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Faster emergence behavior from ketamine/xylazine anesthesia
with atipamezole versus yohimbine

Authors: Lukas Mees¹, Jonathan Fidler^{1,2}, Matthias Kreuzer^{1,2,3}, Jieming Fu¹, Mabelle M. Pardue^{1,4}, Paul S. García*^{1,2}

¹Atlanta VA Center for Visual and Neurocognitive Rehabilitation, Decatur, Georgia, USA

²Department of Anesthesiology, Emory University, Atlanta, Georgia, USA

³Department of Anesthesiology, Technical University of Munich, Munich, Germany

⁴Department of Biomedical Engineering, Georgia Institute of Technology, Atlanta, Georgia, USA

* Corresponding author

E-mail: pgarcia@emory.edu (PSG)

Abstract

24 Recent interest in reversal of the hypnotic effects of anesthesia has mainly focused on
25 overcoming a surge in GABA-mediated inhibitory signaling through activation of subcortical
26 arousal circuits or antagonizing GABA receptors. Here we examine the reversal of anesthesia
27 produced from non-GABA agents ketamine/xylazine and the effects of antagonists of
28 adrenoreceptors. These antagonists vary in selectivity and produce temporally unique waking
29 behavior post-anesthesia. We compared two antagonists with differential selectivity for α_1 - vs. α_2 -
30 receptors, yohimbine (YOH, 1:40 selectivity) and atipamezole (ATI, 1:8500). Adult mice received
31 intraperitoneal injections of either YOH (4.3 mg/kg), ATI (0.4 mg/kg), or saline after achieving
32 sustained loss of righting following injection of ketamine/xylazine (ketamine: 65.0 mg/kg;
33 xylazine: 9.9 mg/kg). Behaviors indicative of the post-anesthesia, re-animation sequence were
34 carefully monitored and the timing of each behavior relative to anesthesia induction was compared.
35 Both YOH and ATI hastened behaviors indicative of emergence, but ATI was faster than YOH to
36 produce certain behaviors, including whisker movement (YOH: 21.9±1.5 min, ATI: 17.5±0.5 min,
37 p=0.004) and return of righting reflex (RORR) (YOH: 40.6±8.8 min, ATI: 26.0±1.2 min, p<0.001).
38 Interestingly, although YOH administration hastened early behavioral markers of emergence
39 relative to saline (whisking), the completion of the emergence sequence (time from first marker to
40 appearance of RORR) was delayed with YOH. We attribute this effect to antagonism of α_1
41 receptors by yohimbine. Also notable was the failure of either antagonist to hasten the re-
42 establishment of coordinated motor behavior (e.g., attempts to remove adhesive tape on the
43 forepaw placed during anesthesia) relative to the end of emergence (RORR). In total, our work
44 suggests that in addition to pharmacokinetic effects, re-establishment of normal waking behaviors
45 after anesthesia involves neuronal circuits dependent on time and/or activity.

46 **Introduction**

47 Reconstruction of consciousness has been studied in the context of anesthesia [1-3] and
48 has been likened to waking from sleep [4]. Despite the active pharmacological reversal of some
49 aspects of anesthesia such as neuromuscular blockade and opioid induced respiratory depression,
50 the recovery of consciousness following clinical anesthesia has traditionally been considered a
51 passive process. Recently, stimulation of arousal pathways [5] and antagonism of inhibitory
52 signaling [6] have been investigated as potential strategies for hastening the arrival of
53 consciousness after isoflurane anesthesia. This work has been extended to reversal of other GABA-
54 ergic anesthetic agents such as propofol [7]. In contrast, the reversal of non-GABA agents (i.e.,
55 ketamine, xylazine, dexmedetomidine) has received much less attention, perhaps because their use
56 as sole agents for maintenance of anesthesia is less common in human clinical practice [8-10].

57 Despite their lack of study, non-GABA agents remain in common use. Ketamine (K) is one
58 of the most popular non-GABA veterinary anesthetic agents. It has been in use for over 50 years,
59 yet there is still much to learn about its pharmacodynamic effects. Although glutamate receptors
60 are known targets of its neurophysiologic effects (NMDA antagonism, AMPA agonism) other
61 receptors involved in neuronal excitability have demonstrated bioactivity.

62 Ketamine is most often administered in combination with other anesthetics, such as
63 xylazine, which is thought to counteract some of ketamine's sympathomimetic effects. The
64 intraperitoneal or intramuscular injection of xylazine in combination with ketamine is a common
65 anesthesia technique used for procedures performed in the laboratory on mice, rats, and other
66 animals [11-13]. Xylazine (X) specifically agonizes the α_2 -adrenoceptor [13]. Its action at the α_2 -
67 receptor in the brain stem produces sedation through increased noradrenergic release throughout
68 the cortex [14].

69 While ketamine/xylazine (K/X) is an effective combination for veterinary anesthesia, it can
70 produce side effects such as acute hyperglycemia [15] and corneal lesions [16] if administered
71 without a reversal agent. Currently, no pharmacologic reversal of ketamine anesthesia exists.
72 However, in veterinary practice, the sedative actions of α_2 -agonists can be pharmacologically
73 reversed with α_2 -antagonists, such as yohimbine and atipamezole [13]. Yohimbine (YOH) is an
74 indole alkaloid derived from the bark of the *Pausinystalia yohimbe* tree. It has highest affinity for
75 the α_2 -receptor, but also antagonizes the α_1 -receptor, as well as some serotonin and dopamine
76 receptors. It is considered a selective α_2 -antagonist, with a 40:1 α_2 : α_1 selectivity ratio. Atipamezole
77 (ATI) is also an α_2 -antagonist, with a higher α_2 : α_1 selectivity ratio 8500:1 [17]. ATI is also more
78 potent than YOH. Ten times the amount of YOH is needed to block central α_2 -receptors to the
79 same level that ATI does [18, 19].

80 The combination of an anesthetic cocktail and a reversal agent (of varying selectivity) can
81 have complex influences on behavior while the animal emerges from anesthesia. A more efficient
82 hastening of emergence with ATI compared to YOH has been suggested in the context of
83 ketamine/xylazine anesthesia [20], but an examination of the behaviors during emergence and
84 recovery from anesthesia with these reversal agents has yet to be studied.

85 Studying anesthesia produced by ketamine and α_2 -agonists has clinical relevance.
86 Ketamine has recently experienced a renaissance in clinical usage [9] as some benefits have been
87 demonstrated in the treatment of depression [21], attenuation of postoperative delirium [22], and
88 reduction of opioid administration for analgesia [23]. Although xylazine is not approved for use in
89 humans, other selective α_2 -agonists, like dexmedetomidine and clonidine, are used for blood
90 pressure control, sedation, and as adjuncts to general anesthesia. Dexmedetomidine in combination

91 with ketamine has been used as a preferred technique intending to minimize post-anesthesia
92 confusion in humans [24, 25].

93 Here we investigated an animal model of recovery from anesthesia in the absence of
94 GABA-ergic anesthetic drugs. We measured the appearance of behavioral markers of emergence
95 and describe their canonical sequence of arrival following K/X anesthesia in the presence and
96 absence of reversal by α_2 -antagonists.

