Title: The scheduling of adolescence with Netrin-1 and UNC5C

Authors

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Abstract

Dopamine axons are the only axons known to grow during adolescence. Here, using rodent models, we examined how two proteins, Netrin-1 and its receptor, UNC5C, guide dopamine axons towards the prefrontal cortex and shape behaviour. We demonstrate in mice (*Mus musculus*) that dopamine axons reach the cortex through a transient gradient of Netrin-1 expressing cells – disrupting this gradient reroutes axons away from their target. Using a seasonal model (Siberian hamsters; *Phodopus sungorus*) we find that mesocortical dopamine development can be regulated by a natural environmental cue (daylength) in a sexually dimorphic manner – delayed in males, but advanced in females. The timings of dopamine axon growth and UNC5C expression are always phase-locked. Adolescence is an ill-defined, transitional period; we pinpoint neurodevelopmental markers underlying this period.

Introduction

Adolescence is a critical developmental period involving dramatic changes in behaviour and brain anatomy. The prefrontal cortex, the brain region responsible for our most complex cognitive functions, is still establishing connections during this time (Gogtay et al., 2004; Petanjek et al., 2011; Sowell et al., 2004). The trajectory of prefrontal cortex development in adolescence determines the vulnerability or resilience of individuals to adolescent-onset psychiatric diseases (Fuhrmann et al., 2015; Keshavan et al., 2014; Kessler et al., 2007, 2005; Lee et al., 2014). The age at which this adolescent development occurs therefore represents a critical window during which the brain is particularly susceptible to environmental influences. Traditionally, the onset of adolescence is thought to coincide with puberty (Hollenstein and

44 Lougheed, 2013). In humans, the age of pubertal onset has been advancing throughout the
45 19th, 20th and 21st centuries, and environmental influences, such as nutrition, can pathologically
46 alter the age of puberty (Wolf and Long, 2016). However, it remains entirely unknown whether
47 the neural and cognitive maturational processes of adolescence can also be plastic. Here we
48 examine how the timing of certain adolescent developmental processes are programmed, and
49 whether this timing can be plastic in response to a natural environmental cue, in parallel with
50 pubertal plasticity.

51 Dopamine innervation to the prefrontal cortex increases substantially across adolescence, and
52 psychopathologies of adolescent origin prominently feature dopamine dysfunction. Evidence
53 continues to emerge that protracted dopamine innervation is a key neural process underlying
54 the cognitive and behavioural changes that characterize adolescence (Larsen and Luna, 2018).
55 The mesocorticolimbic dopamine system – which includes the prefrontal cortex – is unique
56 because not only are connections being formed and lost during adolescence, but there is also
57 long-distance displacement of dopamine axons between brain regions. At the onset of
58 adolescence, both mesolimbic and mesocortical dopamine axons innervate the nucleus
59 accumbens in rodents, but the mesocortical axons leave the accumbens and grow towards the
60 prefrontal cortex during adolescence and early adulthood (Hoops et al., 2018; Reynolds et al.,
61 2018a). This is the only known case of axons growing from one brain region to another so late
62 during development (Hoops and Flores, 2017).

63 The prolonged growth trajectory renders mesocortical dopamine axons particularly vulnerable
64 to disruption. Environmental insults during adolescence (e.g. drug abuse) alter the extent and
65 organization of dopamine innervation in the prefrontal cortex, leading to behavioural and

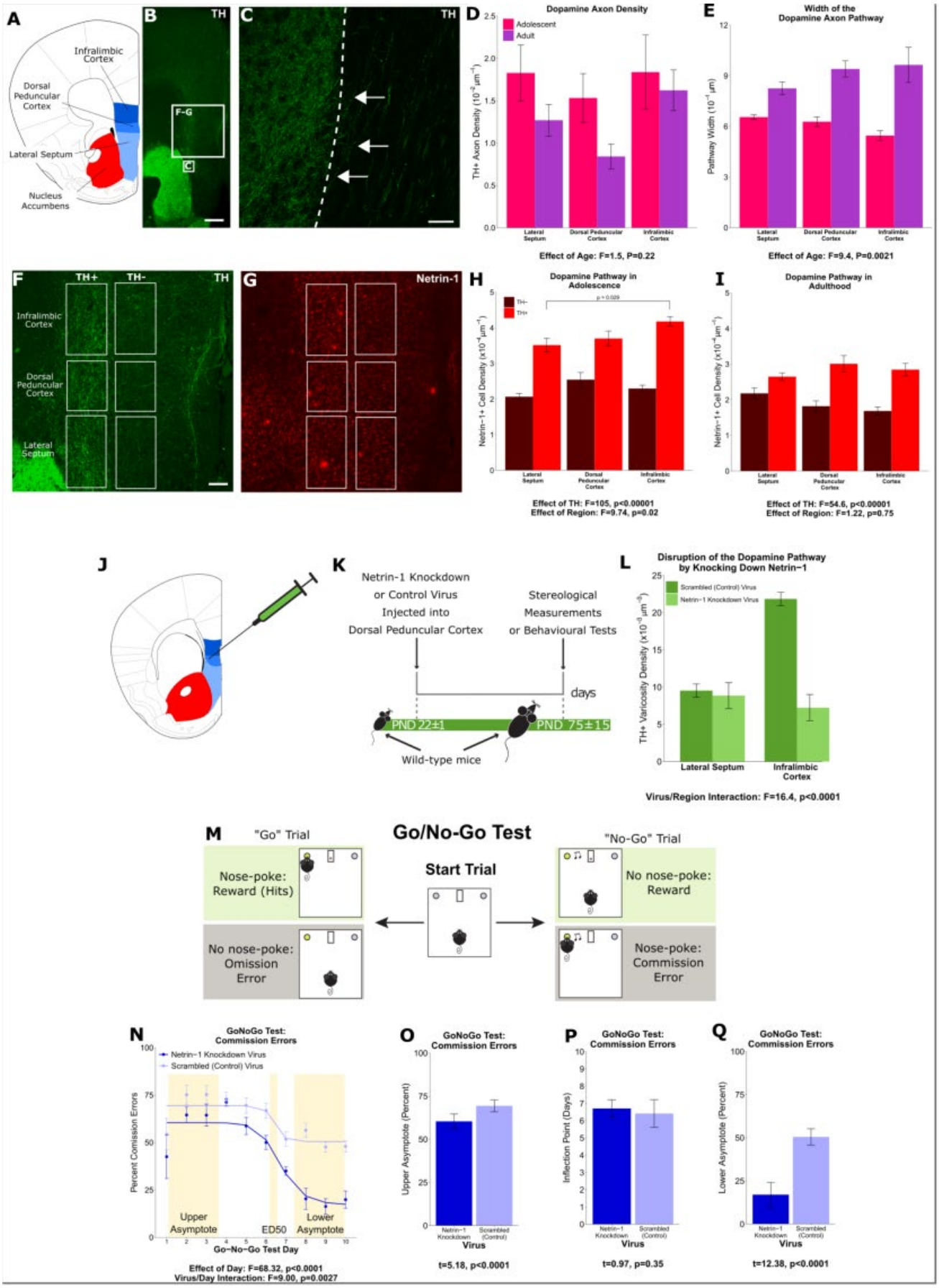
66 cognitive changes in mice throughout adulthood (Drzewiecki and Juraska, 2020; Hoops and
67 Flores, 2017; Reynolds and Flores, 2021). These changes often involve cognitive control, a
68 prefrontal function that develops in parallel with dopamine innervation to the cortex in
69 adolescence (Luna et al., 2015). Disruption of dopamine innervation frequently seems to result
70 in "immature" cognitive control persisting through adulthood (Larsen and Luna, 2018).

