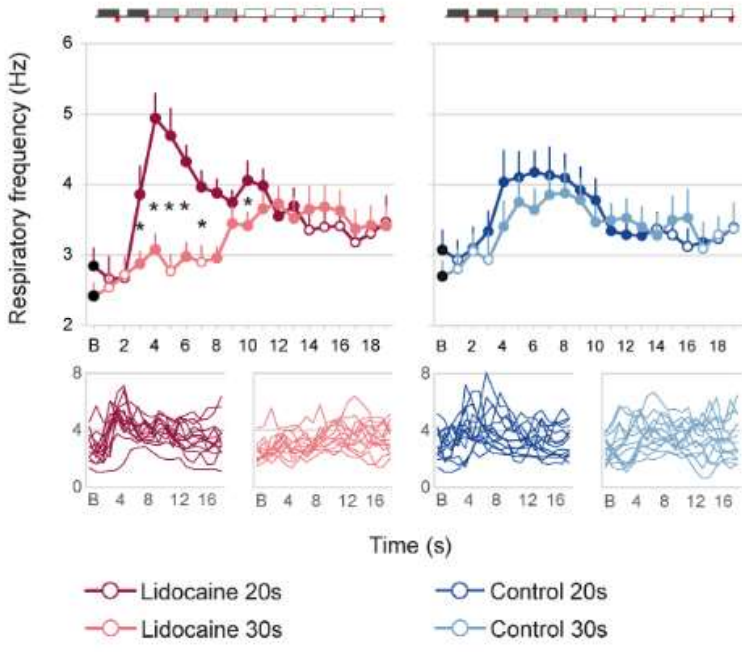
176 For the Shift session, we compared the mean respiratory frequency temporal pattern obtained
177 during the two first trials (in which the two groups expected the shock at 20s and presented similar
178 respiratory patterns, see Figure S3C) with that obtained during the next three odor-shock pairings
179 during which the animals experienced the shock at 30s. The data showed an early adaptation to
180 the new duration in the Lidocaine group, but not in the Control group (Figure 2C). The scalar rule,
181 a hallmark of interval timing according to which the error in estimating a duration is proportional
182 to the timed duration, predicts a superior superposition of patterns of responses to different time

183 intervals when the time axis is normalized than when it is absolute (Gibbon, 1977). To assess
184 this, the time axis for the 30-s data was multiplicatively rescaled to fit that of the 20-s data (Figure
185 S4) and superposition was indexed by eta-squared (η^2) as described before (Boulangier Bertolus
186 et al., 2014; Brown et al., 1992; Shionoya et al., 2013). This analysis indicated that the scalar
187 property was respected for the Lidocaine group, but not for the Control group, further confirming
188 that the shift occurred earlier in the Lidocaine group (Figure S4A). Importantly, by the end of the
189 Shift session the temporal patterns of the respiratory response were not different between groups,
190 and the scalar property was respected for both group (Figure S4B).
191 Together, these data suggest that reversible inactivation of the dorsal striatum hastens an
192 adaptation of the respiratory temporal curve to a newly presented interval duration.
193

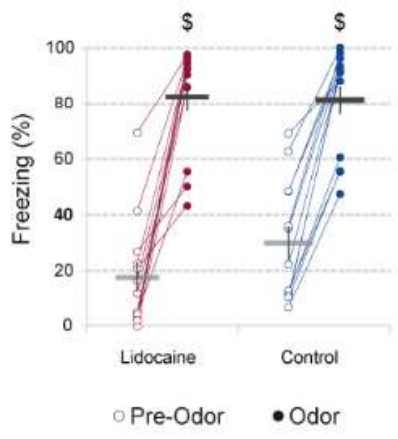
A. Conditioning session



C. Shift session - early trials



B. Retention session



D. Shift session - late trials

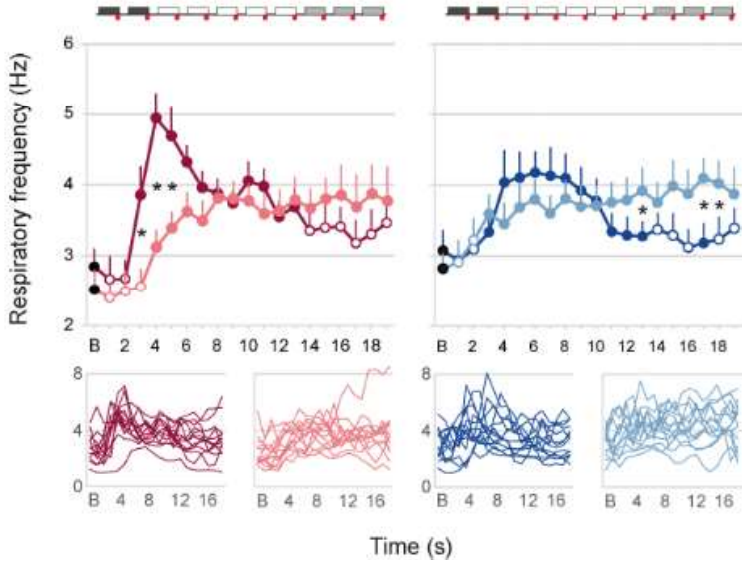


Figure 2: A) Respiratory frequency time course during the 20-s Odor-Shock interval of the Conditioning session, pooled over the 2nd, 3rd and 4th trials (as shown on the schema over the graph), for the Lidocaine (red) and Control (blue) groups. The baseline (B, black dots) corresponds to the average respiratory frequency over the 25s preceding the odor onset. A two-way ANOVA revealed a tendency for a significant Group x Time interaction ($F_{18,450} = 1.54, p = 0.07$). Filled dots are significantly different from baseline, $p < 0.05$. Inserts represent individual curves. # Significant difference between designated points, $p < 0.05$. B) Mean percentage of freezing per minute during the 2min period preceding the first odor

