

The scheduling of adolescence with Netrin-1 and UNC5C

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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.

eLife assessment

This study provides **useful** information regarding the role of Netrin-1 and Unc5c in developing dopaminergic axons. Several findings are **solid**. These include that Netrin-1 and UNC5c can guide dopaminergic innervation from the nucleus accumbens to the cortex during adolescence, that the onset of Unc5 expression is sexually dimorphic in mice, and that in Siberian hamsters environmental effects on development are also sexually dimorphic. While this work is novel and provides **useful** information, the reviewers unanimously agree that support for the main claims remains **incomplete**. Understanding developmental trajectories of adolescence, and how they can be impacted by environmental factors, is an understudied area of neuroscience that is highly relevant to understanding the onset of mental health disorders. These data will be of interest to those interested in these factors.

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

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

The prolonged growth trajectory renders mesocortical dopamine axons particularly vulnerable to disruption. Environmental insults during adolescence (e.g. drug abuse) alter the extent and organization of dopamine innervation in the prefrontal cortex, leading to behavioural and cognitive changes in mice throughout adulthood (Drzewiecki and Juraska, 2020; Hoops and Flores, 2017; Reynolds and Flores, 2021). These changes often involve cognitive control, a prefrontal function that develops in parallel with dopamine innervation to the cortex in adolescence (Luna et al., 2015). Disruption of dopamine innervation frequently seems to result in “immature” cognitive control persisting through adulthood (Larsen and Luna, 2018).

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

Part 1: Netrin-1 “paves the way” for dopamine axons in adolescence

To identify the route by which dopamine axons grow from the nucleus accumbens to the medial prefrontal cortex, we visualized dopamine axons in the adult mouse forebrain. We observed that dopamine axons medial to the nucleus accumbens occupy a distinct area and are oriented dorsally

towards the cortex (**Figure 1A,B** [↗](#)). Individual fibres can be seen crossing the boundary of the nucleus accumbens shell and joining these dorsally-oriented axons (**Figure 1C** [↗](#)). We hypothesized that these are the fibres that grow to the prefrontal cortex during adolescence. If this is correct, the number of dopamine axons oriented dorsally towards the medial prefrontal cortex should continue to increase until adulthood. To test this, we used a modified unbiased stereological approach (Kim et al., 2011 [↗](#)) where axons are counted only if they crossed the upper and lower bounds of a counting probe. We also measured the average width of the area these axons occupy. We found, in both male and female mice, that the density of dopamine axons does not change between adolescence (21 days old) and adulthood (75 days old; **Figure 1D** [↗](#)). However, the width of the area that dopamine axons occupy does change, increasing between adolescence and adulthood (**Figure 1E** [↗](#)). These results indicate that the total number of dopamine axons passing through this area increases over adolescence and that dopamine axons grow to the medial prefrontal cortex via this route.

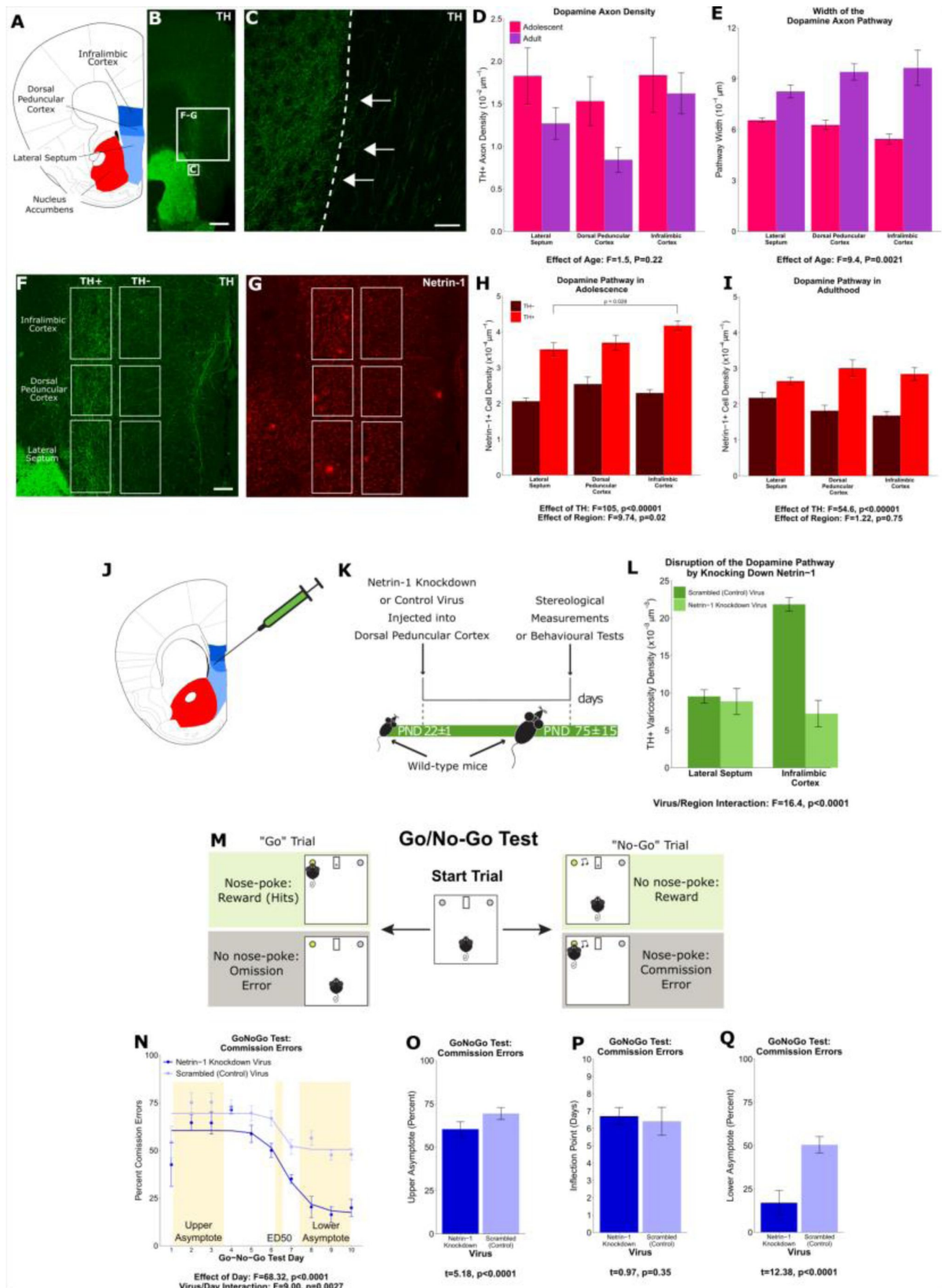


Figure 1.