71 Here, we examine the guidance of growing dopamine axons to the prefrontal cortex, and its
72 timing. The guidance cue molecule Netrin-1, upon interacting with its receptor DCC, determines
73 *which* dopamine axons establish connections in the nucleus accumbens and which ones leave
74 this region to grow to the prefrontal cortex (Hoops and Flores, 2017; Reynolds and Flores,
75 2021). We hypothesized that the answers to *how* and *when* this extraordinary developmental
76 feat is achieved may also lie in the Netrin-1 signalling system.

77 **Part 1: Netrin-1 "paves the way" for dopamine axons in adolescence**

78 To identify the route by which dopamine axons grow from the nucleus accumbens to the
79 medial prefrontal cortex, we visualized dopamine axons in the adult mouse forebrain. We
80 observed that dopamine axons medial to the nucleus accumbens occupy a distinct area and are
81 oriented dorsally towards the cortex (Figure 1A,B). Individual fibres can be seen crossing the
82 boundary of the nucleus accumbens shell and joining these dorsally-oriented axons (Figure 1C).
83 We hypothesized that these are the fibres that grow to the prefrontal cortex during
84 adolescence. If this is correct, the number of dopamine axons oriented dorsally towards the
85 medial prefrontal cortex should continue to increase until adulthood. To test this, we used a
86 modified unbiased stereological approach (Kim et al., 2011) where axons are counted only if

87 they crossed the upper and lower bounds of a counting probe. We also measured the average
88 width of the area these axons occupy. We found, in both male and female mice, that the
89 density of dopamine axons does not change between adolescence (21 days old) and adulthood
90 (75 days old; Figure 1D). However, the width of the area that dopamine axons occupy does
91 change, increasing between adolescence and adulthood (Figure 1E). These results indicate that
92 the total number of dopamine axons passing through this area increases over adolescence and
93 that dopamine axons grow to the medial prefrontal cortex via this route.


A
Infralimbic Cortex
Dorsal Peduncular Cortex
Lateral Septum
Nucleus Accumbens
B
TH
F-G
C
C
TH
D
Dopamine Axon Density
Adolescent
Adult
TH+ Axon Density (10^-2 µm^-1)
Lateral Septum
Dorsal Peduncular Cortex
Infralimbic Cortex
Effect of Age: F=1.5, P=0.22
E
Width of the Dopamine Axon Pathway
Pathway Width (10^-1 µm)
Effect of Age: F=9.4, P=0.0021
F
TH+
TH-
TH
Infralimbic Cortex
Dorsal Peduncular Cortex
Lateral Septum
G
Netrin-1
H
Dopamine Pathway in Adolescence
p = 0.029
Netrin-1+ Cell Density (x10^-1 µm^-1)
Effect of TH: F=105, p<0.00001
Effect of Region: F=9.74, p=0.02
I
Dopamine Pathway in Adulthood
Effect of TH: F=54.6, p<0.00001
Effect of Region: F=1.22, p=0.75
J
K
Netrin-1 Knockdown or Control Virus Injected into Dorsal Peduncular Cortex
Stereological Measurements or Behavioural Tests
days
PND 22±1
PND 75±15
Wild-type mice
L
Disruption of the Dopamine Pathway by Knocking Down Netrin-1
Scrambled (Control) Virus
Netrin-1 Knockdown Virus
TH+ Varicosity Density (x10^-3 µm^-3)
Lateral Septum
Infralimbic Cortex
Virus/Region Interaction: F=16.4, p<0.0001
M
"Go" Trial
Nose-poke: Reward (Hits)
No nose-poke: Omission Error
Go/No-Go Test
Start Trial
"No-Go" Trial
No nose-poke: Reward
Nose-poke: Commission Error
N
GoNoGo Test: Commission Errors
Netrin-1 Knockdown Virus
Scrambled (Control) Virus
Percent Comission Errors
Upper Asymptote
ED50
Lower Asymptote
Go-No-Go Test Day
Effect of Day: F=68.32, p<0.0001
Virus/Day Interaction: F=9.00, p=0.0027
O
GoNoGo Test: Commission Errors
Upper Asymptote (Percent)
Netrin-1 Knockdown
Scrambled (Control)
Virus
t=5.18, p<0.0001
P
GoNoGo Test: Commission Errors
Inflection Point (Days)
Virus
t=0.97, p=0.35
Q
GoNoGo Test: Commission Errors
Lower Asymptote (Percent)
Virus
t=12.38, p<0.0001

95 **Figure 1.** A "pathway" of Netrin-1 expressing cells "paves the way" for dopamine axons growing from the nucleus
96 accumbens to the medial prefrontal cortex during adolescence. **A,** The brain regions containing the of dopamine
97 fibres passing to the medial prefrontal cortex are highlighted in a line drawing of a coronal mouse brain section
98 derived from Paxinos and Franklin (Paxinos and Franklin, 2013). **B,** An image of a coronal section through the
99 forebrain of an adult mouse at low magnification (4x). Green fluorescence indicates immunostaining for tyrosine
100 hydroxylase (TH), used here as a marker for dopamine. The smaller and larger white squares indicate the regions
101 enlarged in panel C and panels F & G, respectively. Scale bar = 500 µm. **C,** The nucleus accumbens (left of the
102 dotted line) is densely packed with TH+ axons (in green). Some of these TH+ axons can be observed extending from
103 the nucleus accumbens medially towards TH+ fibres oriented dorsally towards the medial prefrontal cortex (white
104 arrows). Scale bar = 10 µm. **D,** Modified stereological quantification revealed no significant difference in TH+ axon
105 density between adolescence (21 days old) and adulthood (75 days old). Mixed-effects ANOVA, effect of age:
106 $F=1.53$, $p=0.22$; region by age interaction: $F=1.44$, $p=0.49$. **E,** The average width of the area that dopamine axons
107 occupy increased significantly from adolescence to adulthood, revealing that there is an increase in the total
108 number of fibres passing to the medial prefrontal cortex during this period. Mixed-effects ANOVA, effect of age:
109 $F=9.45$, $p=0.0021$; region by age interaction: $F=5.74$, $p=0.057$. **F,** In order to quantify the Netrin-1 positive cells
110 along the TH+ fibre pathway, the pathway was contoured in each region, and a contour of equal area was placed
111 medial to the dopamine pathway as a negative control. Scale bar = 200 µm. **G,** Using quantitative stereology,
112 Netrin-1 positive cell density was determined along and adjacent to the pathway for each region. Red fluorescence
113 indicates immunostaining for Netrin-1. **H,** In adolescent mice there are more Netrin-1 positive cells along the fibres
114 expressing TH ("TH+") than medial to them ("TH-"). This is what we refer to as the "Netrin-1 pathway". Along the
115 pathway, there is a significant increase in Netrin-1 positive cell density in regions closer to the medial prefrontal
116 cortex, the innervation target. Mixed-effects ANOVA, effect of TH: $F=105$, $p<0.0001$. Effect of region: $F=9.74$,
117 $p=0.021$. A post-hoc Tukey Test revealed a difference ($p = 0.029$) between the densities of the lateral septum and
118 infralimbic cortex, but only within the dopamine pathway. **I,** In adult mice the Netrin-1 pathway is maintained,
119 however there is no longer an increasing density of Netrin-1 positive cells towards the medial prefrontal cortex.
120 Mixed-effects ANOVA, effect of TH: $F=54.56$, $p<0.0001$. Effect of region: $F=1.22$, $p=0.75$. **J,** The virus injection
121 location within the mouse brain. A Netrin-1 knockdown virus, or a control virus, was injected into the dopamine