presentation (Pre-Odor) and during the 6min during which 20-s odor presentations are applied at the beginning of each minute (Odor). The ANOVA confirmed a significant effect of Period ($F_{1,24} = 175.4$, $p < 0.001$), but no effect of Group ($F < 1$) and no significant Period x Group interaction ($F_{1,24} = 2.24$, $p = 0.15$).[§]Significant difference between Pre-Odor and Odor periods, $p < 0.05$. C) Early shift: Respiratory frequency time course during the 20-s (dark colors; first 2 trials) versus 30-s (light colors; following 3 trials). Odor-Shock interval at the early stage of the Shift session, for the Lidocaine group (Left panel, red) and the Control group (Right panel, blue). A three-way ANOVA confirmed a significant Interval x Time x Group interaction ($F_{18,450} = 1.84$, $p = 0.019$). Follow-up analyses showed a significant Interval x Time interaction in the Lidocaine group ($F_{18,216} = 8.57$, $p < 0.001$), but not in the Control group ($F < 1$). (D) Late shift: Respiratory frequency time course during the 20-s (dark colors; first 2 trials) versus 30-s (light colors; last three trials) Odor-Shock intervals at the late stage of the Shift session, for the Lidocaine group (Left panel, in red) and the Control group (Right panel, in blue). The analysis showed an Interval x Time interaction comparing the 20-s trials with the last three trials of the shift ($F_{18,450} = 64.19$, $p < 0.001$), but no Group x Time interaction ($F_{18,450} = 14.57$, $p = 0.13$) nor Group x Interval x Time interaction ($F_{18,450} = 12.41$, $p = 0.54$), confirming that both groups shifted their anticipatory response towards the new interval by the end of the Shift session. Filled dots are significantly different from baseline (i.e. black dots, $p < 0.05$). * Significant between intervals difference ($p < 0.05$).

194

195 **Experiment 2: Modulation of striatal dopamine level during the acquisition of a** 196 **new duration**

197 We then monitored dopamine content in dorsal striatum and nucleus accumbens using
198 intracerebral microdialysis with a 2-min sampling rate, during the acquisition session. The session
199 included seven odor-shock pairings, six pairings with a 20-s odor-shock interval and the last
200 pairing with a 30-s odor-shock interval. Such analysis revealed significant differences in dopamine
201 modulation throughout the session, between the dorsal and ventral (nucleus accumbens) striatum
202 (Figure 3A). Structure-specific analyses showed significant modulation of the dopamine level
203 throughout the conditioning in the dorsal striatum, but not in the nucleus accumbens. Post-hoc
204 analysis in the dorsal striatum showed that dopamine level decreased from the beginning of the
205 conditioning, reaching significant differences from baseline from sample 5 (3rd odor-shock
206 presentation) to 10 (except sample 9) after conditioning onset. Dopamine level then slowly re-
207 increased for samples 11 and 12. Interestingly, the last odor-shock pairing for which the interval
208 was shifted to 30s instead of 20s (sample 13) was associated with a significant drop in dopamine
209 level (significant difference with preceding sample, $p = 0.046$, and following sample, $p = 0.016$,
210 **Figure 3B). More specifically, this drop was observed for 6 out of 7 animals (Figure 3B right panel).**

211

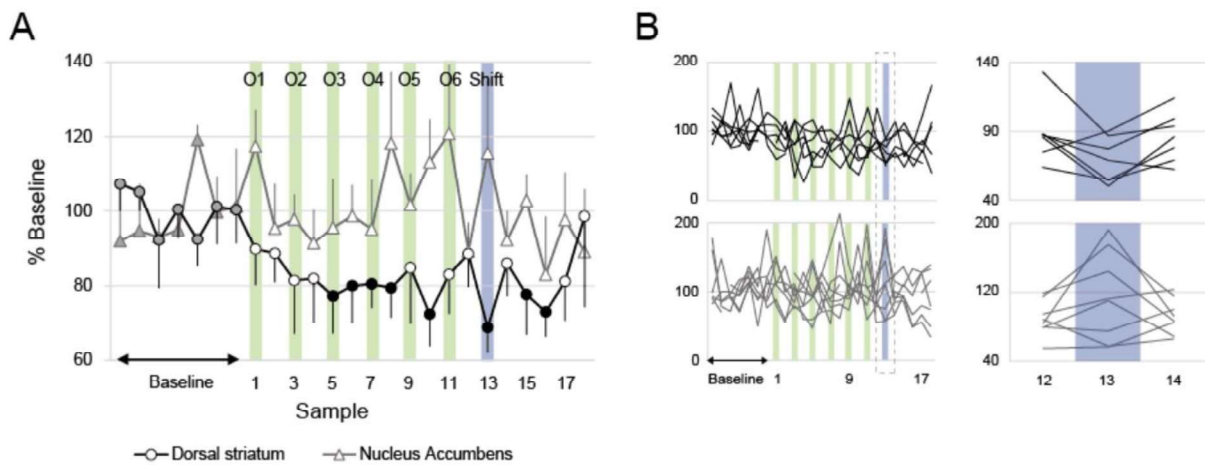


Figure 3: **A.** Dopamine level in the dorsal striatum (circles, black) and the nucleus accumbens (triangles, grey) during Experiment 2. Dopamine level is measured every two minutes and expressed as a percentage of baseline level measured during the 14min preceding the first Odor-Shock presentation (grey filled symbols). The session included six 20-s Odor-Shock pairings (O1 to O6, green vertical lines) and one 30-s Odor-Shock pairing (Shift, blue vertical line). A two-way ANOVA revealed a significant difference between Structures ($F_{1,13} = 9.79$, $p = 0.008$), as well as a significant Structure x Time interaction ($F_{24,312} = 1.55$, $p = 0.050$). Further analyses within each structure revealed a significant effect of Time in the dorsal striatum ($F_{24,144} = 2.17$, $p = 0.003$), but not in the nucleus accumbens ($F_{24,168} = 1.24$, $p = 0.22$). Black filled dots are significantly different from the baseline ($p < 0.05$). **B.** Individual curves of the dopamine levels in the dorsal striatum (black, top) and the nucleus accumbens (grey, bottom). The right panels show a zoom on the 30-s Odor-Shock pairing (Shift).