97

98 **Methods**

99 **Animals**

100 Both male (n = 24, and female (n = 6) adult (approximately 20 – 30 grams) C57BL/6J mice
101 (Jackson Laboratories, Bar Harbor, ME) were used. Animals were experimentally naïve and were
102 used for only one trial each. Mice were housed under a 12:12 light:dark cycle and given standard
103 mouse chow *ad libitum*. All procedures were approved by the Atlanta Veterans Administration
104 Institutional Animal Care and Use Committee.

105 **Emergence procedure, anesthesia challenge, and early behavioral** 106 **markers**

107 Figure 1 depicts the experimental protocol. Animals were first weighed, and then placed in an open
108 top, clear observation box for acclimation. Before induction of anesthesia, each mouse received
109 training on a “sticky dot” test (see below). Following the training trials, mice were given an
110 intraperitoneal injection of K/X cocktail (65 mg/kg of ketamine [Ketaved, Vedco Inc., St. Joseph,
111 MO], 9.9 mg/kg xylazine [Anased, Akorn, Decatur, IL]), and the timer was started. Animals were
112 considered “anesthetized” when they failed to right themselves (by placing all four paws on the
113 surface of the chamber) after being gently placed on their back. This time was noted as the
114 appearance of loss of righting reflex (LORR). To investigate post-anesthesia behaviors in the
115 absence of pharmacological manipulation of alpha receptors, six mice were administered
116 intraperitoneal ketamine (65 mg/kg) and then immediately placed into 4% isoflurane for 60
117 seconds which resulted in until LORR LORR (K/I regimen). Immediately after LORR, each mouse
118 was placed on a heating pad beneath a heat lamp adjusted to maintain body temperature between
119 39.4-40.0 degrees Celsius. While anesthetized, adhesive tape (“sticky dot”) was applied to the right

120 forepaw. At exactly 15 minutes after the injection of anesthesia/sedation each mouse was given
121 either YOH [Yobine, Akorn Inc, Decatur, IL; 4.3 mg/kg], ATI [Antisedan, Orion Corporation,
122 Espoo, Finland; 0.4 mg/kg], ATI and prazosin (ATI+PRA) [Prazosin HCl, Sigma-Aldrich, St.
123 Louis, MO; 2.0 mg/kg], or saline (SAL) [Hospira Inc., Lake Forest, IL; 0.1mL 0.9% solution.
124 Consequently, the groups are depicted SAL, YOH, ATI, ATI+PRA for all groups with
125 ketamine/xylazine and K/I-SAL for the ketamine/isoflurane group in the remainder of the
126 manuscript. The time of the following behavioral markers was taken at their first occurrence:
127 whisker movement (any movement of whiskers), forelimb movement (any movement of either
128 forelimb), and respiration change (either a change in rate or change in breathing depth, judged by
129 the size of the chest excursion with each breath). Next, the time for each mouse to regain its
130 righting reflex (RORR) was recorded. As rodents do not sleep on their backs, it is common to use
131 RORR as an arbitrary marker for cessation of the anesthetized state (end-emergence), and so it is
132 used here to delineate the emergence and recovery periods. Upon righting, an attempt to return the
133 mouse to its back was performed in each mouse to ensure the righting reflex was robust. Following
134 the RORR, each mouse was placed into a clear-walled open top box to enable observation of the
135 recovery period behaviors.

136 **Late behavioral markers and early recovery from anesthesia**

137 The sticky dot test is a complex measure of perception and motor coordination previously
138 used to evaluate animals after ischemic stroke [26]. Briefly, the animals receive a 2.5 x 0.5 cm
139 adhesive tape folded over their forepaw; then the time to investigation of the tape (paw shaking or
140 any purposeful movement towards the tape involving the nose, mouth, or alternate forepaw) is
141 recorded. Prior to the anesthesia challenge, all mice received three trials of the sticky dot test. For
142 our experiments we defined *recovery period* as the time between RORR and the appearance of the

143 final marker of our observed behavioral sequence (sticky dot notice). If the animal did not attempt
144 to remove the tape within 25 minutes after RORR, the trial ended. Ataxia was assessed at five-
145 minute intervals after RORR by testing for splaying of the legs. This was accomplished by lifting
146 the mouse by the tail, suspending both hind limbs and observing the hind limb reflexes after
147 subsequent dropping of the hindquarters. Other ataxic features were recorded if present:
148 ambulation with only the forelimbs, or otherwise uncoordinated movement. Coordinated
149 movement was defined as diagonal cross-matched ambulation, in which the right forelimb
150 movement was followed by movement of the left hind limb. Latency to return of diagonal cross-
151 matched ambulation was recorded. After 25 minutes post-RORR, observation was terminated, and
152 the mouse was sacrificed by cervical dislocation.

153 **Statistics**

154 We applied non-parametric testing for evaluating our data set, because of our modest sample size.
155 We used the Kruskal-Wallis test to evaluate possible differences between all groups and the Mann-
156 Whitney U test as *post-hoc* test. We did not correct for multiple comparisons in order to prevent
157 increase of false negatives [27]. But therefore, additionally to the hypothesis-based tests, we
158 calculated the area under the receiver operating curve (AUC) together with 10000-fold
159 bootstrapped 95% confidence intervals as effect size. As a rough estimate, according to the
160 traditional point system, an effect can be classified as: excellent (very strong) AUC=0.9-1; good
161 AUC=0.8-0.9; fair AUC=0.7-0.8; poor AUC=0.6-0.7; or fail: AUC=0.5-0.6. We used MATLAB
162 R2017 (The Mathworks, Natick, MA) for our statistical tests and the MATLAB-based MES
163 toolbox [28] to calculate AUC and 95% CI. We present our data as raw data together with the
164 mean and the median.

166 **Results**

167 **Baseline testing and induction of anesthesia**

168 During baseline experiments of the sticky dot test all mice notice and removed the adhesive
169 tape in less than 2 minutes by the third trial. No animal noticed or removed the tape before RORR.
170 All 24 mice that received the K/X dose described in the methods experienced LORR in less than
171 5 minutes.

172 **Emergence from ketamine/xylazine anesthesia is hastened with the** 173 **administration of α_2 -antagonists**

174 Among the early behaviors observed before RORR, change in respiration rate, whisker
175 movement, and forelimb movement were recorded. Figure 2 (A-C) contains the detailed
176 information. ATI and YOH produced signs of waking earlier than SAL in all three behaviors.
177 The time to whisker movement was different among the groups ($p=0.0005$, $\chi^2=15.16$). Compared
178 to saline ($n=6$) treated animals, YOH($n=6$, $p=0.0022$, 22.2 [19.3 23.7] min) and ATI ($n=6$, p
179 $=0.0022$, 17.4 [17.0 18.3] min) showed faster recovery to whisker movement than SAL (46.8 [41.0
180 55.3] min). AUC indicated perfect separation (very strong effect) between the groups, i.e., AUC=1.
181 We observed the same result when comparing the ATI with the YOH group ($p=0.0022$; AUC=1,
182 very strong effect). Time required for anesthetized mice to exhibit a change in respiration was also
183 different among groups ($p=0.0013$, $\chi^2=13.35$) depending on reversal agent. We derived very
184 similar statistical results for time to respiration as for time to whisker movement. The SAL group
185 took significantly longer to express respiration signs ($n=6$, 47.1 [41.0-55.3] (median [min max]
186 min) when compared to YOH ($n=5$, 22.2 [19.2-24.5] min) with $p=0.0043$ and when compared to
187 ATI ($n=5$, 17.5 [17.0-18.3] min) with $p=0.0043$. AUC also showed perfect separation (AUC=1)

188 between the groups. When compared to YOH, ATI animals reached this behavioral milestone
189 significantly earlier as well ($p=0.0079$, $AUC=1$). Overall, ATI and YOH have similar profiles in
190 the sequence of early markers of emergence.