122 pathway at the level of the dorsal peduncular cortex. **K,** Our experimental timeline: at the onset of adolescence a
123 Netrin-1 knockdown virus, or a control virus, was injected in wild-type mice. In adulthood the mice were sacrificed
124 and stereological measurements taken. **L,** TH+ varicosity density was quantified in the region below the injection
125 site, the lateral septum, and in the region above the injection site, the infralimbic cortex. There was a significant
126 decrease in TH+ varicosity density only in the infralimbic cortex. Mixed-effects ANOVA, virus by region interaction:
127 $F=16.41$, $p<0.0001$. **M,** The experimental set-up of the final (test) stage of the Go/No-Go experiment. A mouse that
128 has previously learned to nose-poke for a reward in response to a visual cue (illuminated nose-poke hole) must
129 now inhibit this behaviour when the visual cue is paired with an auditory cue (acoustic tone). **N,** Mice injected with
130 the Netrin-1 knockdown virus show improved action impulsivity compared to controls; they incur significantly
131 fewer commission errors across the Go/No-Go task. Mixed-effects ANOVA, effect of day: $F=68.32$, $p<0.0001$. Day
132 by virus interaction: $F=9.00$, $p=0.0027$. A sigmoidal curve is fit to each group of mice to determine how the two
133 groups differ. Points indicate group means and error bars show standard error means. **O,** During the first days of
134 Go/No-Go testing, both groups incur commission errors with high frequency, but the Netrin-1 knockdown group
135 has fewer errors than the control group (T-test, $t=5.18$, $p<0.0001$). **P,** The ED50 – the inflection point in each
136 sigmoidal curve – does not differ between groups, indicating that all mice improve their ability to inhibit their
137 behavior at around the same time (T-test, $t=0.97$, $p=0.35$). **Q,** Mice microinfused with the Netrin-1 knockdown
138 virus incur substantially fewer commission errors in the last days of the Go/No-Go task compared to mice injected
139 with the control virus (T-test, $t=12.38$, $p<0.0001$). For all barplots, bars indicate group means and error bars show
140 standard error means.

141

142 Next, we focussed on Netrin-1, a secreted protein that acts as a guidance cue to growing axons
143 and is important for dopamine axon targeting in the nucleus accumbens in adolescence (Cuesta
144 et al., 2020). Using unbiased stereology, we quantified the number of Netrin-1 expressing cell
145 bodies along the dopamine axon route, and in an adjacent medial region as a control (Figure 1
146 F, G). We found that in adolescence there are more Netrin-1 positive neurons within the

147 dopamine axon route than adjacent to it. Furthermore, along the axon route the density of
148 Netrin-1 positive cells increases towards the medial prefrontal cortex, forming a dorsoventral
149 gradient (Figure 1H). In adulthood, there remains a higher density of Netrin-1 positive cells
150 along the dopamine route compared to the adjacent region, however the dorsoventral gradient
151 is no longer present (Figure 1I).

152 To determine if Netrin-1 along the dopamine axon route is necessary for axon navigation, we
153 silenced Netrin-1 expression in the dorsal peduncular cortex, the transition region between the
154 septum and the medial prefrontal cortex, at the onset of adolescence (Figure 1J,K). In
155 adulthood, we quantified the number of dopamine axon terminals in the regions below and
156 above the injection site. Silencing Netrin-1 did not alter dopamine terminal density below the
157 injection, in the lateral septum (Figure 1L). In the infralimbic cortex, which is the first prefrontal
158 cortical region the axons reach after the injection site, terminal density was reduced in the
159 Netrin-1 knock-down group compared to controls (Figure 1L). The knock-down appears to erase
160 the Netrin-1 path to the prefrontal cortex, resulting in dopamine axons failing to reach their
161 correct innervation target.

162 It remains unknown exactly what types of cells are expressing Netrin-1 along the dopamine
163 axon route, and how this expression is regulated to produce the Netrin-1 gradients that guide
164 the dopamine axons. It also remains unclear where the misrouted axons end up in adulthood.
165 Future experiments aimed at addressing these questions will provide further valuable insight
166 into the nature of the “Netrin-1 pathway”. Nonetheless, our results allow us to conclude that
167 Netrin-1 expressing cells “pave the way” for dopamine axons growing to the medial prefrontal
168 cortex.

169 We next examined how the Netrin-1 pathway may be important for behaviour. Dopamine input
170 to the prefrontal cortex is a key factor in the transition from juvenile to adult behaviours that
171 occurs in adolescence. We hypothesized that cognitive processes involving mesocortical
172 dopamine function would be altered when these axons are misrouted in adolescence. To test
173 our hypothesis, we used the Go/No-Go behavioural paradigm. This test quantifies inhibitory
174 control, which matures in parallel with the innervation of dopamine axons to the prefrontal
175 cortex in adolescence (Casey et al., 2008; Klune et al., 2021; Luna et al., 2015; Paus, 2005;
176 Reynolds and Flores, 2021; Spear, 2000), and it is impaired in adolescent-onset disorders like
177 depression and schizophrenia (Catts et al., 2013; Clementz et al., 2016; McTeague et al., 2016;
178 Millan et al., 2012).