212

213 Discussion

214 In the present study, we investigated how reversible inactivation of the dorsal striatum impacts
 215 the learning of novel time intervals using the respiratory frequency to assess male rat's temporal
 216 expectation in an olfactory fear conditioning paradigm. We further investigated the modulations
 217 of striatal dopamine extracellular level in that task. Contrary to our hypotheses, we found that
 218 reversible inactivation of the dorsal striatum was associated with a hastening of the adaptation of
 219 the respiratory frequency temporal pattern during the odor-shock interval. We also showed that
 220 the fear conditioning and the learning of a novel interval duration were associated with decreases
 221 of dopamine level in the dorsal striatum, while no change in dopamine level was observed in the
 222 nucleus accumbens.

223 In line with the literature (Davis et al., 1989; Diaz-Mataix et al., 2013; Drew et al., 2005; Dupin
 224 et al., 2020; Hegoburu, Parrot, et al., 2014; Laurent-Demir & Jaffard, 2000; Ohyama et al., 2006;
 225 Tallot et al., 2020), our results support the assumption that learning a time interval happens early
 226 in conditioning, and further suggest that the expression of that learning can be modulated by the

227 dorsal striatum. Indeed, inactivation of the dorsal striatum hastened behavioral adaptation of the
228 animal to a novel duration. **Importantly, individual differences observed in response to the shift**
229 **under lidocaine inactivation (Figure 2C) were not associated with differences in the cannulas'**
230 **placement (Figure S1), suggesting they may instead result from interindividual variability in timing**
231 **abilities (see Dupin et al., 2020 for a discussion of timing abilities and associated brain dynamics).**
232 This result suggests that an intact dorsal striatum allows the preservation of the temporal
233 behavioral response pattern to the previously learned time interval at the detriment of the shift of
234 the pattern toward the new duration. This may seem inconsistent with the existing literature.
235 Indeed, electrophysiological recordings in the dorsal striatum during interval timing tasks have
236 consistently shown that the activity of striatal neurons is correlated with the animal's behavioral
237 temporal response (Bakhurin et al., 2017; Gouvêa et al., 2015; Matell et al., 2003; Mello et al.,
238 2015). Furthermore, lesioning or inactivating the striatum abolishes temporal performance of
239 subjects trained to lever-press for food at a fixed interval (Meck, 2006), or to discriminate between
240 durations (Gouvêa et al., 2015). However, these studies have in common that they rely on operant
241 conditioning tasks that require extensive training of the animal. Importantly, lesions of the dorsal
242 striatum impair the expression of both innate and learned motor sequences (Bailey & Mair, 2006;
243 Cromwell & Berridge, 1996). Consequently, in operant tasks, the specific role of the striatum in
244 the encoding of time cannot be investigated independently from the motor execution of a timed
245 response. Here, using a non-motor task - Pavlovian fear conditioning - and assessing the animals'
246 behavioral temporal response through respiration, allowed us to overcome this confound,
247 uncovering a previously undescribed role for the striatum. Our results also concur with previous
248 findings indicating that measuring the respiratory response is relevant to understand the brain
249 activity associated with emotions (Dupin et al., 2019; Moberly et al., 2018), including in the context
250 of interval timing (Dupin et al., 2020). **As animals were first conditioned to a 20-s interval duration**
251 **and then shifted to a 30-s duration, one may wonder whether the respiratory patterns observed**
252 **for these two durations could reflect non-specific changes in performance with trials repetition**
253 **instead of temporal adaptation to the new duration. However, this is unlikely, as we have shown**
254 **previously that the respiration curves obtained in animals trained only with a 30-s interval were**
255 **similar to those of animals shifted to a 30-s interval after training with a 20-s interval as in the**
256 **present study (Boulangier Bertolus et al., 2014; Dupin et al., 2020).**

257 Importantly, another difference between the present work and previous studies is that most of
258 them used appetitive conditioning to probe the role of striatum in interval timing, while we used
259 an aversive one. Interestingly, Dallérac et al (2017) used a Pavlovian aversive conditioning task,
260 and reported a decreased synaptic plasticity in the striatum when the expected time interval was

261 shifted to a new duration. That study also showed that the amygdala was activated by a shift to a
262 novel interval to time (Dallérac et al., 2017; Diaz-Mataix et al., 2013), and its inhibition resulted in
263 a faster shift toward the new learned duration. Here, we show that, akin to amygdala's inhibition,
264 inhibiting the striatum resulted in a speeding of the shift of the behavioral pattern towards the new
265 duration. Together, our data and those of Dallérac et al (2017) suggest that the disengagement
266 of the dorsal striatum, possibly under the control by the amygdala, might facilitate flexible temporal
267 expectancy's behaviors in Pavlovian aversive conditioning.