191 **Yohimbine increases the time to completion of the emergence** 192 **sequence**

193 The timing of RORR was significantly different among treatments ($p=0.0011$; $\chi^2=13.66$) as shown
194 in Figure 3. The SAL group took longer to right themselves after ketamine/xylazine anesthesia
195 (56.4 [46.0-63.2] min) compared to YOH (38.7 [29.8-51.2] min) with $p=0.0260$ and $AUC=0.89$
196 [0.67 1] (strong effect) and ATI (25.9 [24.6-27.4]) with $p=0.0022$ and $AUC=1$ (very strong effect).
197 ATI animals also exhibited RORR faster than YOH animals ($p=0.0022$ and $AUC=1$ (very strong
198 effect). Interestingly, the time delay between the first exhibited behavior and RORR showed
199 significant difference among groups ($p=0.0248$; $\chi^2=7.40$), but with a different pattern. YOH
200 showed a significantly longer delay in completion of the emergence sequence ($n=6$, 17.1 [7.9-29.3]
201 min) compared to SAL ($n=6$, 8.8 [1.0-13.6] min) with $p=0.0152$ and $AUC=0.92$ [0.67 1] and ATI
202 ($n=6$, 8.1 [7.2-10.3] min) with $p=0.0260$ and $AUC=0.89$ [0.58 1]. There was no significant
203 difference between SAL and ATI ($p=1$; $AUC=0.5$ [0.17 0.83]). This suggests that although
204 yohimbine hastened the start of emergence behaviors, completion of the entire sequence of
205 emergence behaviors was lengthened by administration of yohimbine (Figure 3B).

206 **Atipamazole and yohimbine hasten recovery from ketamine/xylazine** 207 **anesthesia**

208 The recovery of locomotor activity in an uncoordinated fashion (uncoordinated movement)
209 was significantly different among treatment groups ($p=0.0008$, $\chi^2=14.36$). YOH mice exhibited

210 uncoordinated movement faster (n=6, 41.3 [34.3-51.2] min) than SAL mice (n=6, 59.6 [49.7-65.7]
211 min) with $p=0.0087$ and $AUC =0.94 [0.78 1]$ (very strong effect), while ATI mice showed
212 uncoordinated movement earlier (n=6, 28.6 [25.4-32.4] min) than both SAL ($p=0.0022$; $AUC=1$
213 (very strong effect)) and YOH ($p=0.0022$; $AUC=1$ (very strong effect); Figure 4A). Latency to the
214 first notice of the sticky dot was different between groups ($p=0.0057$, $\chi^2=10.32$). ATI mice were
215 faster (n=6, 36.1 [26.1-40.4] min) to identify the sticky dot compared to both SAL (n=4, 67.1 [54.1
216 70.0] min) with $p=0.0095$ and $AUC=1$ (very strong effect) as well as and YOH (n=6; 59.0 [39.2-
217 62.7] min) with $p=0.0087$ and $AUC=0.97 [0.8 1]$ (very strong effect). YOH mice were statistically
218 indistinguishable from SAL mice in noticing the sticky dot ($p=0.19$, $AUC=0.8 [0.4 1]$ (strong, but
219 not significant effect); Figure 4B). Two saline-treated mice failed to show diagonally cross-
220 matched ambulation, notice the sticky dot, or show ataxia attenuation within 25 minutes after
221 RORR and one yohimbine-treated mouse did not notice the sticky dot within 25 minutes after
222 RORR. These three animals were removed from the analysis in Figure 4B (see also supplemental
223 Figures). But for complete presentation of the results without removed animals, we set the times
224 of appearance of behavioral markers these animals to the maximum RORR+25 min. The results
225 were similar to the reduced data set. The time to sticky dot notice was significantly different among
226 the groups ($p=0.0016$, $\chi^2=12.88$). Time to event was 69.2 [54.1 88.2] min for SAL, 57.0 [39.2
227 62.7] min for YOH, and 36.1 [26.1 40.4] min for ATI. The pairwise comparison led to $p=0.0411$,
228 $AUC=0.86 [0.58 1]$ for SAL vs. YOH, to $p=0.0022$, $AUC=1$ for SAL vs. ATI and to $p=0.0043$,
229 $AUC=0.97 [0.83 1]$ for YOH vs. ATI. The recovery of more coordinated (diagonally cross-
230 matched ambulation) locomotor efforts was different between groups ($p=0.0005$, $\chi^2=10.65$). YOH
231 mice (n=5, 49.1 [39.3-54.3] min) showed a trend towards faster diagonally cross-matched
232 ambulation than SAL controls (n=4, 66.7 [51.0-71.8] min) with $p=0.0635$ and $AUC =0.9 [0.6 1]$

233 (very strong effect), while ATI mice (n=6, 36.4 [30.4-37.4] min) were faster than SAL (p=0.0159,
234 AUC=1, very strong effect) and YOH mice (p=0.0080, AUC=1, very strong effect); Figure S1).
235 The time delay to ataxia attenuation was similarly dependent on treatment (p=0.0043, $\chi^2=10.88$).
236 YOH mice exhibited ataxia attenuation earlier (n=6, 47.7 [39.3-56.2] min) than SAL mice (n=4,
237 66.4 [51.0-71.7] min) with p=0.0381 and AUC=0.92 [0.67 1] (very strong effect), while ATI mice
238 (n=6, 36.8 [29.6-42.4] min) were faster to show ataxia attenuation compared to both SAL p=0.0095
239 and AUC=1 (very strong effect) and YOH (p=0.0152, AUC=0.92 [0.70 1], very strong effect;
240 Figure S1).

241 **Emergence and recovery behaviors are influenced by activity at α_1 -** 242 **receptors.**

243 Three animals (2, SAL and 1, YOH) did not regard the sticky dot before the experiment
244 timed out (25 minutes after RORR) so a maximum of 25 minutes was assigned as their recovery
245 period. We did not find a significant different distribution in times from RORR to sticky dot notice
246 between the groups (p=0.5273, $\chi^2=1.28$). Figure 5 graphs the latencies which were 11.1 [7.5-25]
247 min for SAL (n=6), 13.2 [4.9-25.0] min for YOH (n=6), and 8.8 [1.2-15.0] min for ATI (n=6). The
248 pairwise *post hoc* comparisons did not reveal any significant changes between the groups, neither
249 did the AUC analysis reveal any trends. As an additional piece of information, the latencies
250 between RORR to uncoordinated movement, diagonally cross matched ambulation and ataxia
251 attenuation can be found in Figure S2. The observed difference between ATI and YOH during
252 emergence (Figure 3B) and the observation that no animal timed out during recovery for ATI, but
253 one did for YOH (Figure 5) prompted us to further examine the role of α_1 -receptors in these post-
254 anesthesia behaviors.