A “pathway” of Netrin-1 expressing cells “paves the way” for dopamine axons growing from the nucleus accumbens to the medial prefrontal cortex during adolescence. **A**, The brain regions containing the of dopamine fibres passing to the medial prefrontal cortex are highlighted in a line drawing of a coronal mouse brain section derived from Paxinos and Franklin (Paxinos and Franklin, 2013). **B**, An image of a coronal section through the forebrain of an adult mouse at low magnification (4x). Green fluorescence indicates immunostaining for tyrosine hydroxylase (TH), used here as a marker for dopamine. The smaller and larger white squares indicate the regions enlarged in panel C and panels F & G, respectively. Scale bar = 500 μ m. **C**, The nucleus accumbens (left of the dotted line) is densely packed with TH+ axons (in green). Some of these TH+ axons can be observed extending from the nucleus accumbens medially towards TH+ fibres oriented dorsally towards the medial prefrontal cortex (white arrows). Scale bar = 10 μ m. **D**, Modified stereological quantification revealed no significant difference in TH+ axon density between adolescence (21 days old) and adulthood (75 days old). Mixed-effects ANOVA, effect of age: $F=1.53$, $p=0.22$; region by age interaction: $F=1.44$, $p=0.49$. **E**, The average width of the area that dopamine axons occupy increased significantly from adolescence to adulthood, revealing that there is an increase in the total number of fibres passing to the medial prefrontal cortex during this period. Mixed-effects ANOVA, effect of age: $F=9.45$, $p=0.0021$; region by age interaction: $F=5.74$, $p=0.057$. **F**, In order to quantify the Netrin-1 positive cells along the TH+ fibre pathway, the pathway was contoured in each region, and a contour of equal area was placed medial to the dopamine pathway as a negative control. Scale bar = 200 μ m. **G**, Using quantitative stereology, Netrin-1 positive cell density was determined along and adjacent to the pathway for each region. Red fluorescence indicates immunostaining for Netrin-1. **H**, In adolescent mice there are more Netrin-1 positive cells along the fibres expressing TH (“TH+”) than medial to them (“TH-”). This is what we refer to as the “Netrin-1 pathway”. Along the pathway, there is a significant increase in Netrin-1 positive cell density in regions closer to the medial prefrontal cortex, the innervation target. Mixed-effects ANOVA, effect of TH: $F=105$, $p<0.0001$. Effect of region: $F=9.74$, $p=0.021$. A post-hoc Tukey Test revealed a difference ($p=0.029$) between the densities of the lateral septum and infralimbic cortex, but only within the dopamine pathway. **I**, In adult mice the Netrin-1 pathway is maintained, however there is no longer an increasing density of Netrin-1 positive cells towards the medial prefrontal cortex. 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 location within the mouse brain. A Netrin-1 knockdown virus, or a control virus, was injected into the dopamine pathway at the level of the dorsal peduncular cortex. **K**, Our experimental timeline: at the onset of adolescence a Netrin-1 knockdown virus, or a control virus, was injected in wild-type mice. In adulthood the mice were sacrificed and stereological measurements taken. **L**, TH+ varicosity density was quantified in the region below the injection site, the lateral septum, and in the region above the injection site, the infralimbic cortex. There was a significant decrease in TH+ varicosity density only in the infralimbic cortex. Mixed-effects ANOVA, virus by region interaction: $F=16.41$, $p<0.0001$. **M**, The experimental set-up of the final (test) stage of the Go/No-Go experiment. A mouse that has previously learned to nose-poke for a reward in response to a visual cue (illuminated nose-poke hole) must now inhibit this behaviour when the visual cue is paired with an auditory cue (acoustic tone). **N**, Mice injected with the Netrin-1 knockdown virus show improved action impulsivity compared to controls; they incur significantly fewer commission errors across the Go/No-Go task. Mixed-effects ANOVA, effect of day: $F=68.32$, $p<0.0001$. Day by virus interaction: $F=9.00$, $p=0.0027$. A sigmoidal curve is fit to each group of mice to determine how the two groups differ. Points indicate group means and error bars show standard error means. **O**, During the first days of Go/No-Go testing, both groups incur commission errors with high frequency, but the Netrin-1 knockdown group has fewer errors than the control group (T-test, $t=5.18$, $p<0.0001$). **P**, The ED50 – the inflection point in each sigmoidal curve – does not differ between groups, indicating that all mice improve their ability to inhibit their behavior at around the same time (T-test, $t=0.97$, $p=0.35$). **Q**, Mice microinfused with the Netrin-1 knockdown virus incur substantially fewer commission errors in the last days of the Go/No-Go task compared to mice injected with the control virus (T-test, $t=12.38$, $p<0.0001$). For all barplots, bars indicate group means and error bars show standard error means.

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

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

To determine if Netrin-1 along the dopamine axon route is necessary for axon navigation, we silenced Netrin-1 expression in the dorsal peduncular cortex, the transition region between the septum and the medial prefrontal cortex, at the onset of adolescence (**Figure 1J,K** [↗](#)). In adulthood, we quantified the number of dopamine axon terminals in the regions below and above the injection site. Silencing Netrin-1 did not alter dopamine terminal density below the injection, in the lateral septum (**Figure 1L** [↗](#)). In the infralimbic cortex, which is the first prefrontal cortical region the axons reach after the injection site, terminal density was reduced in the Netrin-1 knock-down group compared to controls (**Figure 1L** [↗](#)). The knock-down appears to erase the Netrin-1 path to the prefrontal cortex, resulting in dopamine axons failing to reach their correct innervation target. It remains unknown whether these axons are misrouted to a different target. We conclude that Netrin-1 expressing cells “pave the way” for dopamine axons growing to the medial prefrontal cortex.

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

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

Mice injected with the Netrin-1 silencing virus differed from controls in their performance during “No-Go” trials. As the mice learn to withhold their responses over the course of the test, the number of commission errors they made in No-Go trials decreased in a sigmoidal fashion (**Figure 1N** [↗](#)). The upper and lower asymptotes of the sigmoidal curve quantify the number of commission errors committed during early and late test days, respectively, while the inflection point (ED50) indicates when mice start improving their ability to inhibit their behavior. At the start of the Go/No part of the task, the Netrin-1 silencing group make slightly fewer commission errors (**Figure 1O** [↗](#)) than control groups, although both groups begin to improve in the No-Go task at around the same time. However, the Netrin-1 silencing group achieved a substantially higher level of behavioural inhibition, quantified as a lower percentage of commission errors in the last test days (**Figure 1Q** [↗](#)), indicating an improved ability to withhold their behaviour on cue. These behavioural results demonstrate that the maturation of action impulsivity is sensitive to the

organization of the ventro-dorsal Netrin-1 path that guides mesocortical dopamine axon growth. Deviations in this route associate with striking changes in the cognitive development that is characteristic of adolescence. In this case, the deviation leads to improved action impulsivity, suggesting that these dopamine axons may end up ectopically innervating a forebrain region other than the medial prefrontal cortex, enhancing cognitive control.

Part 2: UNC5C expression coincides with the onset of adolescence

When axons leave the nucleus accumbens during adolescence, they follow a Netrin-1 “path” through intermediate brain regions to reach their intended innervation target. However, only a small subset of the dopamine axons that have reached the nucleus accumbens by early adolescence leave; the vast majority stay and form connections in the accumbens throughout life (Reynolds et al., 2018a [↗](#)). The “decision making” process of whether to “stay” (in the accumbens) or “go” (to the cortex via the Netrin-1 path) happens during a narrow developmental window at the onset of adolescence (Reynolds et al., 2018b [↗](#)). It remains unknown how the timing of this process is determined.