268 Dallérac et al suggested (2017) that this disengagement of the dorsal striatum could be
269 facilitated by dopamine-regulated plasticity (Calabresi et al., 2007; Tritsch & Sabatini, 2012).
270 Using intracerebral microdialysis of dopamine, we were able to show that dopamine level in the
271 dorsal striatum, but not in the nucleus accumbens, decreases when the animal learns the odor-
272 shock association, and when the time interval is changed. A few studies have shown that the
273 dorsal striatum might be involved in fear conditioning (Jeanblanc et al., 2003; Kathirvelu &
274 Colombo, 2013; Matsumoto & Hikosaka, 2009; White & Salinas, 2003). On the other hand,
275 although a few studies have suggested its possible involvement in Pavlovian fear conditioning
276 (Fadok et al., 2010; Wendler et al., 2014), the nucleus accumbens has been mostly implicated in
277 appetitive conditioning (for a review see Schultz, 2016). **It might be argued that the observed**
278 **decreases in dopamine levels in the dorsal striatum were related to non-temporal cognitive**
279 **processes, such as the mere learning of the odor-shock association or the novelty of the**
280 **experience. However, changing the interval duration without changing other elements of the**
281 **association resulted in a further decrease in dopamine level (highlighted in figure 3B), supporting**
282 **the assumption that dopamine in the dorsal striatum is indeed modulated by the temporal**
283 **manipulation.** Of note, in our study, dopamine level was analyzed every two minutes, which does
284 not allow to ascribe the observed changes to the arrival of the odor or of the shock specifically.
285 Nevertheless, this sampling rate permitted a dynamic **assessment** of dopamine level that revealed
286 a transient change in response to the shift in duration that had not been described before. These
287 data suggest that the dopamine decrease observed in the dorsal striatum could contribute to
288 support two distinct phenomena: the learning of the association itself (or the development of the
289 behavioral response to that learning), which is associated with a progressive decrease in
290 dorsostriatal dopamine, and the detection of a change in the temporal relationships of the
291 elements of the association, which is associated with a transient dopamine decrease.

292

293 Conclusion

294 The findings of our study **suggest** that inactivation of the dorsal striatum hastens the behavioral
295 adaptation of the rat's respiratory response to the time interval embedded in an aversive
296 conditioning. They further **indicate** that this behavioral adaptation is **likely** associated with a
297 decreased release of dopamine in the dorsal striatum. Furthermore, they are in stark contrast to
298 our initial predictions based on the existing literature and suggest a need to rethink the role of the
299 striatum and striatal dopamine in interval timing. This discrepancy further calls attention to the
300 advantage for the field to diversify the tasks and behaviors used to study interval timing in order
301 to discriminate between the brain structures involved in the learning of time intervals and those
302 necessary for the expression of these learned time intervals.

303

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312

313 **Author contributions**

314 JBB, SP and AMM designed the study, did the experiments, carried out the analyses and wrote
315 the manuscript; VD discussed the data, commented and edited the manuscript.

316

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467

468 Supplementary Material

469 Supplementary Methods

470 **Experiment 1: Inactivation of the dorsal striatum during odor fear conditioning**

471 **Animals**

472 Data were collected from 27 male Long Evans rats (Janvier, France), weighing 250-300 g at
473 the start of the experimentation (2 animals were used and excluded due to wrong positioning of
474 the injection cannulae). They were housed in pairs at 23°C and maintained under a 12h light–
475 dark cycle (lights on from 7:00 a.m. to 7:00 p.m.). Experiments were performed during the light
476 phase. Food and water were available *ad libitum*. All experiments and surgical procedures were
477 conducted in strict accordance with the 2010/63/EU Council Directive Decree and the French
478 National Committee (87/848) for care and use of laboratory animals. The experiments were
479 carried out under the approval of Direction of Veterinary Service (#69000692), and care was taken
480 at all stages to minimize stress and discomfort to the animals.

481 **Surgery**

482 Animals were anesthetized with Equithesin, a mixture of chloral hydrate (127 mg/kg, i.p.) and
483 sodium pentobarbital (30 mg/kg, i.p.), administrated by intraperitoneal injection, and placed in a
484 stereotaxic frame (Stoelting, USA). Before head skin incision, lidocaine (1% solution; Sigma-
485 Aldrich, Saint-Quentin Fallavier, France) was administered subcutaneously for local anesthesia.
486 Rats were implanted bilaterally with stainless steel guide cannulae (23G, Phymep, France)
487 positioned 1.5 mm above the targeted location of the injection needle tips in the dorsal striatum,
488 whose final stereotaxic coordinates from Paxinos and Watson (2014) relative to bregma, were as
489 follows: AP: 0.6 mm; L: 2.8 mm; DV: -4.5 mm from dura. The cannulae were fixed to the skull with
490 dental acrylic cement and anchored with surgical screws. Stylets of the length of the guide cannula
491 were inserted in it to prevent clogging. The animals were allowed two weeks of post-surgical
492 recovery.

493 **Experimental apparatus**

494 The apparatus has been described in a previous study (Hegoburu et al., 2011). It consisted of
495 a whole-body customized plethysmograph (diameter 20cm, height 30cm, EmkaTechnologies,
496 France) placed in a sound-attenuating cage. The ceiling of the plethysmograph was equipped
497 with a tower which allowed the introduction of three Tygon tubing connected to a programmable
498 custom olfactometer. Deodorized air flowed constantly through the cage (2L/min), a ventilation
499 pump drawing air out of the bottom of the plethysmograph. When programmed, an odor
500 (McCormick Pure Peppermint; 2 L/min; 1:10 peppermint vapor to air) was introduced in the air

501 stream through the switching of a solenoid valve (Fluid automation systems, CH-1290 Versoix,
502 Switzerland). The bottom of the plethysmograph was equipped with a shock floor connected to a
503 programmable Coulbourn shocker (Bilaney Consultants GmbH, Düsseldorf, Germany). Animal's
504 behavior was monitored with four video cameras (B/W CMOS PINHOLE camera, Velleman,
505 Belgium) placed at each corner of the sound-attenuating cage.