255

256 **Prazosin, a selective α_1 inverse agonist does not hasten emergence**
257 **from ketamine/xylazine anesthesia in the presence of atipamezole**

258 Co-administration of prazosin (2.0 mg/kg) with atipamezole does not hasten RORR but prolongs
259 sticky dot removal in the recovery from ketamine/xylazine anesthesia. Time to RORR for
260 ATI+PRA was 26.1 [18.2 52.1] min and hence not significantly different from the ATI group (25.9
261 [24.6 27.4] min; $p=0.7922$; $AUC=0.57$ [0.20 0.93]; Fig. 6A). The time to sticky dot notice for
262 ATI+PRA was 60.8 [33.3 81.7] min and also not significantly different from the ATI group (36.1
263 [26.1 40.4] min; $p=0.2468$; $AUC=0.73$ [0.33 1] (fair effect); Fig. 6B). However, the $AUC>0.7$ may
264 indicate a trend towards a longer time to sticky dot notice with ATI+PRA. The time from the first
265 marker of emergence to RORR was 9.3 [1.3 34.9] min for the ATI+PRA group and hence not
266 significantly different from the ATI group (8.1 [7.2 10.3] min; $p=0.8918$; $AUC=0.53$ [0.13 0.90],
267 Fig. 6C). The recovery period, i.e., the time from RORR to sticky dot notice, was 25.4 [13.2 54.5]
268 min for the ATI+PRA group. This was significantly slower (8.8 [1.2 15.0] min; $p=0.0173$;
269 $AUC=0.93$ [0.73 1] (very strong effect)) compared to the ATI group (Fig. 6D).

270 Through a disruption of normal α_1 -receptor activity, the recovery from ketamine/xylazine in the
271 ATI+PRA group is lengthened. In similar fashion, the emergence period (whisking to RORR) is
272 increased for animals given YOH for reversal (Figures 6C, 6D). This highlights the importance
273 of alpha receptor pharmacology in emergence and recovery from this ketamine/xylazine regimen.

274 **Lengthy recovery is associated with effects on α_1 receptors**

275 These mice were given a brief exposure to isoflurane to induce LORR and an equivalent amount
276 of ketamine to the K/X regimen. Figure 7 compares the latency to noticing the sticky dot for the
277 ketamine/isoflurane regimen with sham (saline) reversal (K/I-SAL). The Kruskal-Wallis test

278 indicated a significant difference among groups ($p=0.0052$, χ^2 : 14.79). Time to sticky dot notice
279 for KET was significantly shorter when compared to SAL ($p=0.010$, AUC=1, very strong effect),
280 YOH ($p=0.009$, AUC=0.97 [0.80 1], very strong effect), and ATI+PRA ($p=0.0303$, AUC=0.9
281 [0.67 1], strong to very strong effect). There was no significant difference when compared to the
282 ATI group ($p=0.180$, AUC=0.75 [0.42 1], fair effect). This is the group that was given only α_2 -
283 selective agonists and antagonists and like the K/I-SAL regimen no α_1 -antagonism. Figure 8 is a
284 summary of all the emergence and recovery observations for all 5 regimens. Qualitatively, the
285 appearance of emergence and recovery behaviors indicative of a return to neurocognitive baseline
286 varied in time but the order of these behaviors was largely unaltered across groups. To complete
287 the picture, Figure S3 presents a model of the pharmacodynamic effects and the latencies of
288 emergence and recovery period for all groups.

290 **Discussion**

291 In this study, behavioral milestones indicative of the approach to normal neurocognitive
292 function after anesthesia with ketamine/xylazine were observed following injection of reversal
293 agents. As predicted, both YOH and ATI effectively shortened the time required to reach these
294 milestones during emergence from anesthesia compared to saline.

295 The behavioral profile between YOH and ATI mice during emergence and recovery from
296 anesthesia was not identical, in agreement with previous studies [20]. Differential pharmacology
297 between YOH and ATI offers some possible explanations for this. First, there are slight differences
298 between YOH and ATI affinity for α_2 -receptor subtypes. ATI has an equal affinity for α_{2a} , α_{2b} , α_{2c} ,
299 and α_{2d} , while YOH has similar affinity for all the α_2 -subtypes except for α_{2d} , for which it has a
300 lower affinity. Xylazine indiscriminately targets all of the α_2 -subtypes [29]. Due to YOH's lower
301 affinity for the α_{2d} -subtype, it is possible that some α_2 -receptors could still be agonized by xylazine
302 resulting in a prolonged emergence as compared to ATI.

303 While both drugs predominately target α_2 -receptors, YOH interacts with other systems.
304 Unlike ATI, which has a negligible affinity for serotonin (5-HT) [17, 18], β_1/β_2 -adrenergic,
305 muscarinic, dopamine₂, tryptamine, GABA, opiate or benzodiazepine receptors [17], YOH is less
306 discriminatory. High doses of YOH (>1 mg/kg; the current study used 4.3 mg/kg) have been
307 shown to have 5-HT_{1A} agonistic properties, which lead to decreases in heart rate, blood pressure,
308 activity level, and body temperature [30]. Similarly, in doses approximating those used in the
309 current study, YOH has been shown to decrease ambulation, an effect not seen with more selective
310 α_2 -antagonists [19]. During the post-RORR period in the observation box, YOH and ATI animals
311 exhibited qualitatively different levels of exploratory behavior.

312 In an attempt to characterize non- α_2 -interactions, the current study dosed the reversal
313 agents in order to equalize α_2 blockade between the two reversal agents. Pertovaara et al. found
314 that,in order to block α_2 receptors to the same level that ATI does, about ten times the amount of
315 YOH needs to be administered [18, 19].We dosed 10.8 times more YOH compared to ATI. This
316 provides some evidence that non- α_2 -interactions in YOH are slowing down the emergence process,
317 while possibly leading to hypoactivity during recovery.

318 Because YOH has an $\alpha_2:\alpha_1$ selectivity ratio over 200 times smaller than ATI [17], α_1
319 antagonism could be implicated in YOH mice. Antagonism of α_1 -receptors is mechanistically
320 involved in the sedating effects of some anti-psychotics (quetiapine, risperidone) [31] as well as
321 anti-hypertensives. Some of the behavioral effects of co-administering the selective α_1 -inverse
322 agonist prazosin [32] with atipamezole mimicked the yohimbine reversal, specifically the time to
323 notice of sticky dot, the final behavioral marker we observed. This suggests that the slowing of
324 recoveryafter ketamine/xylazine anesthesia after yohimbine reversal (relative to ATI) could be
325 mediated by antagonism of α_1 -receptors and likely not subsequent hypoactivity due to the effect
326 of yohimbine on 5-HT, or other receptors. Our experiments with ketamine in the absence of
327 xylazine or any other adrenoreceptor manipulation further support the notion that α_1 -receptor
328 antagonism can delay complex behaviors indicative of the restoration of normal behaviors post-
329 anesthesia (notice of sticky dot). In situ hybridization experiments reveal that neurons in layers II-
330 V of most areas of the cerebral cortex the lateral amygdala, hippocampus, and reticular thalamus
331 all have high density of α_1 receptors [33]. Distribution of normal signaling of these regions may
332 contribute to failure to achieve a normal recovery sequence efficiently.