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

Using immunohistochemistry, we assessed the expression of UNC5C on nucleus accumbens dopamine axons across development. In male mice, we found little expression of UNC5C on dopamine axons at the onset of adolescence (Figure 2A [↗](#)), while we did find UNC5C expression on dopamine axons in adults (Figure 2B [↗](#)). Remarkably, when we assessed this in females, we found dopamine axons already expressing UNC5C in the nucleus accumbens at the onset of adolescence (Figure 2D [↗](#)), similar to adult females (figure 2E [↗](#)), indicating that the onset of UNC5C expression on dopamine axons in the nucleus accumbens is sexually dimorphic, with an earlier emergence in females. We examined the nucleus accumbens in pre-adolescent female mice and indeed found little UNC5C expression on dopamine axons (Figure 2C [↗](#)). The onset of UNC5C expression in mesocorticolimbic dopamine axons is therefore peri-adolescent but occurs earlier in females than in males, consistent with the earlier emergence of adolescence in female rodents and the earlier onset of adolescence and puberty in humans (Wolf and Long, 2016 [↗](#)).

Part 3: Environmental control of the timing of adolescence

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

Siberian hamsters (*Phodopus sungorus*) regulate many aspects of their behavior and physiology to meet the changing environmental demands of seasonality (Paul et al., 2008; Stevenson et al., 2017). In winter, they increase the thickness of their fur, exchange their brown summer coats for white winter ones, and undergo a daily torpor to conserve energy (Scherbarth and Steinlechner, 2010 [↗](#)). In addition, adults suppress reproduction and juveniles delay puberty (Pevet, 1988 [↗](#); Yellon and Goldman, 1984 [↗](#)), including developmental changes in gonadotropin releasing hormone neurons in the hypothalamus (Buchanan and Yellon, 1991 [↗](#); Heywood and Yellon, 1997 [↗](#)). Reproductive organ development is delayed as part of pubertal postponement (Darrow et al., 1980 [↗](#); Ebling,

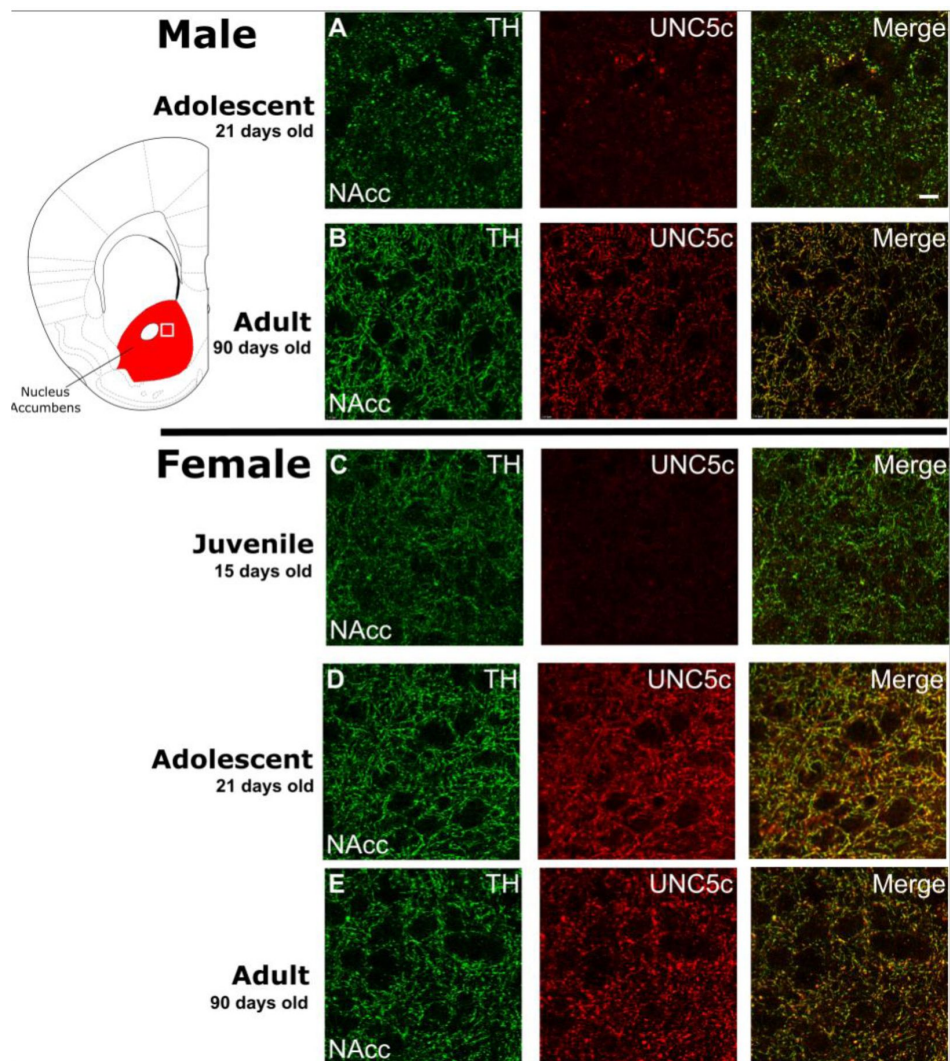


Figure 2

The age of onset of UNC5C expression by dopamine axons in the nucleus accumbens of mice is sexually dimorphic. Images are representative of observed immunofluorescence patterns in the nucleus accumbens (approx. location highlighted as a white square in the coronal mouse brain section Plate 19, modified from Paxinos & Franklin, 2013 [\[1\]](#)). No qualitative differences were noted between the shell and core of the nucleus accumbens. For each row, six individuals were sampled. In males (**A-B**), UNC5C expression on dopamine fibres (here identified by immunofluorescent staining for tyrosine hydroxylase, TH) in the nucleus accumbens appears during adolescence. **A**, At the onset of adolescence (21 days old) dopamine fibres do not express UNC5C. Scale bar = 10 μ m. **B**, By adulthood (90 days old), dopamine fibres express UNC5C. In females (**C-E**), UNC5C expression on dopamine fibres in the nucleus accumbens appears prior to adolescence. **C**, In juvenile (15 day old) mice, there is no UNC5C expression on dopamine fibres. **D**, By adolescence, dopamine fibres express UNC5C. **E**, In adulthood, dopamine fibres continue to express UNC5C.

1994 [↗](#); Timonin et al., 2006 [↗](#)). This seasonal plasticity is regulated by long or short periods of daylight (Heldmaier and Steinlechner, 1981 [↗](#); Hoffmann, 1978 [↗](#)) and raises the possibility that aspects of adolescent development are sensitive to these environmental cues. To our knowledge, adaptive variation in the timing of adolescent neural development has never been recorded in any animal.