506 **Experimental paradigm**

507 Subjects were submitted to a 7d experimental paradigm consisting of 4d of Habituation to the
508 conditioning cage (20min/day), followed by a Conditioning session, a Retention test and a Shift
509 session (Figure 1A) at 24h intervals. During the Conditioning session, the animals were allowed
510 free exploration for 4min, then received ten odor-shock trials during which the conditioned
511 stimulus (CS), a peppermint odor, was introduced into the cage for 20s, the last second of which
512 overlapped with the delivery of a 0.4 mA foot-shock, the unconditioned stimulus (US), with an
513 intertrial interval of 4 minutes. During the Retention test, after a 2-min odor-free period in the
514 experimental cage (equipped with new visual cues and with a plastic floor to avoid contextual fear
515 expression), the CS was then presented 6 times for 20s with a 1-min intertrial interval. During the
516 Shift session, the animals were re-conditioned by receiving a first odor-shock trial using the
517 previously learned 20s interval, after which nine odor-shock trials were carried out with a new
518 (30s) CS-US interval. During the different steps of the experiment, the animal's behavior and
519 respiration were continuously monitored and recorded for offline analysis.

520 **Pharmacological inactivation of the dorsal striatum**

521 Five minutes prior to the Conditioning and Shift sessions, animals were injected with 0.5 μ L of
522 either lidocaine (2%, Sigma-Aldrich France, dissolved in sterile saline 0.9%, injection rate 0.5
523 μ L/min, Lidocaine group, n = 13) or saline (Control group, n = 14). Injection needles (30G)
524 extended 1.5 mm from the tip of the guide cannulae, and were connected via polyethylene tubing
525 to two 10-mL Hamilton microsyringes driven by an automated microinfusion pump (Harvard
526 Apparatus, France). After the injection, the needles were left in position for an additional minute
527 to enable diffusion of the solution into the brain tissue.

528 At the end of the experiment, the animals were sacrificed with a lethal dose of pentobarbital
529 for histological verification of the cannulae tips.

530 **Data acquisition and pre-processing**

531 The respiratory signal collected from the plethysmograph was amplified and sent to an
532 acquisition card (MC-1608FS, Measurement Computing, USA; Sampling rate = 1000 Hz) for
533 storage and offline analysis. The detection of the respiratory cycles was achieved using an
534 algorithm described in a previous study (Roux et al., 2006). Momentary respiratory frequency was

535 determined as the inverse of the respiratory cycle (inspiration plus expiration) duration, averaged
536 on a second by second basis, and synchronized to the odor arrival and shock delivery using TTL
537 signals. The video signal collected through the four video cameras was acquired with homemade
538 acquisition software using the Matrox Imaging Library and a Matrox acquisition card (Morphis QxT
539 16VD/M4, Matrox video, UK). Offline, the animal's freezing behavior was automatically encoded
540 via a LabView homemade software that had been validated by comparison to hand scoring by an
541 experimenter blind to the rat's group. Data were analyzed using scripts in Python.

542 **Data analysis**

543 We assessed the effects of treatment on the temporal dynamics of the respiratory frequency
544 in presence of the CS odor. For this analysis, the time course, in 1-second time bins, of the
545 respiratory frequency during the 19 first seconds of the odor presentation was compared using a
546 two-way ANOVA with the group (Lidocaine vs Control) as an independent factor and the time
547 (seconds 1 to 19) as a repeated measure factor. Post-hoc pairwise comparisons were then carried
548 out when allowed by the ANOVA results. For all statistical comparisons performed, the
549 significance level was set at 0.05.

550 During the Retention test, the conditioned fear response was assessed by comparing the
551 amount of freezing per minute before and during the odor introduction, using a two-way ANOVA
552 with the group as an independent factor and the period (Pre-Odor vs Odor) as a repeated measure
553 factor. Pairwise comparisons were then carried out when allowed by the ANOVA results.

554

555 **EXPERIMENT 2: Microdialysis during odor fear conditioning**

556 **Animals**

557 Twenty-one male Long Evans rats (Janvier Labs, France), weighing 250-300 g at the start of
558 the experimentation were used for this experiment (6 animals were excluded for wrong positioning
559 of the probe or technical problem during the session). They were individually housed in the
560 environmental conditions described above.

561 **Surgery**

562 The animals were anesthetized with ketamine (70 mg/kg) and xylazine (6 mg/kg) administrated
563 by intraperitoneal injection, and placed in a stereotaxic frame (Stoelting, USA). Before head skin
564 incision, lidocaine (1% solution; Sigma-Aldrich, Saint-Quentin Fallavier, France) was
565 administered subcutaneously for local anesthesia. The rats were implanted unilaterally (left side)
566 with a guide cannula for microdialysis probe (CMA12, Phymep, France) and positioned 3.5 mm
567 above the targeted location of the dialysis membrane tip, whose final stereotaxic coordinates from
568 (Paxinos & Watson, 2014), relative to bregma, were as follows: dorsal striatum AP: 0.6 mm; L:

569 3.5 mm; DV: -5.7 mm from dura) or nucleus accumbens (AP: 2 mm; L: 1.2 mm; DV: -7.6 mm from
570 dura). The cannula was fixed to the skull with dental acrylic cement and anchored with two surgical
571 screws. Stylets of the length of the guide cannula were inserted in it to prevent clogging. The
572 animals were allowed 2 weeks of postsurgical recovery during which they were regularly handled
573 and habituated to the experimental chamber for 20 min daily during the four days preceding the
574 microdialysis experiment.