333 While ATI mice were more active than YOH mice, they showed a profound lack of
334 coordination once righted. This ataxia did not completely resolve during the 25-minute

335 observation. α_2 -adrenoceptors are known to be present in the cerebellum [34]. Further studies will
336 be necessary to determine if this prolonged ataxia is related to the hastening of emergence, causing
337 enhanced locomotion before coordination is re-established, or a lingering effect of atipamezole on
338 cerebellar function.

339 A limitation of our study is the failure to pharmacologically antagonize the effects of
340 ketamine. Although ketamine is known to inhibit NMDA receptors, it is pharmacologically
341 promiscuous and its exact mechanism for producing surgical anesthesia is unknown. Unlike
342 xylazine, dexmedetomidine, opioids, and benzodiazepines, no pharmacologic agent specifically
343 reverses all of the pharmacodynamic effects of ketamine. Some evidence suggests that ketamine
344 minimally interacts with adrenoceptors [35], but these interactions have yet to be thoroughly
345 examined. While it is not possible from this data to distinguish the effects of residual ketamine
346 from lingering effects of the antagonists, our observations of mild hyperactive ataxic behavior in
347 ATI animals are similar to the clinical situation often described for recovery from ketamine
348 anesthesia characterized by excitation and features of emergence delirium [36]. This supports the
349 notion that ATI treated animals may be exhibiting behavior typical of ketamine after effectively
350 eliminating xylazine's pharmacodynamic effects (Figure S3). Interestingly, ataxia appeared to be
351 attenuated after approximately the same delay (in reference to RORR as opposed to anesthesia
352 injection) in all groups. In parallel, if measured in reference to RORR, other measures of
353 coordination recovery (uncoordinated movement, diagonally cross-matched ambulation) and
354 higher perception and motor processing (sticky dot notice) had the same delay across groups. It
355 appears that, although ATI produces a more efficient emergence from ketamine/xylazine
356 anesthesia, it does not improve late recovery compared to YOH or even no reversal agent at all
357 (Figure 8). It is possible that the differences in waking behaviors between ATI and YOH are arising

358 from a differential clearance in ketamine and xylazine, rather than off-target interactions. It would
359 be excellent to characterize these effects further with additional experiments examining the
360 potential for a dose-effect of YOH and/or ATI. Based on our findings we conclude that proper
361 reconstruction of network activity, requiring specific activation of networks involving
362 adrenoceptors, underlies restoration of coordinated movement, as opposed to this being solely a
363 consequence of ketamine pharmacokinetics. Future studies involving careful blood sampling over
364 time would be necessary to determine which has the greatest influence.

365 Although both YOH and ATI produced waking behaviors before saline, ATI was slightly
366 quicker to elicit several markers. These differences are likely attributable to the differential affinity
367 between the two drugs for α_2 -subtypes, as well as α_1 -interactions. Because the effects of
368 adrenoceptor antagonism on behavior are dose-dependent [19, 30], further experiments are needed
369 to compare these results to lower doses of these drugs. During recovery from anesthesia it is
370 difficult to determine if the animal is attempting to explore their environment versus exhibiting an
371 escape response, however motoric behaviors can still be observed and measured. Quantification
372 of arousal, exploratory behavior, and balance should be done given the observations made during
373 the current experiment. In total, our results highlight that an efficient emergence is not necessarily
374 a preferred trajectory for the immediate post-anesthesia recovery. In addition to pharmacokinetic
375 effects, the re-establishment of normal behaviors after anesthesia likely involves neuronal circuits
376 dependent on time and/or activity.

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482

483

484 **Figure Legends**

485

486 **Figure 1. Waking behavior observation protocol.** Timeline describing observation protocol.
487 Procedures listed on top, with behaviors listed below. K/X = ketamine/xylazine, LORR = loss of
488 righting reflex, RORR = return of righting reflex.

489

490 **Figure 2. Both YOH and ATI reduce the time required to exhibit the first behavioral signs**
491 **of emergence from ketamine/xylazine anesthesia.** Time for first incidence of individual waking
492 behaviors is plotted, including whisker movement (A), respiratory change (B), and forelimb
493 movement (C). Time measurements are from ketamine/xylazine injection. * indicate significance
494 between different groups; solid bar: median, dashed bar: mean.

495

496 **Figure 3. Both $\alpha 2$ antagonist treated groups recover righting reflex faster, but YOH has**
497 **increased delay between early markers and RORR.** (A) Return of righting reflex. (B) Delay
498 from first behavioral marker to RORR. Time measurement in (A) is from ketamine/xylazine
499 injection and from the first exhibited behavior in (B). * indicate significance between different
500 groups; solid bar: median.

501

502 **Figure 4. Appearance of motoric behaviors during recovery show ATI elicits activity faster**
503 **than both YOH and SAL.** Time delay to post-RORR behaviors are compared, including
504 uncoordinated movement (A) and notice of sticky dot (B). Time measurements are from
505 ketamine/xylazine injection. * indicate significance between different groups; solid bar: median.

506

507 **Figure 5. Time from RORR to sticky dot notice (recovery period) between treatment groups.**

508 Latency from RORR to the first notice of the sticky dot. * indicate significance between different
509 groups; solid bar: median.

510

511 **Figure 6. Latency to RORR and sticky dot notice and the emergence and recovery period**

512 **duration for the ATI and ATI+PRA group.** There were no significant differences between the

513 ATI and ATI+PRA group in the latency to RORR and sticky dot notice. There was no difference

514 in the duration of the emergence period as well. The recovery period was significantly shorter for

515 ATI than for ATI+PRA.

516

517 **Figure 7. Timing of waking behavior incidence varies with reversing agent, while order is**

518 **generally maintained.** Animals that did not receive alpha receptor agonists or antagonists

519 (KET+ISO+SAL) notice the sticky dot earlier than animals from the SAL and YOH group The

520 Kruskal-Wallis test indicated a significant difference among groups ($p=0.0052$, χ^2 : 14.79). The

521 significant differences between the groups exclusive K/I-SAL are reported in the results section.

522 Time to sticky dot notice for K/I-SAL was significantly shorter when compared to SAL ($p=0.010$,

523 $AUC=1$), YOH ($p=0.009$, $AUC=0.97$ [0.80 1]). There was no significant difference when

524 compared to the ATI group ($p=0.180$, $AUC=0.75$ [0.42 1])

525

526 **Figure 8. Timing of waking behavior incidence varies with reversing agent, while order is**

527 **generally maintained.** All measured waking behavior hallmarks compared between mice

528 receiving SAL (blue), YOH (red), or ATI (purple). The gray line indicates time of reversal

529 injection, 15 minutes post-ketamine/xylazine injection. Solid lines connect the medians and dotted
530 lines connect the means.