179 At the onset of adolescence, we injected the Netrin-1 silencing, or a scrambled control virus,
180 bilaterally into the dorsal peduncular cortex; in adulthood we tested the mice in the Go/No-Go
181 task. This paradigm first involves discrimination learning and reaction time training
182 (Supplementary Figure 1a), followed by a Go/No-Go test consisting of “Go” trials where mice
183 respond to a cue as previously trained and “No-Go” trials where mice must abstain from
184 responding to the cue (Figure 1M). Correct responses to both trial types are reinforced with a
185 food reward. We quantified the percent of “No-Go” trials where the mice incorrectly responded
186 to the cue (“Commission Errors”) and the percent of “Go” trials where the mice correctly
187 responded (“Rewards” or “Hits”; Supplementary Figure 1b). The ability of mice to respond
188 correctly overall to both trial types is quantified as the Correct Response Rate (Supplementary
189 Figure 1c) (Cuesta et al., 2019; Reynolds et al., 2018a, 2018b; Vassilev et al., 2021).

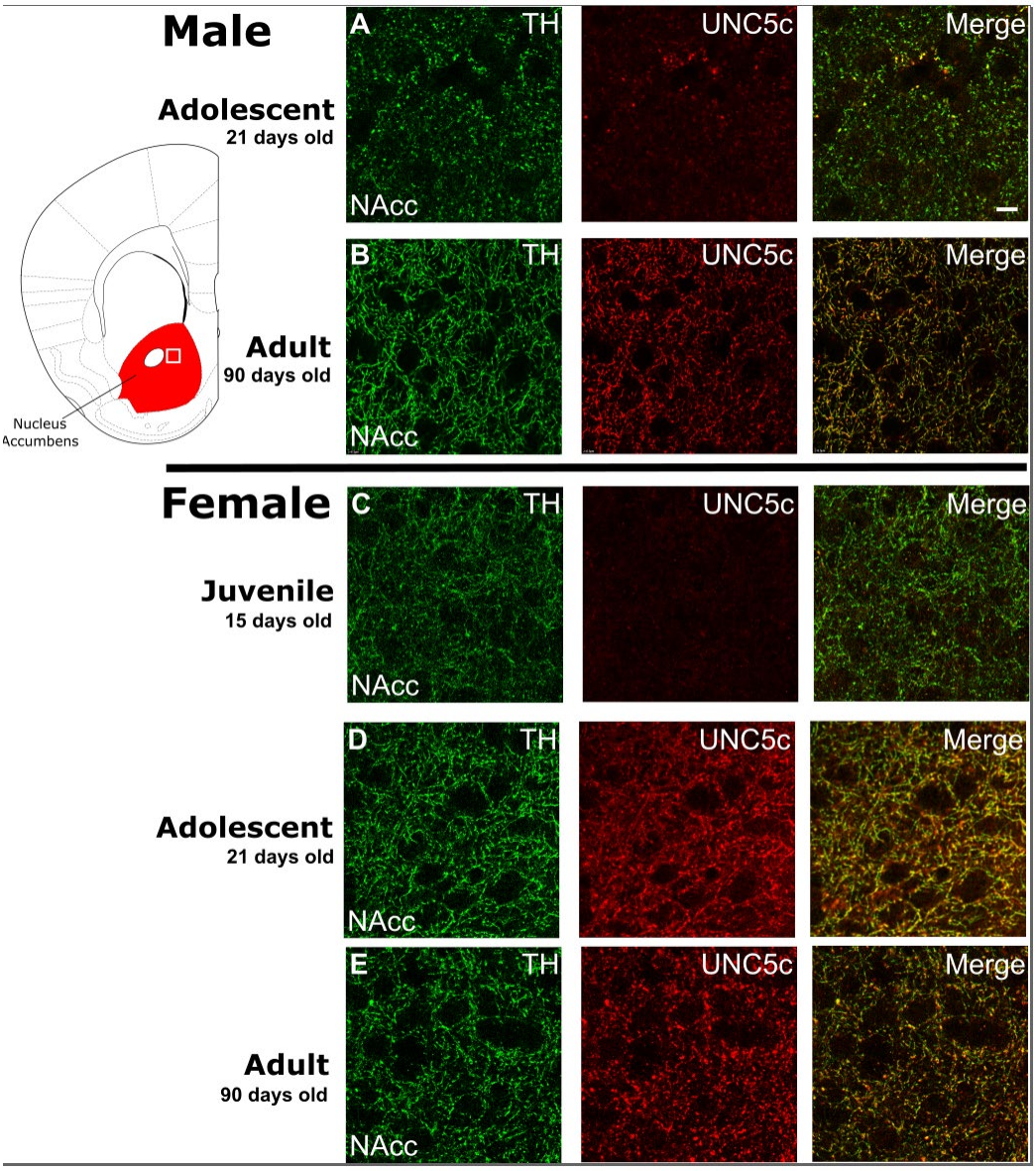
190 Mice injected with the Netrin-1 silencing virus differed from controls in their performance
191 during “No-Go” trials. As the mice learn to withhold their responses over the course of the test,
192 the number of commission errors they made in No-Go trials decreased in a sigmoidal fashion
193 (Figure 1N). The upper and lower asymptotes of the sigmoidal curve quantify the number of
194 commission errors committed during early and late test days, respectively, while the inflection
195 point (ED50) indicates when mice start improving their ability to inhibit their behavior. At the
196 start of the Go/No part of the task, the Netrin-1 silencing group make slightly fewer commission
197 errors (Figure 1O) than control groups, although both groups begin to improve in the No-Go
198 task at around the same time. However, the Netrin-1 silencing group achieved a substantially
199 higher level of behavioural inhibition, quantified as a lower percentage of commission errors in
200 the last test days (Figure 1Q), indicating an improved ability to withhold their behaviour on cue.
201 These behavioural results demonstrate that the maturation of action impulsivity is sensitive to
202 the organization of the ventro-dorsal Netrin-1 path that guides mesocortical dopamine axon
203 growth. Deviations in this route associate with striking changes in the cognitive development
204 that is characteristic of adolescence. In this case, the deviation leads to improved action
205 impulsivity, suggesting that these dopamine axons may end up ectopically innervating a
206 forebrain region other than the medial prefrontal cortex, enhancing cognitive control.

207

208 **Part 2: UNC5C expression coincides with the onset of adolescence**

209 When axons leave the nucleus accumbens during adolescence, they follow a Netrin-1 “path”
210 through intermediate brain regions to reach their intended innervation target. However, only a

211 small subset of the dopamine axons that have reached the nucleus accumbens by early
212 adolescence leave; the vast majority stay and form connections in the accumbens throughout
213 life (Reynolds et al., 2018a). The “decision making” process of whether to “stay” (in the
214 accumbens) or “go” (to the cortex via the Netrin-1 path) happens during a narrow
215 developmental window at the onset of adolescence (Reynolds et al., 2018b). It remains
216 unknown how the timing of this process is determined.