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

3.i The seasonality of adolescence

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

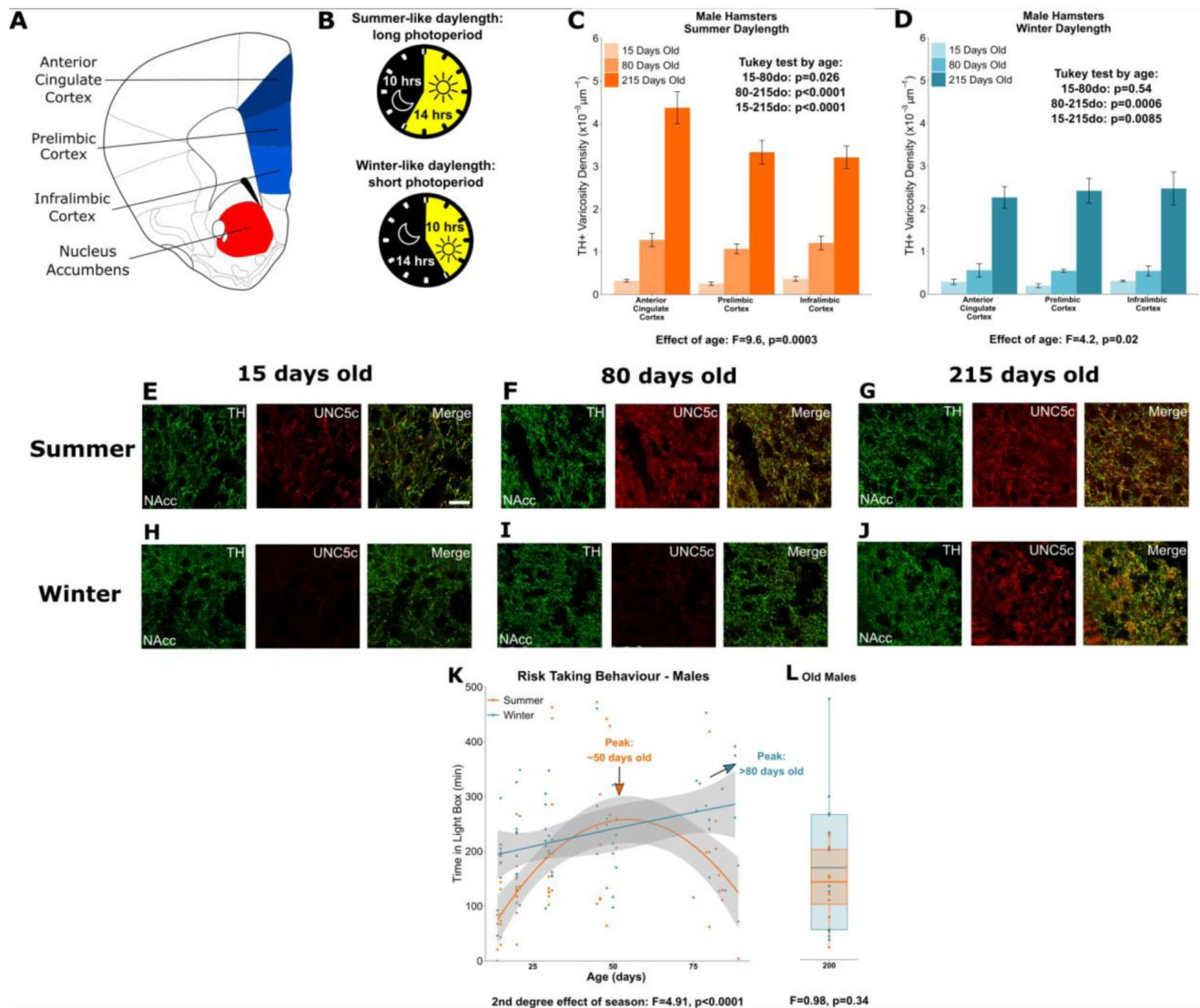


Figure 3

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

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

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

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

A behavioural characteristic of adolescence is increased willingness to enter a novel environment, a behaviour that assumes an increased amount of risk (Arrant et al., 2013; Lynn and Brown, 2009). To measure this, we used the light/dark test (Bourin and Hascoët, 2003). Time spent in the light compartment is dopamine-dependent (Bahi and Dreyer, 2019; Gao and Cutler, 1993) and peaks in adolescence (Arrant et al., 2013). We will refer to this behaviour as “risk taking”. We assessed the developmental profile of risk taking in the light/dark box test in summer and

winter hamsters across adolescence. In male summer hamsters, the risk taking increases across adolescence, peaks around 50 days, then subsequently declines (**Figure 3K**). However, the adolescent increase in risk taking is protracted in winter hamsters: across the age range examined we observe a gradual, consistent increase in risk taking rather than a peak and decline.

We next assessed a cohort of 215-day old hamsters, for which both summer and winter male hamsters have undergone puberty and exhibit high levels of dopamine innervation of the prefrontal cortex (**Figure 3C,D**, **Figure 3G,J**, Supplementary Figure 3a). In these hamsters, we find no difference in risk taking between the male summer and winter groups (**Figure 3L**), demonstrating that, after 80 days, risk taking begins to decline in male winter hamsters and that by 215 days it has declined to the same level as in summer hamsters. Male hamsters raised under summer-mimicking long days and winter-mimicking short days both ultimately make the transition to the adult behavioral phenotype.