531

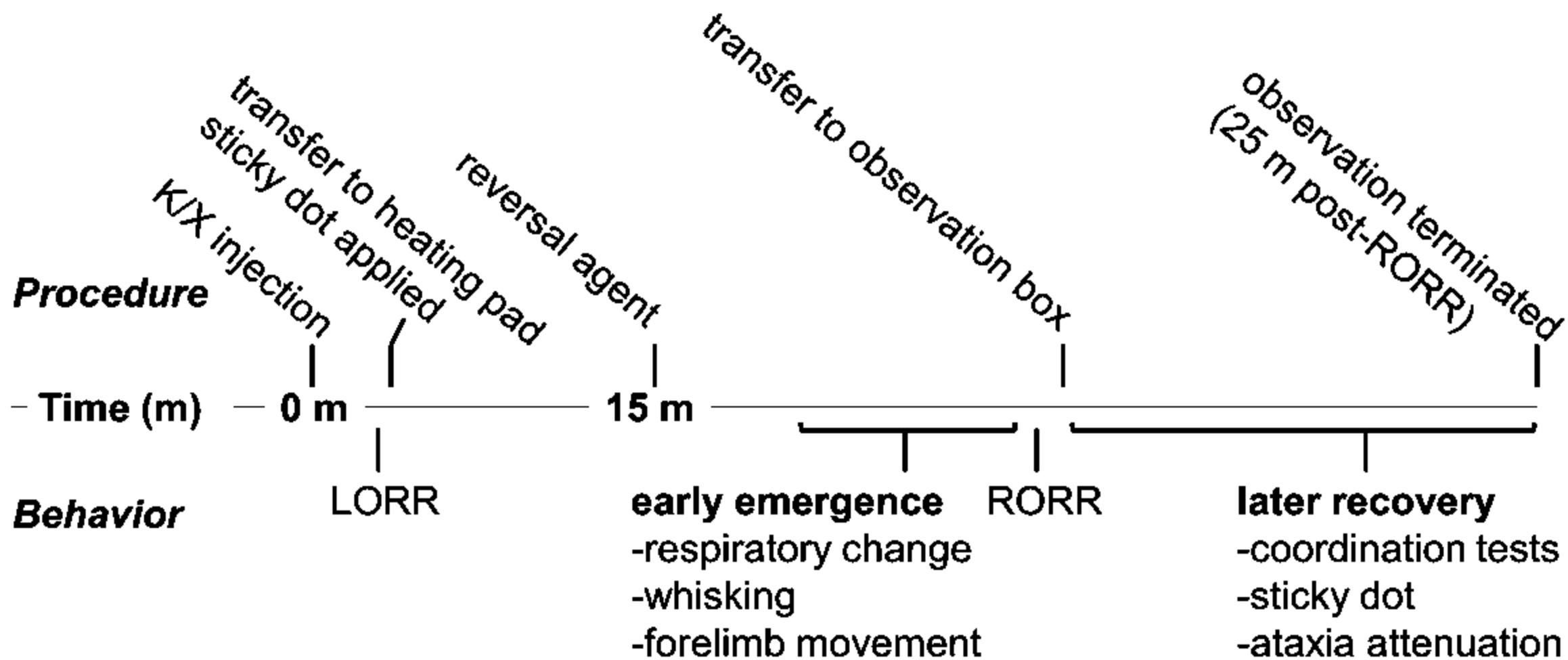
532 **Figure S1. Relative to ketamine/xylazine injection, ATI hastens diagonally cross-matched**
533 **ambulation and ataxia attenuation faster than both SAL and YOH.** A) latency to diagonally
534 cross-matched ambulation; B) latency to ataxia attenuation. Time measurements are from
535 ketamine/xylazine injection. Individual animals are plotted as translucent shapes overlaying lines
536 representing the mean (dashed) and the median (solid). * indicate significance between different
537 groups; the (*) indicates a non-significant $p=0.063$ (MWU), but a very strong effect as indicated
538 by an $AUC=0.90$ [0.60 1].

539

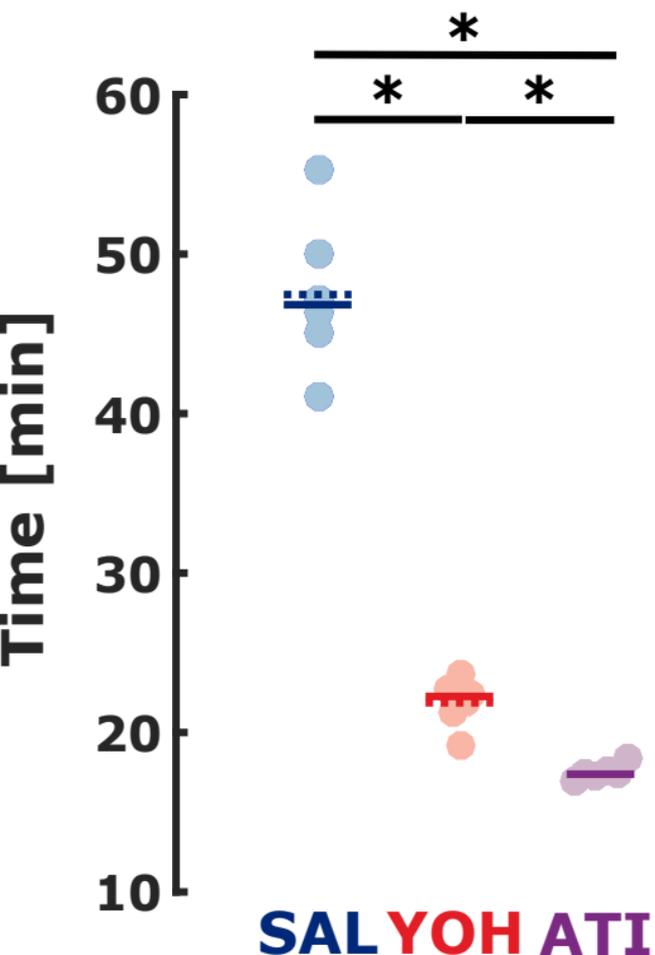
540 **Figure S2. Time from RORR to sticky dot notice (recovery period) shows no difference**
541 **between treatment groups.** Latency from RORR to the first notice of the sticky dot. * indicate
542 significance between different groups; solid bar: median

543

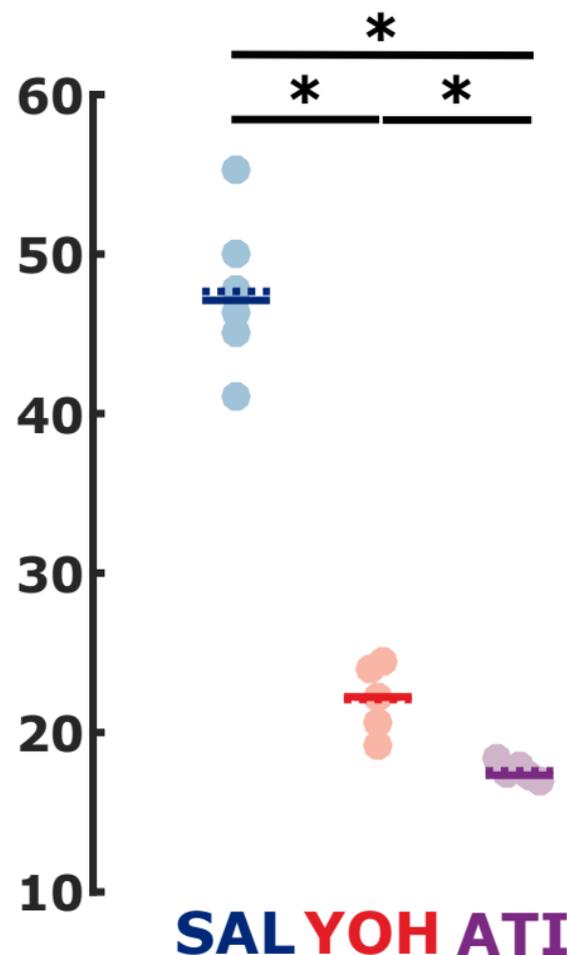
544 **Figure S3. YOH lengthens time to complete emergence and recovery.** The upper graph is an
545 idealized model that schematically depicts the expected pharmacodynamic effects (estimated
546 overall effect of the drugs on the animal) of anesthetic agents over time (x-axis on the same scale
547 as lower graph). Ketamine = yellow line, xylazine = green, xylazine with reversal agent = light
548 green line. Mean latency for emergence period (whisker movement to RORR, solid lines) and
549 recovery period (RORR to sticky dot notice, dashed lines) is plotted on the lower graph. SAL =
550 blue, YOH = red, ATI = purple, K/I-SAL = yellow, ATI+PRA = green. Mean values are plotted.
551 Vertical dashed gray line represents time of reversal agent injection.