217

218 **Figure 2** The age of onset of UNC5C expression by dopamine axons in the nucleus accumbens of mice is sexually
219 dimorphic. Images are representative of observed immunofluorescence patterns in the nucleus accumbens
220 (approx. location highlighted as a white square in the coronal mouse brain section Plate 19, modified from Paxinos
221 & Franklin, 2013). No qualitative differences were noted between the shell and core of the nucleus accumbens. For
222 each row, six individuals were sampled. In males (**A-B**), UNC5C expression on dopamine fibres (here identified by
223 immunofluorescent staining for tyrosine hydroxylase, TH) in the nucleus accumbens appears during adolescence.
224 **A,** At the onset of adolescence (21 days old) dopamine fibres do not express UNC5C. Scale bar = 10 µm. **B,** By
225 adulthood (90 days old), dopamine fibres express UNC5C. In females (**C-E**), UNC5C expression on dopamine fibres
226 in the nucleus accumbens appears prior to adolescence. **C,** In juvenile (15 day old) mice, there is no UNC5C
227 expression on dopamine fibres. **D,** By adolescence, dopamine fibres express UNC5C. **E,** In adulthood, dopamine
228 fibres continue to express UNC5C.

229

230 In adolescence, dopamine neurons begin to express the repulsive Netrin-1 receptor UNC5C,
231 particularly when mesolimbic and mesocortical dopamine projections segregate in the nucleus
232 accumbens (Manitt et al., 2010; Reynolds et al., 2018a). In contrast, dopamine axons in the
233 prefrontal cortex do not express UNC5c, except in very rare cases (Supplementary Figure 2a). In
234 adult male mice with *Unc5c* haploinsufficiency, there appears to be ectopic growth of
235 mesolimbic dopamine axons to the prefrontal cortex (Auger et al., 2013). This miswiring is
236 associated with alterations in prefrontal cortex-dependent behaviours (Auger et al., 2013).

237 Using immunohistochemistry, we assessed the expression of UNC5C on nucleus accumbens
238 dopamine axons across development. In male mice, we found little expression of UNC5C on
239 dopamine axons at the onset of adolescence (Figure 2A), while we did find UNC5C expression
240 on dopamine axons in adults (Figure 2B). Remarkably, when we assessed this in females, we

241 found dopamine axons already expressing UNC5C in the nucleus accumbens at the onset of
242 adolescence (Figure 2D), similar to adult females (figure 2E), indicating that the onset of UNC5C
243 expression on dopamine axons in the nucleus accumbens is sexually dimorphic, with an earlier
244 emergence in females. We examined the nucleus accumbens in pre-adolescent female mice
245 and indeed found little UNC5C expression on dopamine axons (Figure 2C). The onset of UNC5C
246 expression in mesocorticolimbic dopamine axons is therefore peri-adolescent but occurs earlier
247 in females than in males, consistent with the earlier emergence of adolescence in female
248 rodents and the earlier onset of adolescence and puberty in humans (Wolf and Long, 2016).
249 Differences in the precise timing of dopamine innervation to the PFC in adolescence have been
250 suggested by findings reported in male and female rats (Willing et al., 2017)".

251 **Part 3: Environmental control of the timing of adolescence**

252 We hypothesize that at the emergence of adolescence, UNC5C expression by dopamine axons
253 in the nucleus accumbens signals the initiation of the growth of dopamine axons to the
254 prefrontal cortex. We therefore examined whether the developmental timings of UNC5C
255 expression and dopamine innervation of the prefrontal cortex are similarly affected by an
256 environmental cue known to delay pubertal development in seasonal species.

257 Siberian hamsters (*Phodopus sungorus*) regulate many aspects of their behavior and physiology
258 to meet the changing environmental demands of seasonality (Paul et al., 2008; Stevenson et al.,
259 2017). In winter, they increase the thickness of their fur, exchange their brown summer coats
260 for white winter ones, and undergo a daily torpor to conserve energy (Scherbarth and
261 Steinlechner, 2010). In addition, adults suppress reproduction and juveniles delay puberty

262 (Pevet, 1988; Yellon and Goldman, 1984), including developmental changes in gonadotropin
263 releasing hormone neurons in the hypothalamus (Buchanan and Yellon, 1991; Heywood and
264 Yellon, 1997). Reproductive organ development is delayed as part of pubertal postponement
265 (Darrow et al., 1980; Ebling, 1994; Timonin et al., 2006). This seasonal plasticity is regulated by
266 long or short periods of daylight (Heldmaier and Steinlechner, 1981; Hoffmann, 1978) and
267 raises the possibility that aspects of adolescent development are sensitive to these
268 environmental cues. To our knowledge, adaptive variation in the timing of adolescent neural
269 development has never been recorded in any animal.

270 Here, we tested whether day length regulates *when* dopamine axons grow to the cortex, and
271 whether the timing of UNC5C expression in the nucleus accumbens and adolescent changes in
272 behavior are similarly affected.