3.ii An extraordinary case of decoupling puberty and adolescence

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

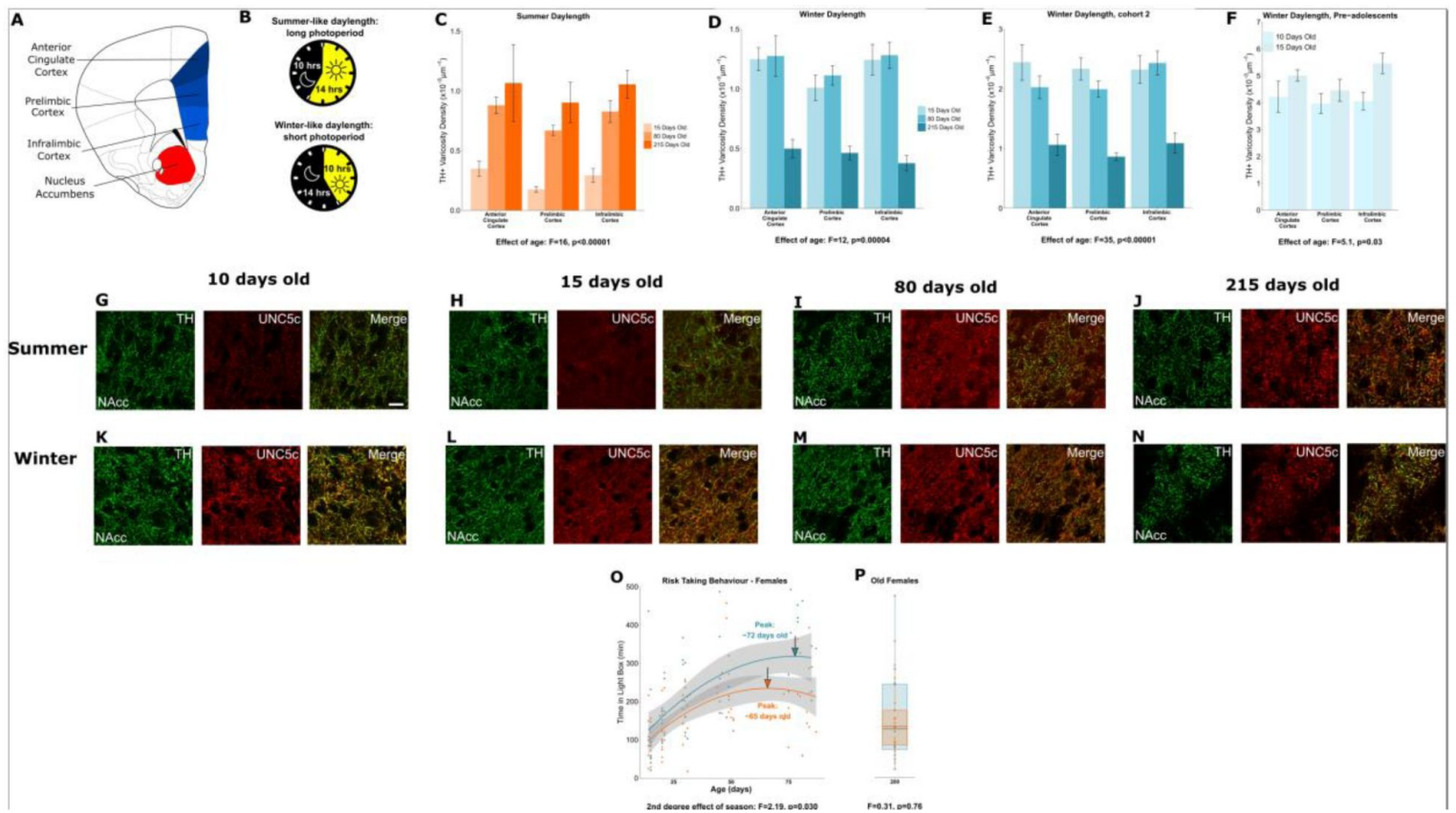


Figure 4

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: $F=12.33$, $p=0.00043$. **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-H**, In 10- and 15-day-old female summer hamsters there is little UNC5C expression in nucleus accumbens dopamine axons (here identified by immunofluorescent staining for tyrosine hydroxylase, TH). Sample size: 4 (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 the nucleus accumbens express UNC5C in female summer hamsters. Sample sizes: 6. Scale bar = 20um (panel G bottom right). **K-N**, At all ages which winter female hamsters were examined, dopamine axons in the nucleus accumbens express UNC5C in winter female hamsters. Sample sizes: 4 (panel K) or 6 (panels L-N). **O**, In female hamsters, those raised under summer and winter daylengths both show an increase in risk taking over time. The winter hamsters peak later compared to the summer daylength hamsters. Arrows indicate the ages at which risk taking peaks in summer (orange) and winter (blue) hamsters. Polynomial regression, effect of season: $F=3.305$, $p=0.00126$. **P**, In female hamsters, at 215 days of age, there is no difference in risk taking between hamsters raised under summer and winter photoperiods. T-test, effect of season: $t=0.309$, $p=0.76$. For all barplots, bars indicate group means and error bars show standard error means.

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

We then measured dopamine axon density in female winter hamsters at two earlier ages: 10 and 15 days old. Dopamine innervation increases during this period (**figure 4F**), well before normal adolescence and long before pubertal development. This is an extraordinary phenomenon: a key marker of adolescent neurodevelopment is accelerated and dissociated from puberty in female hamsters raised under winter-mimicking short days (Supplementary Figure 4a,4b).

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

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

We used the light/dark box test to examine potential risk taking implications of the extraordinary developmental trajectory we observed in the prefrontal cortex of female hamsters. In female summer and winter hamsters, the adolescent increase and peak in risk taking occurs between the ages of 15 and 80 days, as it does in summer daylength males (**Figure 4O**). However, contrary to what we would expect, the peak in winter females is delayed compared to summer females. When we assessed an independent cohort of 215-day-old female hamsters, we found no difference in risk taking between groups (**Figure 4R**), indicating that, like males, female summer and winter hamsters both eventually reach the same adult level of risk taking.

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

Conclusion

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

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

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

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Author contributions (Brand et al., 2015 [↗](#))

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Competing interests

Authors declare that they have no competing interests.

Data and code availability

All data and code use in these analyses are available through the Open Science Framework (DIO 10.17605/OSF.IO/DU3H4).

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Reviewer #1 (Public Review):

In this study, Hoops et al. showed that Netrin-1 and UNC5c can guide dopaminergic innervation from nucleus accumbens to cortex during adolescence in rodent models. They found that these dopamine axons project to the prefrontal cortex in a Netrin-1 dependent manner and knocking down Netrin-1 disrupted motor and learning behaviors in mice. Furthermore, the authors used hamsters, a seasonal model that is affected by the length of

daylight, to demonstrate that the guidance of dopamine axons is mediated by the environmental factor such as daytime length and in sex dependent manner.

Regarding the cell type specificity of Netrin-1 expression, the authors began by stating "this question is not the focus of the study and we consider it irrelevant to the main issue we are addressing, which is where in the forebrain regions we examined Netrin-1+ cells are present." This statement contradicts the exact issue regarding the specificity issue I raised. They then went on to show the RNAscope data for Netrin-1 in Figure 2, which showed Netrin-1 mRNA was actually expressed quite ubiquitously in anterior cingulate cortex, dorsopeduncular cortex, infralimbic cortex, prelimbic cortex, etc. In addition, contrary to the authors' statement that Netrin-1 is a "secreted protein", the confocal images in Figure 1 in the rebuttal letter actually show Netrin-1 present in "granule-like" organelles inside the cytoplasm of neurons. Finally, the authors presented Figure 7 to indicate the location where virus expressing Netrin-1 shRNA might be located. Again, the brain region targeted was quite focal and most likely did not cover all the Netrin-1+ brain regions in Figure 2. Collectively, these results raised more questions regarding the specificity of Netrin-1 expression in brain regions that are behaviorally relevant to this study.

With respect to the effectiveness of Netrin-1 knockdown in the animals in this study, the authors cited data in HEK293 cells (Figure 5), which did not include any statistics, and previously published in vivo data in a separate, independent study (Figure 6). They do not provide any data regarding the effectiveness of Netrin-1 knockdown in THIS study.

Similar concerns regarding UNC5C knockdown (points #6, #7, and #8) were not adequately addressed.

In brief, while this study provides a potential role of Netrin-1-UNC5C in target innervation of dopaminergic neurons and its behavioral output in risk-taking, the data lack sufficient evidence to firmly establish the cause-effect relationship.

<https://doi.org/10.7554/eLife.88261.2.sa2>

Reviewer #2 (Public Review):

In this manuscript, Hoops et al., using two different model systems, identified key developmental changes in Netrin-1 and UNC5C signaling that correspond to behavioral changes and are sensitive to environmental factors that affect the timing of development. They found that Netrin-1 expression is highest in regions of the striatum and cortex where TH+ axons are travelling, and that knocking down Netrin-1 reduces TH+ varicosities in mPFC and reduces impulsive behaviors in a Go-No-Go test. Further, they show that the onset of Unc5 expression is sexually dimorphic in mice, and that in Siberian hamsters, environmental effects on development are also sexually dimorphic. This study addresses an important question using approaches that link molecular, circuit and behavioral changes. Understanding developmental trajectories of adolescence, and how they can be impacted by environmental factors, is an understudied area of neuroscience that is highly relevant to understanding the onset of mental health disorders. I appreciated the inclusion of replication cohorts within the study.

<https://doi.org/10.7554/eLife.88261.2.sa1>

Reviewer #3 (Public Review):

This study from the Flores group aims at understanding neuronal circuit changes during adolescence which is an ill-defined, transitional period involving dramatic changes in

behavior and anatomy. They focus on DA innervation of the prefrontal cortex, and their interaction with the guidance cue Netrin-1. They propose DA axons in the PFC increase in the postnatal period, and their density is reduced in a Netrin 1 knockdown, suggesting that Netrin abets the development of this mesocortical pathway. In such mice impulsivity gauged by a go-no go task is reduced. They then provide some evidence that Unc5c is developmentally regulated in DA axons. Finally they use an interesting hamster model, to study the effect of light hours on mesocortical innervation, and make some interesting observations about the timing of innervation and Unc5c expression, and the fact that females housed in winter day length conditions display an accelerated innervation of the prefrontal cortex.