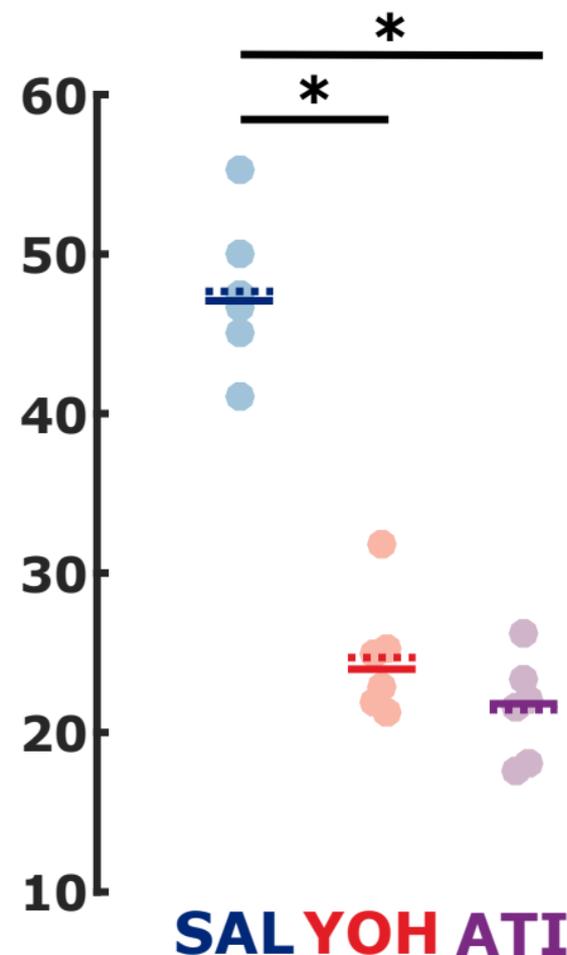
A Whisking



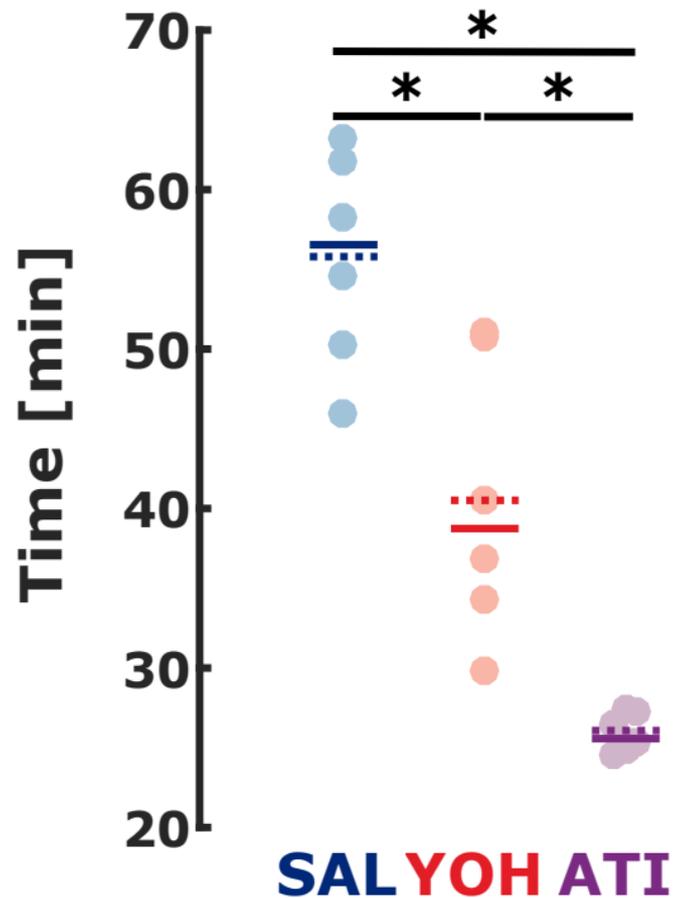
B Respiratory Change



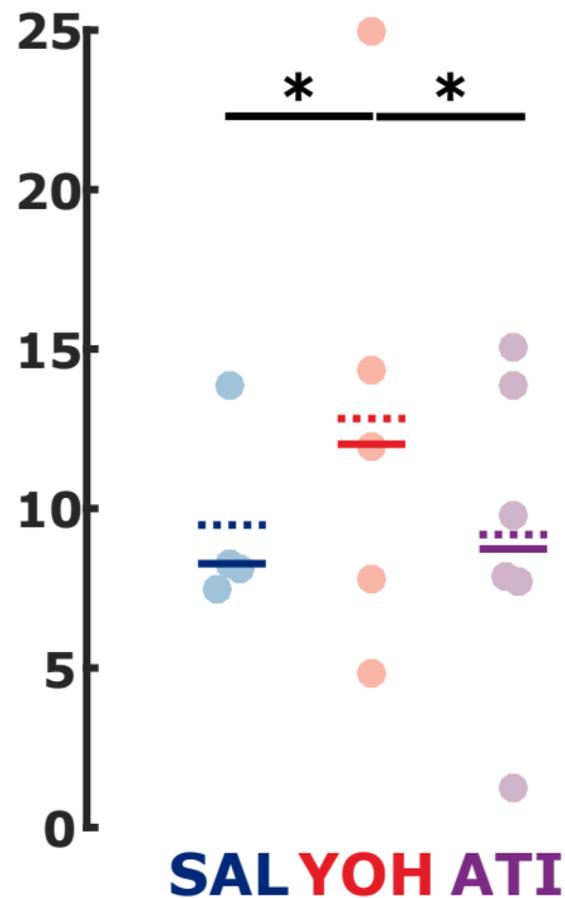
C Forelimb Movement



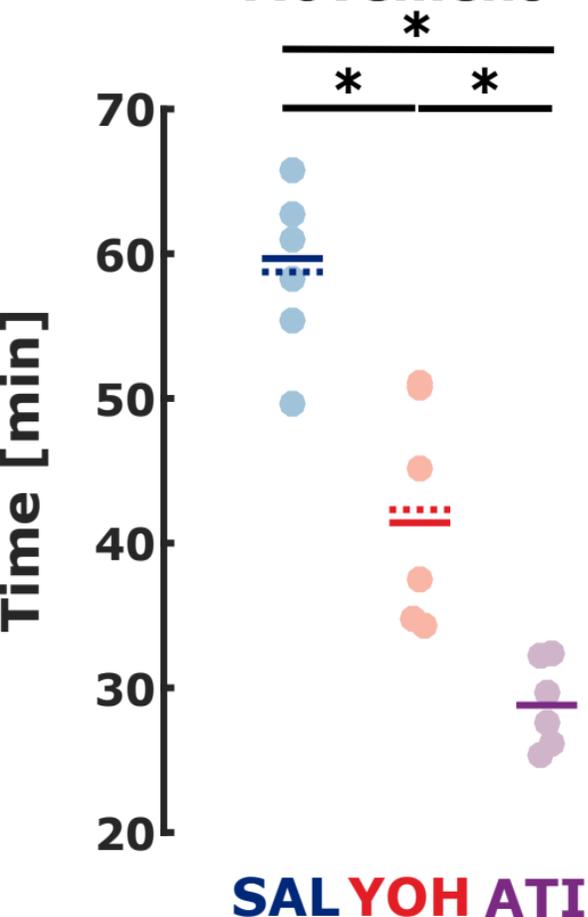
A RORR



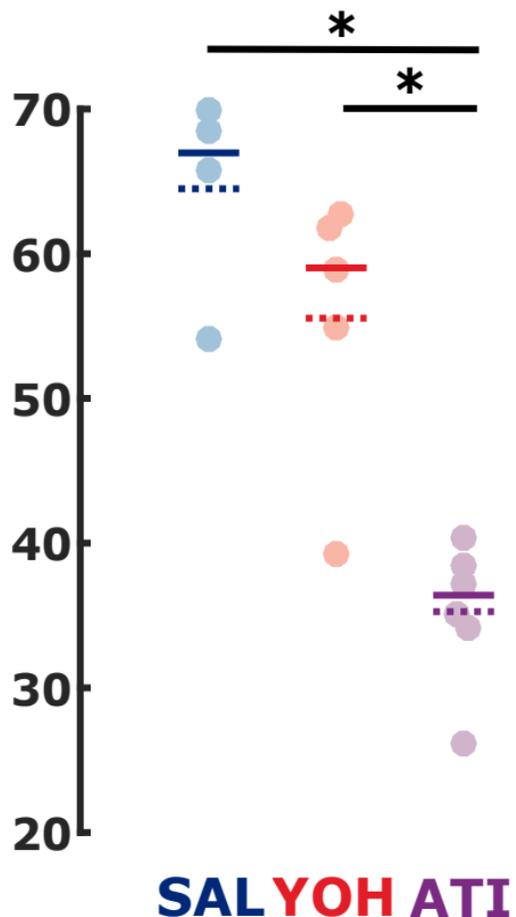