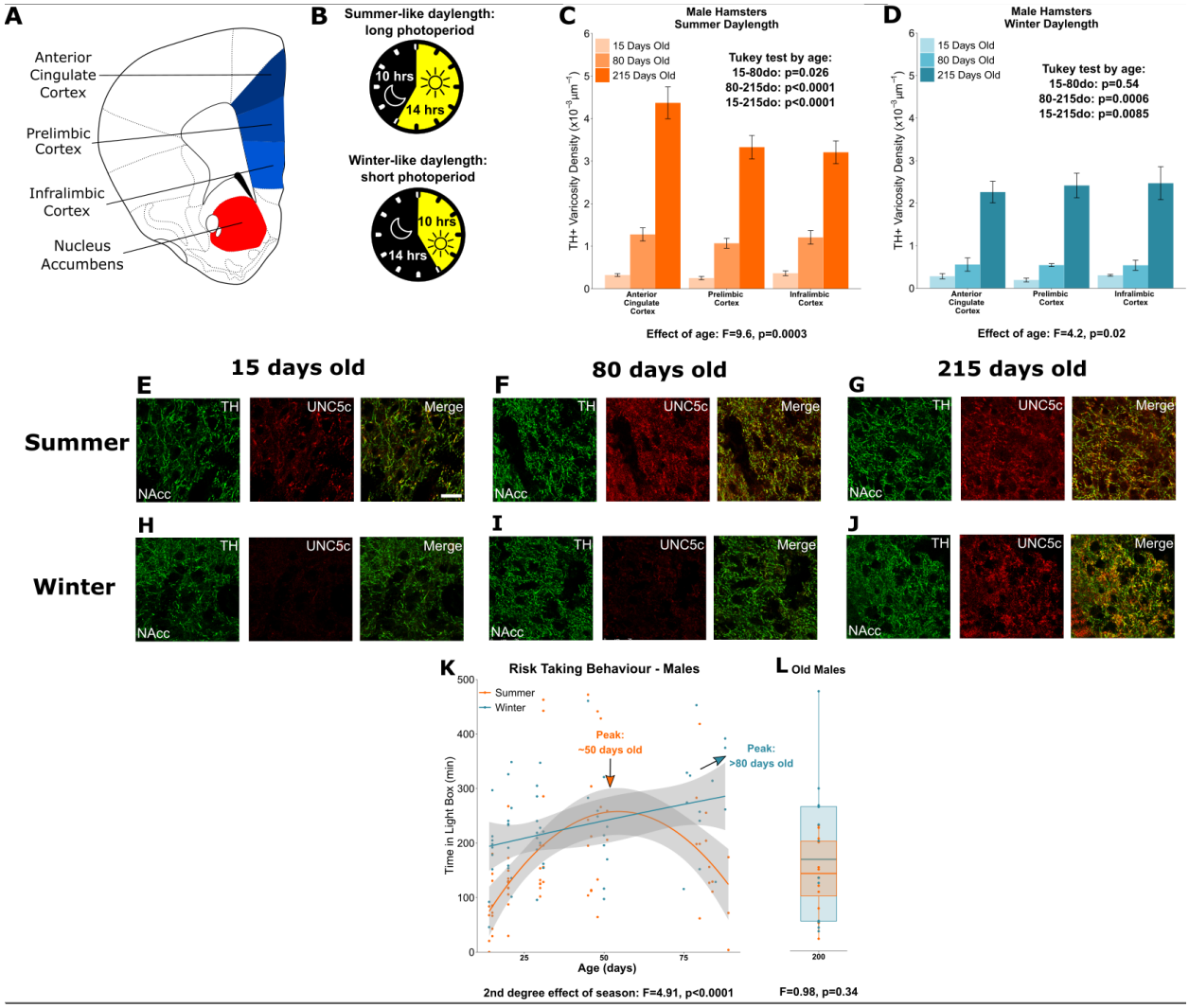
273

274 **3.i The seasonality of adolescence**

275 Male hamsters were examined at three ages: 15 days old (±1), 80 days old (±10), and 215 days
276 old (±20). We compared the density of the dopamine innervation to the medial prefrontal
277 cortex in male hamsters housed under lighting conditions that replicate summer daylengths
278 (long days, short nights) or winter daylengths (short days, long nights) (Figure 3A,B). We will
279 refer to these two groups as “summer hamsters” and “winter hamsters” to emphasize the
280 natural stimulus we are replicating in the laboratory environment. We confirmed that puberty
281 is delayed in male winter hamsters compared to summer hamsters in the present experiment
282 by measuring their gonadal weights across ages (Supplementary Figure 3a).

283 In male summer hamsters, dopamine input density to the prefrontal cortex increases during
284 adolescence, after 15 days old and before 80 days old (Figure 3C), consistent with dopamine
285 axon growth in mice (Manitt et al., 2013, 2011; Reynolds et al., 2018a). Prefrontal cortex
286 dopamine innervation in summer hamsters continues to increase after 80 days old (Figure 3C).

287 In male winter hamsters, dopamine innervation to the prefrontal cortex is delayed until after 80
288 days, which coincides with their delayed pubertal onset (Figure 3D, Supplementary Figure 3a).
289 This demonstrates that an environmental cue can determine the timing of adolescent brain
290 development.



291

292 **Figure 3** Plasticity of adolescent development in male Siberian hamsters according to seasonal phenotype. All
293 results illustrated in this figure refer to results in male hamsters. **A,** Dopamine innervation was quantified in three
294 subregions of the medial prefrontal cortex, highlighted in blue. UNC5C expression was examined in the nucleus
295 accumbens, highlighted in red. Line drawing of a coronal section of the mouse brain was derived from Plate 19 of
296 Paxinos and Franklin (Paxinos and Franklin, 2013). **B,** Hamsters were housed under either summer-mimicking long
297 days and short nights ("summer hamsters") or winter-mimicking short days and long nights ("winter hamsters"). **C,**
298 In male hamsters housed under a summer-mimicking daylength there is an increase in dopamine varicosity density
299 in the medial prefrontal cortex between 15 and 80 days old. Mixed-effects ANOVA, effect of age: F=9.6,
300 p=0.000255. Tukey Test, 15-80 days old (do): p=0.026; 80-215do: p<0.0001; 15-215do: p<0.0001. **D,** In male
301 hamsters housed under a winter-mimicking daylength there is no increase in dopamine varicosity density until
302 hamsters have reached 215 days of age. Mixed-effects ANOVA, effect of age: F=4.17, p=0.0205. Tukey Test, 15-
303 80do: p=0.54; 80-215do: p=0.0006; 15-215do: p=0.0085. **E,** At 15 days old, dopamine axons (here identified by
304 immunofluorescent staining for tyrosine hydroxylase, TH) in the nucleus accumbens of male summer-daylength
305 hamsters largely do not express UNC5C. Scale bar = 20um (bottom right). **F-G,** At 80 (**F**) and 215 (**G**) days old,
306 dopamine axons in the nucleus accumbens express UNC5C. **H-I,** At 15 (**H**) and 80 (**I**) days old, dopamine axons in
307 the nucleus accumbens of male winter hamsters largely do not express UNC5C. **J,** By 215 days old there is UNC5C
308 expression in dopamine axons in the nucleus accumbens of male winter hamsters. **E-J**, Representative images of
309 the nucleus accumbens shell, 6 individuals were examined per group. **K,** Male hamsters house under a summer-
310 mimicking daylength show an adolescent peak in risk taking in the light/dark box apparatus. Those raised under a
311 winter-mimicking photoperiod show a steady increase in risk taking over the same age range. Arrows indicate the
312 ages at which risk taking peaks in summer (orange) and winter (blue) hamsters. Polynomial regression, effect of
313 season: F=3.551, p=0.00056. **L,** In male hamsters, at 215 days of age, there is no difference in risk taking between
314 hamsters raised under summer and winter photoperiods. T-test, effect of season: t=0.975, p=0.341. For all
315 barplots, bars indicate group means and error bars show standard error means.

316

317 We then examined UNC5C expression by dopamine axons in the nucleus accumbens in male
318 summer and winter hamsters across age classes. UNC5C expression was apparent only after the
319 onset of adolescence in summer hamsters (Figure 3E,F,G), as observed in male mice. However,
320 UNC5C expression was delayed in male winter hamsters – this group did not show UNC5C
321 expression in dopamine axons in the nucleus accumbens until after 80 days old (Figure 3H,I,J).
322 This aligns with the delayed timing of mesocortical dopamine axon growth and pubertal onset
323 in male winter hamsters and demonstrates that the emergence of UNC5C is a marker of
324 adolescent onset in male mice.