Comments on the revision. Several points were addressed; some remain to be addressed.

#4. It's not clear to me that TH doesn't stain noradrenergic axons in the PFC. See Islam and Blaess, 2021, and references therein.

#6. The Netrin knockdown data provided is from a previous study/samples.

#8. While the authors make the argument that the behavior is linked to DA, they still haven't formally tested it, in my opinion.

#13. Fig 3, UNC 5c levels are not yet quantified. Furthermore, I agree with the previous reviewer that UNC5C knockdown would corroborate key aspects of the model.

New - Developmental trajectory of prefrontal TH-positive axons from early adolescence to adulthood is similar in male and female rats, (Willing Juraska et al., 2017). This needs discussion.

<https://doi.org/10.7554/eLife.88261.2.sa0>

Author Response

The following is the authors' response to the original reviews.

Public Comments

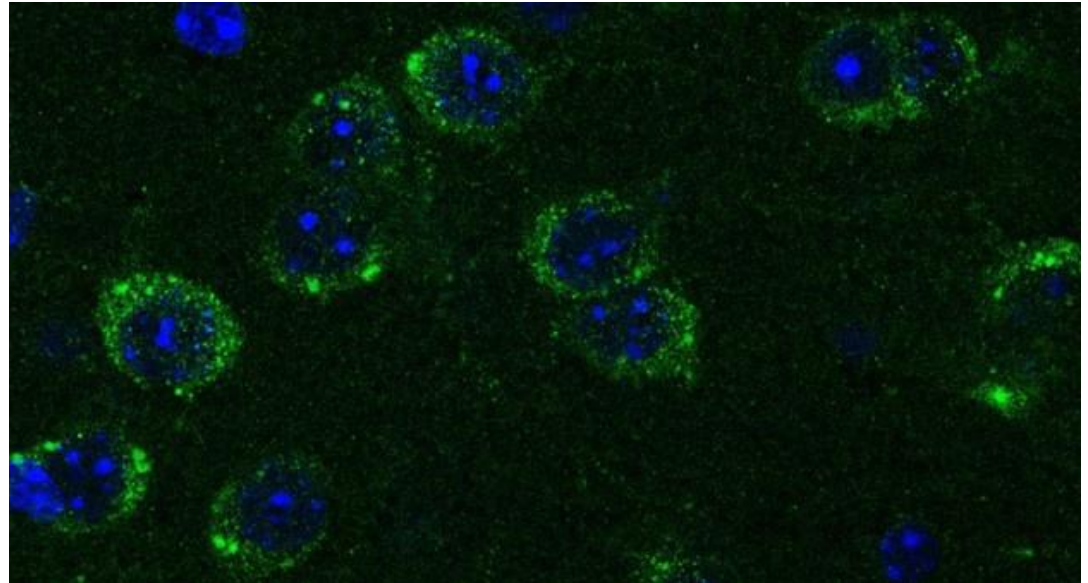
Reviewer 1

(1) Despite the well-established role of Netrin-1 and UNC5C axon guidance during embryonic commissural axons, it remains unclear which cell type(s) express Netrin-1 or UNC5C in the dopaminergic axons and their targets. For instance, the data in Figure 1F-G and Figure 2 are quite confusing. Does Netrin-1 or UNC5C express in all cell types or only dopamine-positive neurons in these two mouse models? It will also be important to provide quantitative assessments of UNC5C expression in dopaminergic axons at different ages.

Netrin-1 is a secreted protein and in this manuscript we did not examine what cell types express Netrin-1. This question is not the focus of the study and we consider it irrelevant to the main issue we are addressing, which is where in the forebrain regions we examined Netrin-1+ cells are present. As per the reviewer's request we include below images showing Netrin-1 protein and Netrin-1 mRNA expression in the forebrain. In Figure 1 below, we show a high magnification immunofluorescent image of a coronal forebrain section showing Netrin-1 protein expression.

Author response image 1.

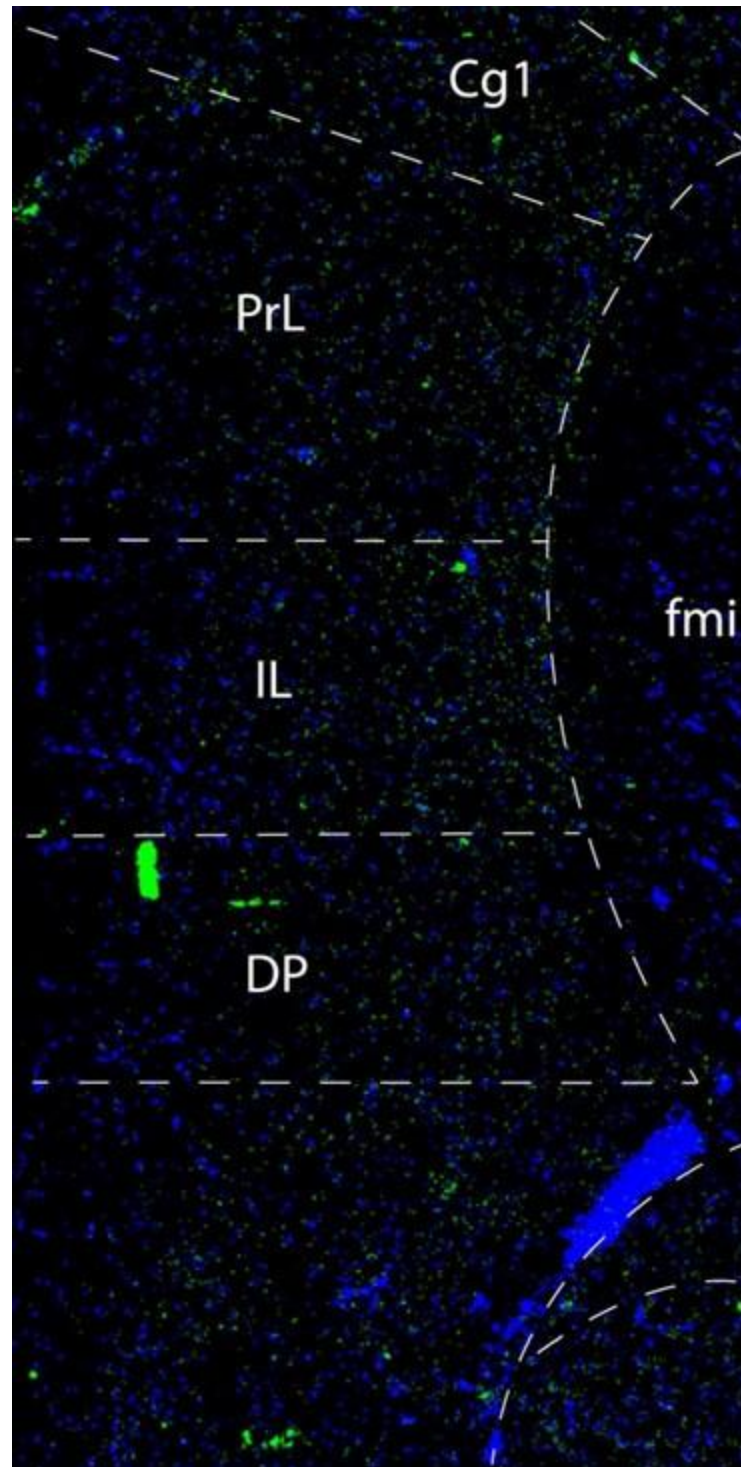
This confocal microscope image shows immunofluorescent staining for Netrin-1 (green) localized around cell nuclei (stained by DAPI in blue). This image was taken from a coronal section of the lateral septum of an adult male mouse. Scale bar = 20µm



In Author response images 2 and 3 below we show low and high magnification images from an RNAscope experiment confirming that cells in the forebrain regions examined express Netrin-1 mRNA.