575 **Microdialysis procedure**

576 Concentric microdialysis probes were constructed in our laboratory from regenerated cellulose
577 dialysis tubing (Spectra/Por hollow fiber; ref #132274, 225 mm O.D., 2 mm as active length,
578 Spectrum Medical Industries) and fused-silica capillary tubing (90 cm and 80 cm long for inlet and
579 outlet, respectively, 40 mm i.d., 105 mm O.D., Polymicro Technology). The body of the probe
580 consisted of a 26-G stainless steel tubing that was glued on a flat probe holder (Harvard)
581 adaptable to the CMA12 cannula-guide. After being flushed, the probes were continuously infused
582 with artificial cerebrospinal fluid (aCSF: 145.0 mmol/L NaCl, 2.70 mmol/L KCl, 1.0 mmol/L MgCl₂,
583 1.20mmol/L CaCl₂, 0.45mmol/L NaH₂PO₄, 2.33mmol/L Na₂HPO₄, pH 7.4) using a 500- μ L
584 Hamilton syringe mounted on an infusion pump (Harvard Model PHD 2000 Infuse). The aCSF
585 was infused at 1 μ L/min for the present experiments to avoid ultrafiltration.

586 Microdialysis on behaving animals requires long tubing (0.9- 1.2 m). At a given flow rate, the
587 dead volume of these tubing (i.e. the tubing volume between the dialysis membrane and the outlet
588 of the probe) results in a dead time that must be taken into account to accurately correlate the
589 neurochemical data with the behavioral events (Parrot et al., 2004; Hegoburu et al., 2009;
590 Hegoburu, Denoroy, et al., 2014), estimated to be ~ 2 min.

591 **Experimental apparatus**

592 The apparatus has been described in a previous study (Hegoburu et al., 2009). It consisted of
593 a Plexiglas transparent cylinder (diameter = 21 cm, height = 21.5 cm) with a lateral door placed
594 in a sound-attenuating cage. The ceiling of the cage was perforated with a central aperture
595 allowing the passage of microdialysis tubing and the branching of three Tygon tubing connected
596 to the programmable custom olfactometer described for experiment 1. The bottom of the cage
597 was equipped with the shock floor as described above and connected to an exhaust fan allowing
598 continuous evacuation of the odorant stream from the cage. Animal's behavior was monitored
599 with four video cameras (B/W CMOS PINHOLE camera, Velleman, Belgium) placed at each
600 corner of the sound-attenuating cage.

601 **Experimental paradigm**

602 On the day of the experiment, the microdialysis probe was inserted into the guide-cannula
603 (Dorsal striatum group : n = 7, Nucleus Accumbens group: n = 8) and the animal was introduced
604 in the experimental cage described in experiment 1. After a 3-hour probe equilibration period, the
605 conditioning session was initiated (Figure 1B). Six odor-shock pairings with a 20-s CS-US interval
606 were presented, with an intertrial interval of 4 minutes. An additional pairing was then presented
607 using a 30s CS-US interval. All along the session, dialysates were collected every 2 min in PCR
608 tubes previously rinsed with an acidic preservative medium. The samples were immediately
609 stored at -20°C. Once all the samples were collected, they were transferred into a -30°C freezer
610 until analysis. These procedures permit to limit greatly the degradation of DA from oxidation due
611 to heat, light and the non-acidic aCSF matrix. Of note, even though the paradigm was conducted
612 in a plethysmograph, the microdialysis tubing prevented the sealing of the plethysmograph and
613 thus the collection of the respiratory signal in this experiment.

614 **Microdialysis samples analysis**

615 The dialysates were analyzed using ultra-high-performance liquid chromatography (UHPLC).
616 Dopamine, 1-octanesulfonic acid (OSA), triethylamine (TEA), ethylene–diamine–tetra-acetic acid
617 (EDTA) disodium salt, and sodium hydroxide were purchased from Sigma (St. Louis, MO, USA),
618 potassium dihydrogenphosphate and methanol U-HPLC gradient grade from Fisher Scientific
619 (Loughborough, UK). Ultrapure water was produced using a Milli-Q system (Millipore, Bedford,
620 MA, USA). Standard solutions of 1 mmol/L of dopamine were stored at -30°C as aliquots in 0.1
621 mol/L hydrochloric acid.

622 The UHPLC system consisted of a Prominence degasser, a LC-30 AD pump and a SIL-30AC
623 autosampler (Shimadzu, Tokyo, Japan). Detection was carried out at 40°C using a Decade II
624 electrochemical detector fitted with a 0.7 mm glass carbon working electrode, a salt-bridge
625 Ag/AgCl reference electrode, and a 25 m spacer (cell volume 80 nL, Antec, Leyden, The
626 Netherlands). Separations were performed at 40°C (in detector oven) using a 100 × 0.32 mm
627 Kappa Hypersil Gold 1.9 m C18 column (Thermo Scientific). The mobile phase, which was
628 adapted from Ferry et al. (2014), consisted of 0.14 mol/L potassium phosphate, 0.1 mmol/L EDTA,
629 6 mmol/L OSA, 0.01% TEA (v/v), pH adjusted to 5 with 10 mmol/L sodium hydroxide, 6%
630 methanol, filtered through a 0.22 m cellulose acetate membrane before elution at 8.5 µL/ min.
631 Analytes were detected at an oxidation potential of 700 mV versus the reference electrode.
632 Chromatograms were acquired at a rate of 10 Hz using Lab Solutions software. The acquisition
633 time was 22 min. The injection volume was 1 µL. The sample analysis started the same day of
634 the microdialysis collection to prevent dopamine degradation as much as possible, as 25 samples
635 were collected per animal. In some rare cases of chromatographic matters, the analysis was

636 postponed, but not later than 2 days after collection. On the day of analysis, the samples were
637 placed in the autosampler and kept at +4°C before injection. Concentrations of dopamine were
638 calculated using a day calibration curve. Data were expressed as percentage (mean ± SEM) of
639 the baseline obtained by averaging the dopamine concentrations measured in the seven samples
640 collected before the start of the conditioning session. Changes in dopamine concentration were
641 then analyzed using a two-way ANOVA with the structure (dorsal striatum or nucleus accumbens)
642 as an independent factor and the time as a repeated measure factor. Post-hoc pairwise
643 comparisons were then carried out when allowed by the ANOVA results.