325 A behavioural characteristic of adolescence is increased willingness to enter a novel
326 environment, a behaviour that assumes an increased amount of risk (Arrant et al., 2013; Lynn
327 and Brown, 2009). To measure this, we used the light/dark test (Bourin and Hascoët, 2003).
328 Time spent in the light compartment is dopamine-dependent (Bahi and Dreyer, 2019; Gao and
329 Cutler, 1993) and peaks in adolescence (Arrant et al., 2013). We will refer to this behaviour as
330 "risk taking". We assessed the developmental profile of risk taking in the light/dark box test in
331 summer and winter hamsters across adolescence. In male summer hamsters, the risk taking
332 increases across adolescence, peaks around 50 days, then subsequently declines (Figure 3K).
333 However, the adolescent increase in risk taking is protracted in winter hamsters: across the age
334 range examined we observe a gradual, consistent increase in risk taking rather than a peak and
335 decline.

336 We next assessed a cohort of 215-day old hamsters, for which both summer and winter male
337 hamsters have undergone puberty and exhibit high levels of dopamine innervation of the
338 prefrontal cortex (Figure 3C,D,G,J, Supplementary Figure 3a). In these hamsters, we find no

339 difference in risk taking between the male summer and winter groups (Figure 3L),
340 demonstrating that, after 80 days, risk taking begins to decline in male winter hamsters and
341 that by 215 days it has declined to the same level as in summer hamsters. Male hamsters raised
342 under summer-mimicking long days and winter-mimicking short days both ultimately make the
343 transition to the adult behavioral phenotype.

344 **3.ii An extraordinary case of decoupling puberty and adolescence**

345 In parallel with males, we conducted equivalent experiments in female hamsters (Figure 4A,B).
346 Under a summer-mimicking daylength, dopamine innervation to the medial prefrontal cortex
347 increases between 15 and 80 days old, similar to male summer hamsters (Figure 4C). There is
348 no further increase in innervation density after 80 days old, consistent with earlier adolescent
349 development in females observed in other rodent species (Juraska and Willing, 2017; Kopec et
350 al., 2018; Reynolds and Flores, 2021; Spear, 2000; Westbrook et al., 2018). We confirmed that
351 puberty is delayed in female winter hamsters compared to summer hamsters by measuring
352 their uterine weights (Supplementary Figure 4a) and vaginal opening (Supplementary Figure 4b)
353 across ages.

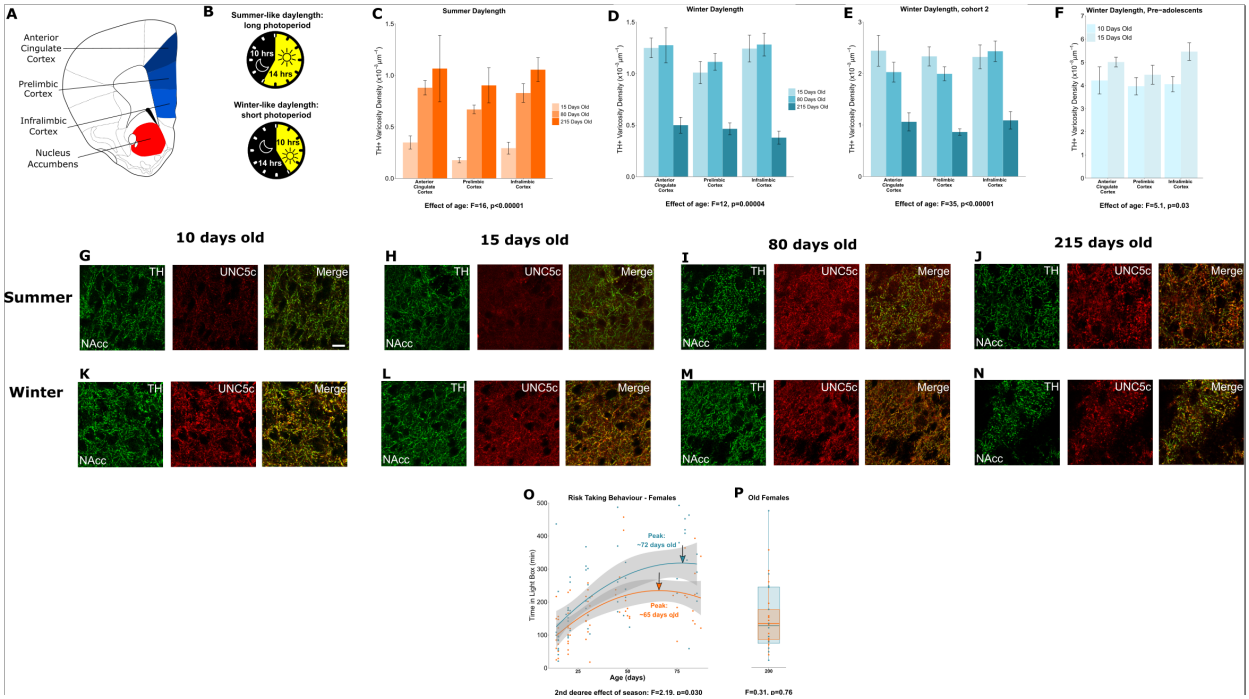


Figure 4 Plasticity of adolescent development in female Siberian hamsters according to seasonal phenotype. All results illustrated in this figure refer to results in female hamsters. **A,** Dopamine innervation was quantified in three subregions of the medial prefrontal cortex, highlighted here in blue. UNC5C expression was examined in the nucleus accumbens, highlighted in red. Line drawing of a coronal section of the mouse brain was derived from Paxinos and Franklin (Paxinos and Franklin, 2013). **B,** Hamsters were housed under either a summer-mimicking or winter-mimicking daylength. **C,** In female hamsters housed under a summer daylength dopamine varicosity density in the medial prefrontal cortex increases between 15 and 80 days of age. Mixed-effects ANOVA, effect of age: F=16.72, p<0.0001 **D,** In female hamsters housed under a winter daylength there is no increase in dopamine varicosity density post-adolescence. Instead, there is a steep decline in density between 80 and 215 days of age. Mixed-effects ANOVA, effect of age: F=12.33, p=0.000043. **E,** As our results in panel D were unexpected, we replicated them with a second cohort of hamsters and found qualitatively identical results. Mixed-effects ANOVA, effect of age: F=34.871, p<0.0001. **F,** To try and determine when dopamine varicosities innervate the medial prefrontal cortex, we examined a cohort of 10- and 15-day-old hamsters. We found that varicosity density increases in the medial prefrontal cortex during this time, indicating that dopamine innervation to the medial prefrontal cortex is accelerated in female winter hamsters. Mixed-effects ANOVA, effect of age: F=5.05, p=0.03. **G-**

370 **H,** In 10- and 15-day-old female summer hamsters there is little UNC5C expression in nucleus accumbens
371 dopamine axons (here identified by immunofluorescent staining for tyrosine hydroxylase, TH). Sample size: 4
372 (panel G) or 6 (panel H). **I-J,** By 80 days old (panel I), and continuing at 215 days old (panel J), dopamine axons in
373 the nucleus accumbens express UNC5C in female summer hamsters. Sample sizes: 6. Scale bar = 20um (panel G
374 bottom right). **K-N,** At all ages which winter female hamsters were examined, dopamine axons in the nucleus
375 accumbens express UNC5C in winter female hamsters. Sample sizes: 4 (panel K) or 6 (panels L-N). **O,** In female
376 hamsters, those raised under summer and winter daylengths both show an increase in risk taking over time. The
377 winter hamsters peak later compared to the summer daylength hamsters. Arrows indicate the ages at which risk
378 taking peaks in summer (orange) and winter (blue) hamsters. Polynomial regression, effect of season: F=3.305,
379 p=0.00126. **P,** In female hamsters, at 215 days of age, there is no difference in risk taking between hamsters raised
380 under summer and winter photoperiods. T-test, effect of season: t=0.309, p=0.76. For all barplots, bars indicate
381 group means and error bars show standard error means.