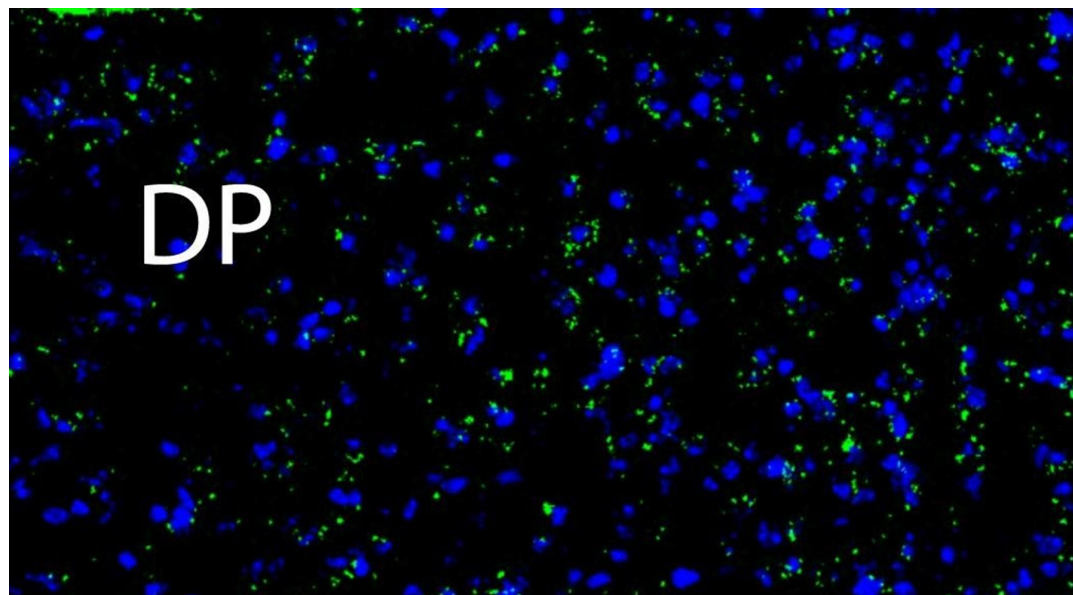
Author response image 2.

This confocal microscope image of a coronal brain section of the medial prefrontal cortex of an adult male mouse shows Netrin-1 mRNA expression (green) and cell nuclei (DAPI, blue). Brain regions are as follows: Cg1: Anterior cingulate cortex 1, DP: dorsopeduncular cortex, fmi: forceps minor of the corpus callosum, IL: Infralimbic Cortex, PrL: Prelimbic Cortex



Author response image 3.

A higher resolution image from the same sample as in Figure 2 shows Netrin-1 mRNA (green) and cell nuclei (DAPI; blue). DP = dorsopeduncular cortex



Regarding UNC5c, this receptor homologue is expressed by dopamine neurons in the rodent ventral tegmental area (Daubaras et al., 2014; Manitt et al., 2010; Phillips et al., 2022). This does not preclude UNC5c expression in other cell types. UNC5c receptors are ubiquitously expressed in the brain throughout development, performing many different developmental functions (Kim and Ackerman, 2011; Murcia-Belmonte et al., 2019; Srivatsa et al., 2014). In this study we are interested in UNC5c expression by dopamine neurons, and particularly by their axons projecting to the nucleus accumbens. We therefore used immunofluorescent staining in the nucleus accumbens, showing UNC5 expression in TH⁺ axons. This work adds to the study by Manitt et al., 2010, which examined UNC5 expression in the VTA. Manitt et al. used Western blotting to demonstrate that UNC5 expression in VTA dopamine neurons increases during adolescence, as can be seen in the following figure:

References:

- Daubaras M, Bo GD, Flores C. 2014. Target-dependent expression of the netrin-1 receptor, UNC5C, in projection neurons of the ventral tegmental area. *Neuroscience* 260:36–46. doi:10.1016/j.neuroscience.2013.12.007
- Kim D, Ackerman SL. 2011. The UNC5C Netrin Receptor Regulates Dorsal Guidance of Mouse Hindbrain Axons. *J Neurosci* 31:2167–2179. doi:10.1523/jneurosci.5254-10.2011
- Manitt C, Labelle-Dumais C, Eng C, Grant A, Mimee A, Stroth T, Flores C. 2010. Peri-Pubertal Emergence of UNC-5 Homologue Expression by Dopamine Neurons in Rodents. *PLoS ONE* 5:e11463-14. doi:10.1371/journal.pone.0011463
- Murcia-Belmonte V, Coca Y, Vegar C, Negueruela S, Romero C de J, Valiño AJ, Sala S, DaSilva R, Kania A, Borrell V, Martinez LM, Erskine L, Herrera E. 2019. A Retino-retinal Projection Guided by Unc5c Emerged in Species with Retinal Waves. *Current Biology* 29:1149-1160.e4. doi:10.1016/j.cub.2019.02.052
- Phillips RA, Tuscher JJ, Black SL, Andraka E, Fitzgerald ND, Ianov L, Day JJ. 2022. An atlas of transcriptionally defined cell populations in the rat ventral tegmental area. *Cell Reports* 39:110616. doi:10.1016/j.celrep.2022.110616
- Srivatsa S, Parthasarathy S, Britanova O, Bormuth I, Donahoo A-L, Ackerman SL, Richards LJ, Tarabykin V. 2014. Unc5C and DCC act downstream of Ctip2 and Satb2 and contribute to

corpus callosum formation. Nat Commun 5:3708. doi:10.1038/ncomms4708

(2) Figure 1 used shRNA to knockdown Netrin-1 in the Septum and these mice were subjected to behavioral testing. These results, again, are not supported by any valid data that the knockdown approach actually worked in dopaminergic axons. It is also unclear whether knocking down Netrin-1 in the septum will re-route dopaminergic axons or lead to cell death in the dopaminergic neurons in the substantia nigra pars compacta?

First we want to clarify and emphasize, that our knockdown approach was not designed to knock down Netrin-1 in dopamine neurons or their axons. Our goal was to knock down Netrin-1 expression in cells expressing this guidance cue gene in the dorsal peduncular cortex.

We have previously established the efficacy of the shRNA Netrin-1 knockdown virus used in this experiment for reducing the expression of Netrin-1 (Cuesta et al., 2020). The shRNA reduces Netrin-1 levels in vitro and in vivo.

We agree that our experiments do not address the fate of the dopamine axons that are misrouted away from the medial prefrontal cortex. This research is ongoing, and we have now added a note regarding this to our manuscript.