B Delay from first behavior to RORR

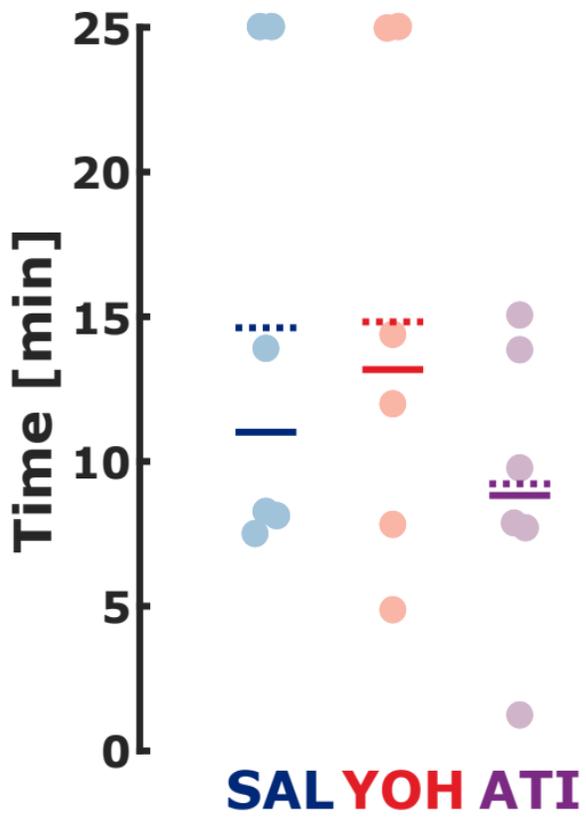


A Uncoordinated Movement

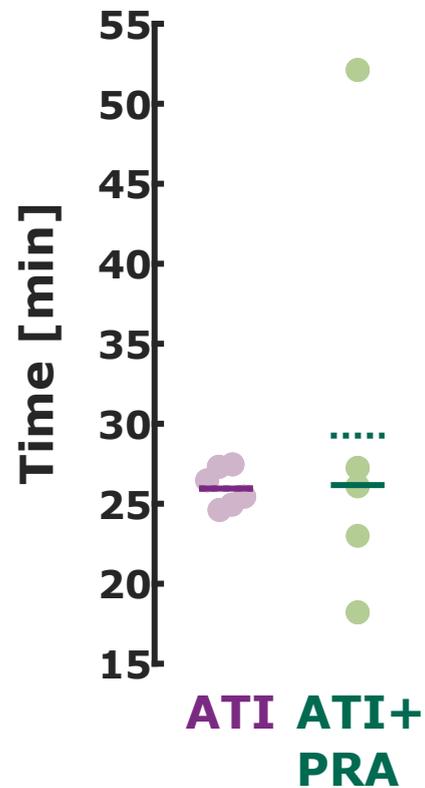


B Sticky Dot Notice

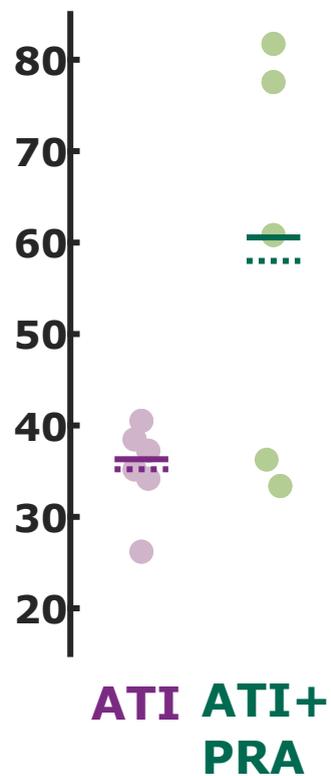




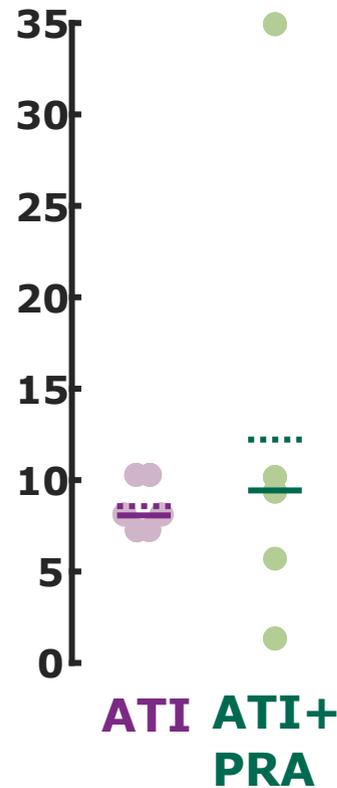
A RORR



B Sticky Dot



C Emergence



D Recovery