382

383 When housed under a winter-mimicking daylength, dopamine input density in the prefrontal
384 cortex of female hamsters is *not* delayed as in males, but rather reaches adult levels prior to 15
385 days old (Figure 4D). We replicated this unexpected finding in a separate, independent cohort
386 of female winter hamsters (Figure 4E). This surprising result shows an intervention that
387 accelerates adolescent cortical development.

388 We then measured dopamine axon density in female winter hamsters at two earlier ages: 10
389 and 15 days old. Dopamine innervation increases during this period (figure 4F), well before
390 normal adolescence and long before pubertal development. This is an extraordinary
391 phenomenon: a key marker of adolescent neurodevelopment is accelerated and dissociated

392 from puberty in female hamsters raised under winter-mimicking short days (Supplementary
393 Figure 4a,4b).

394 The early increase in prefrontal cortex dopamine terminals in winter females is followed by a
395 dramatic reduction between 80 and 215 days old (Figure 4D,E). This overlaps with the delayed
396 timing of puberty in these females (Butler et al., 2007; Supplementary Figure 4a,4b). Synaptic
397 pruning in the cortex is a well-known component of adolescent neural development across
398 species (Huttenlocher, 1984; Koss et al., 2013; Petanjek et al., 2011). Under normal conditions,
399 the effect of pruning on dopamine synapses is likely masked by the growth of new dopamine
400 axons to the prefrontal cortex (Manitt et al., 2013, 2011; Reynolds et al., 2018a). In the case of
401 female winter hamsters, we hypothesize that the growth of dopamine axons to the prefrontal
402 cortex occurs early while synaptic pruning, including of dopamine synapses, appears to occur
403 later. This leads to a remarkable dissociation between two cortical developmental processes
404 that are normally simultaneous, the behavioural implications of which are unclear.

405 If the developmental onset of UNC5C expression determines the timing of dopamine
406 innervation of the prefrontal cortex, then onset of UNC5C expression should also be advanced
407 in female winter hamsters. Hence, we examined UNC5C expression at the same ages as we
408 examined dopamine axon growth in female hamsters. At 10 and 15 days old, UNC5C
409 expression is present *only* in the winter hamsters (Figure 4G,H,K,L), but at 80 and 215 days old,
410 UNC5C expression is apparent in both summer and winter hamsters (Figure 4I,J,M,N).

411 We used the light/dark box test to examine potential risk taking implications of the
412 extraordinary developmental trajectory we observed in the prefrontal cortex of female

413 hamsters. In female summer and winter hamsters, the adolescent increase and peak in risk
414 taking occurs between the ages of 15 and 80 days, as it does in summer daylength males
415 (Figure 4O). However, contrary to what we would expect, the peak in winter females is delayed
416 compared to summer females. When we assessed an independent cohort of 215-day-old
417 female hamsters, we found no difference in risk taking between groups (Figure 4R), indicating
418 that, like males, female summer and winter hamsters both eventually reach the same adult
419 level of risk taking.

420 In both sexes, hamsters housed under a summer-mimicking daylength showed an adolescent
421 peak in risk taking at an age that we would predict based on results from other rodents (Arrant
422 et al., 2013; Pietropaolo et al., 2004; Tanaka, 2015). When raised under a winter-mimicking
423 daylength, hamsters of either sex show a protracted peak in risk taking. In males, it is delayed
424 beyond 80 days old, but the delay is substantially less in females. This is a counterintuitive
425 finding considering that dopamine development in winter females appears to be accelerated.
426 Our interpretation of this finding is that the timing of the risk taking peak in females may reflect
427 a balance between different adolescent developmental processes. The fact that dopamine axon
428 growth is accelerated does not imply that all adolescent maturational processes are
429 accelerated. Some may be delayed, for example those that induce axon pruning in the cortex.
430 The timing of the risk taking peak in winter female hamsters may therefore reflect the
431 amalgamation of developmental processes that are advanced with those that are delayed –
432 producing a behavioural effect that is timed somewhere in the middle. Disentangling the effects
433 of different developmental processes on behaviour will require further experiments in

434 hamsters, including the direct manipulation of dopamine activity in the nucleus accumbens and
435 prefrontal cortex.

436 **Conclusion**

437 Here we describe how the gradual growth of mesocortical dopamine axons marks adolescent
438 development, and how this process uses guidance cues and is sensitive to sex and environment.
439 Netrin-1 signalling provides the “stay-or-go” “decision making” conducted by dopamine axons
440 that innervate the nucleus accumbens at the onset of adolescence (Cuesta et al., 2020). UNC5C
441 expression by these dopamine axons marks the timing at which this decision is made. In mice,
442 UNC5C expression coincides with sex differences in both adolescent and pubertal development.
443 Females, which develop earlier, show earlier UNC5C expression in dopamine axons compared
444 to males.

445 In hamsters, behavioural and developmental shifts in response to environmental cues occur in
446 parallel with alterations in the timing of dopamine axon growth. As we show here, male
447 hamsters raised under a winter-mimicking daylength delay not only puberty, but also
448 adolescent dopamine and behavioural maturation. In contrast, female hamsters under identical
449 conditions delay puberty but accelerate dopamine axon growth, a key marker of adolescent
450 brain development. Behavioural shifts during adolescence appear to be delayed in these
451 females, but less substantially than in male hamsters. Notably, under all conditions, the
452 developmental timing of UNC5C expression corresponds to the timing of dopamine innervation
453 of the prefrontal cortex.

454 In both mice and hamsters, the emergence of UNC5C expression coincides with the onset of
455 dopamine axon growth to the prefrontal cortex, a key characteristic of the adolescent transition
456 period. While previously we have shown that the Netrin-1 signalling in the nucleus accumbens
457 is responsible for coordinating *whether* dopamine axons grow in adolescence (Reynolds and
458 Flores, 2021), here we propose that Netrin-1 signalling is also key to determining *how* and *when*
459 this marker of adolescence occurs.

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468 Formal Analysis: DH
469 Investigation: DH, RFK, SS, EE, AH, TO, AD, CP, JZ, KCS, LPL, QEC
470 Investigation (post-review experiments): DM, RGA
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475 Visualization: DH
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