644

645 Supplementary Figures

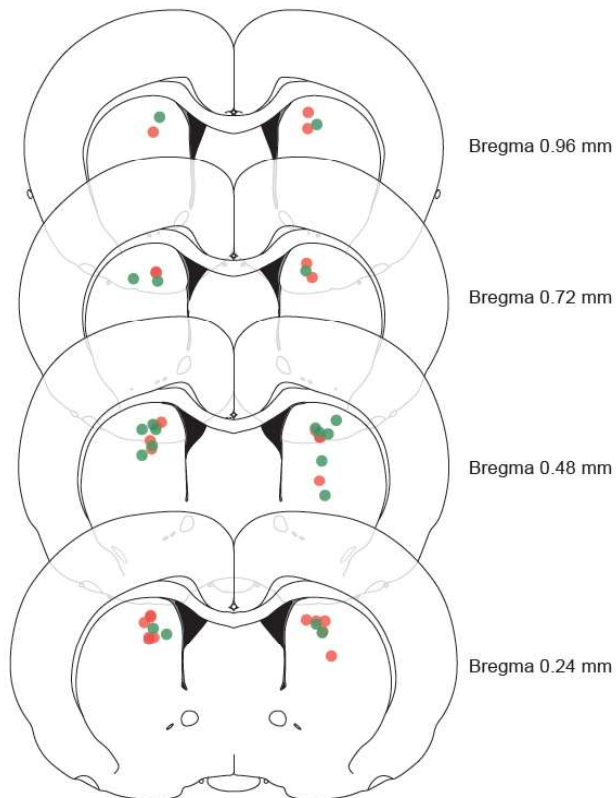


Figure S1: Localization of the tip of the injectors in Experiment 1 for rats in the Lidocaine group (red) and the Control group (green).

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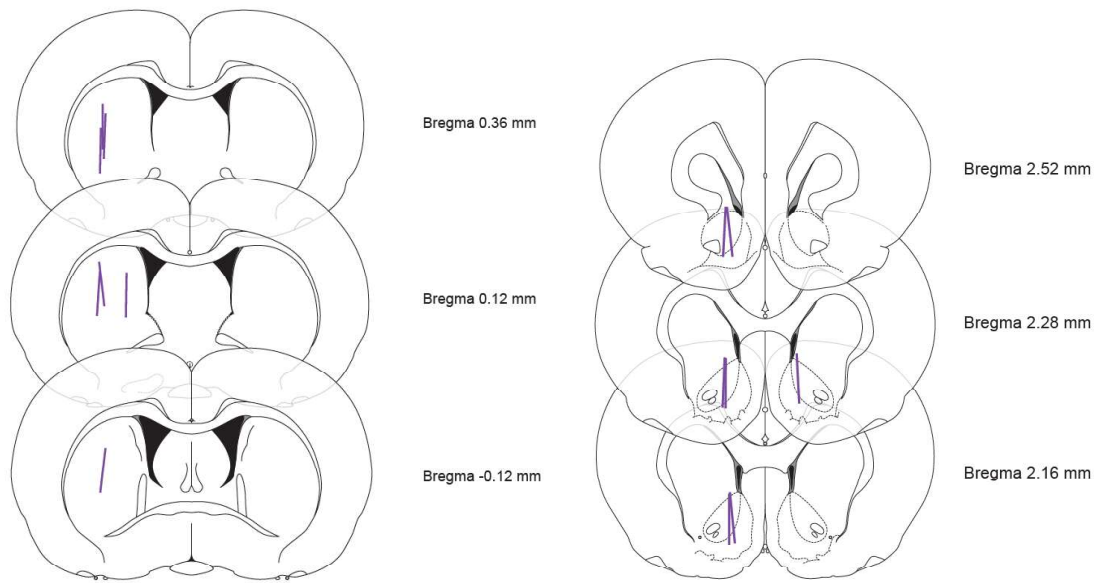
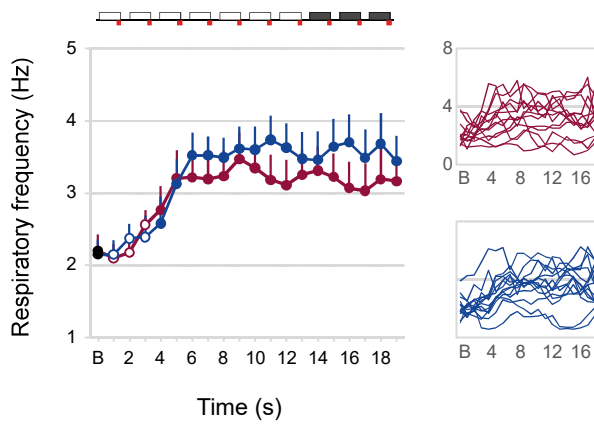


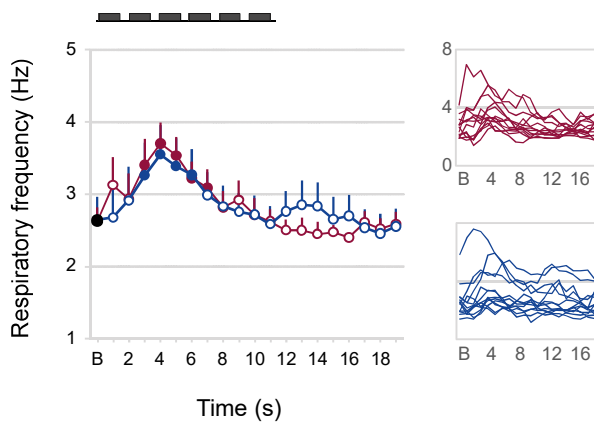
Figure S2: Localization of the microdialysis membranes in Experiments 2 for the rats implanted in the dorsal striatum (left panel) and the nucleus accumbens (right panel).

647

A. Conditioning session - last trials



B. Retention session



C. Shift session - first trials

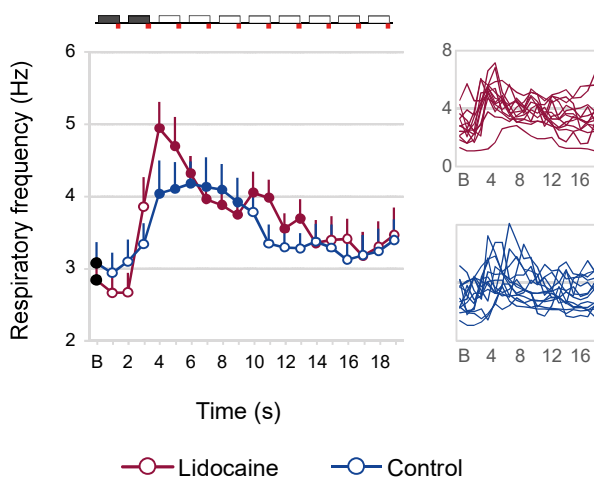


Figure S3: Time course of the respiratory frequency during (A) the last 3 trials of the conditioning session, (B) the 6 trials of the retention test, (C) the first 2 trials of the shift session (as shown on the schema over the graphs), for the Lidocaine (red) and Control groups (blue). ANOVAs with independent factor Group and repeated factor Time show no Group x Time interaction for the last three trials of the Conditioning session: $F_{18,432} = 1.08$, $p = 0.37$; or for the 2 first trials of the Shift session: $F_{18,450} = 1.16$, $p = 0.29$. Filled dots are significantly different from baseline ($p < 0.05$), inserts are the respiratory frequency of individual rats.

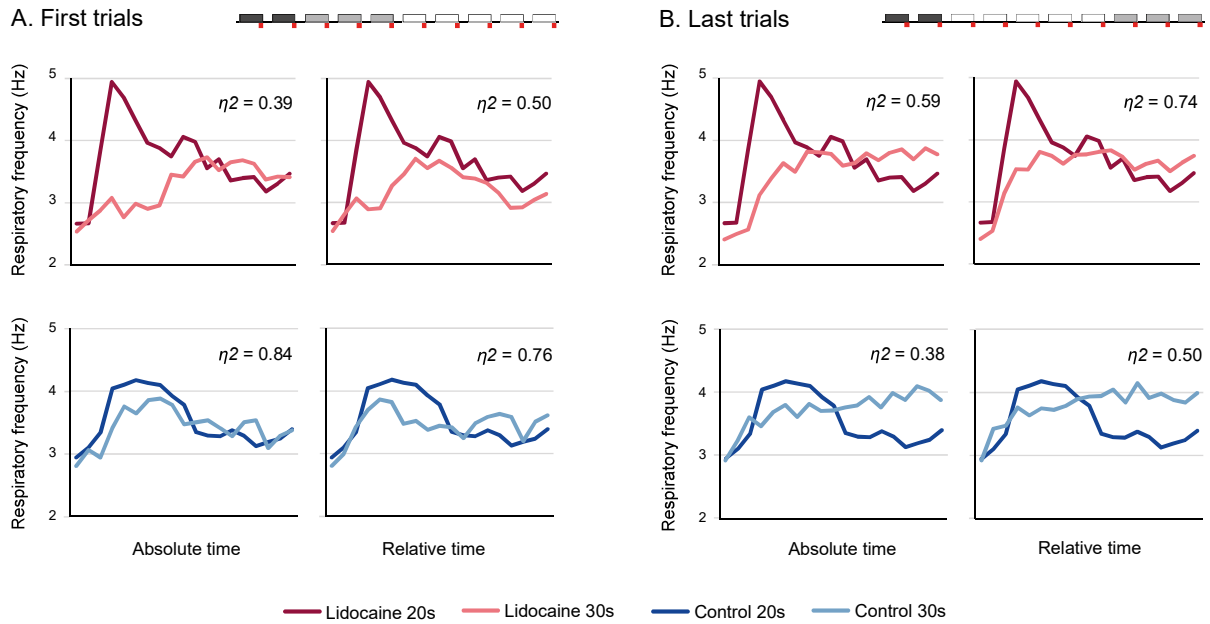


Figure S4: Testing the scalar property of the respiratory frequency curve during the 20-s (in dark colors; first 2 trials) versus 30-s (in light colors) Odor-Shock interval at (A) the early stage of the Shift session of Experiment 1 (first 3 trials after the rats have been exposed to the 30s interval), and (B) the last trials of the shift, for the Lidocaine group (upper panel, red) and the Control group (lower panel, blue). In order to assess scalar timing quantitatively, in each group, the time axis 30-s Odor-shock interval curves was multiplicatively rescaled to superpose with the 20-s interval (relative time). Superposition between the two curves was indexed by eta-square (η^2), compared with the superposition index of non-rescaled curves (absolute time), and indicated in the upper right corner of each graph.

Author contributions

JBB, SP and AMM designed the study, did the experiments, carried out the analyses and wrote the manuscript; VD discussed the data, commented and edited the manuscript.