Our current hypothesis, based on experiments being conducted as part of another line of research in the lab, is that these axons are rerouted to a different brain region which they then ectopically innervate. In these experiments we are finding that male mice exposed to tetrahydrocannabinol in adolescence show reduced dopamine innervation in the medial prefrontal cortex in adulthood but increased dopamine input in the orbitofrontal cortex. In addition, these mice show increased action impulsivity in the Go/No-Go task in adulthood (Capolicchio et al., Society for Neuroscience 2023 Abstracts)

References:

Capolicchio T., Hernandez, G., Dube, E., Estrada, K., Giroux, M., Flores, C. (2023) Divergent outcomes of delta 9 - tetrahydrocannabinol in adolescence on dopamine and cognitive development in male and female mice. Society for Neuroscience, Washington, DC, United States [abstract].

Cuesta S, Nouel D, Reynolds LM, Morgunova A, Torres-Berrío A, White A, Hernandez G, Cooper HM, Flores C. 2020. Dopamine Axon Targeting in the Nucleus Accumbens in Adolescence Requires Netrin-1. Frontiers Cell Dev Biology 8:487. doi:10.3389/fcell.2020.00487

(3) Another issue with Figure1J. It is unclear whether the viruses were injected into a WT mouse model or into a Cre-mouse model driven by a promoter specifically expresses in dorsal peduncular cortex? The authors should provide evidence that Netrin-1 mRNA and proteins are indeed significantly reduced. The authors should address the anatomic results of the area of virus diffusion to confirm the virus specifically infected the cells in dorsal peduncular cortex.

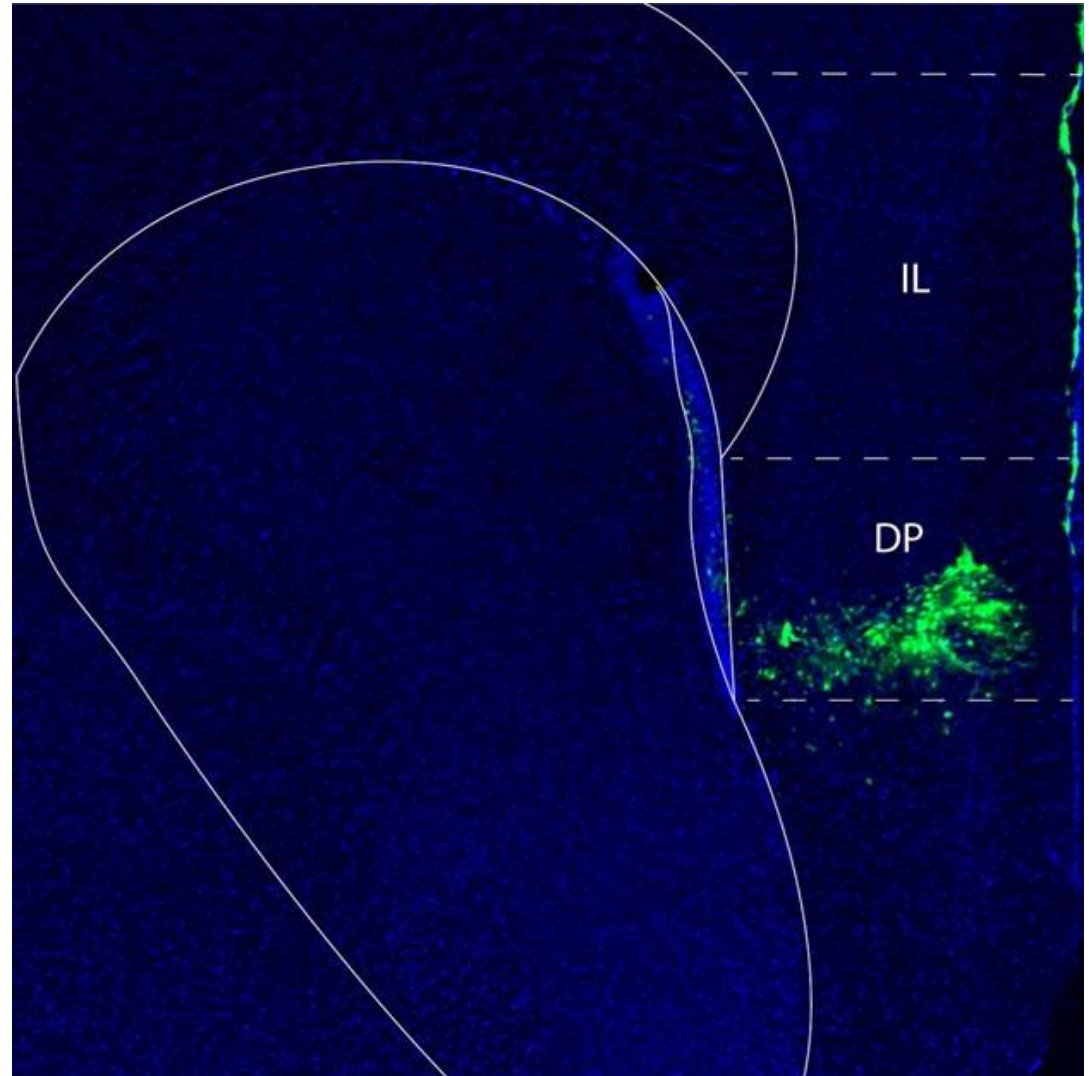
All the virus knockdown experiments were conducted in wild type mice, we added this information to Figure 1k.

The efficacy of the shRNA in knocking down Netrin-1 was demonstrated by Cuesta et al. (2020) both in vitro and in vivo, as we show in our response to the reviewer's previous comment above.

We also now provide anatomical images demonstrating the localization of the injection and area of virus diffusion in the mouse forebrain. In Author response image 4 below the area of virus diffusion is visible as green fluorescent signal.

Author response image 4.

Fluorescent microscopy image of a mouse forebrain demonstrating the localization of the injection of a virus to knock down Netrin-1. The location of the virus is in green, while cell nuclei are in blue (DAPI). Abbreviations: DP: dorsopeduncular cortex IL: infralimbic cortex



References:

Cuesta S, Nouel D, Reynolds LM, Morgunova A, Torres-Berrío A, White A, Hernandez G, Cooper HM, Flores C. 2020. Dopamine Axon Targeting in the Nucleus Accumbens in Adolescence Requires Netrin-1. *Frontiers Cell Dev Biology* 8:487. doi:10.3389/fcell.2020.00487

(4) The authors need to provide information regarding the efficiency and duration of knocking down. For instance, in Figure 1K, the mice were tested after 53 days post injection, can the virus activity in the brain last for such a long time?

In our study we are interested in the role of Netrin-1 expression in the guidance of dopamine axons from the nucleus accumbens to the medial prefrontal cortex. The critical window for these axons leaving the nucleus accumbens and growing to the cortex is early adolescence (Reynolds et al., 2018b). This is why we injected the virus at the onset of adolescence, at

postnatal day 21. As dopamine axons grow from the nucleus accumbens to the prefrontal cortex, they pass through the dorsal peduncular cortex. We disrupted Netrin-1 expression at this point along their route to determine whether it is the Netrin-1 present along their route that guides these axons to the prefrontal cortex. We hypothesized that the shRNA Netrin-1 virus would disrupt the growth of the dopamine axons, reducing the number of axons that reach the prefrontal cortex and therefore the number of axons that innervate this region in adulthood.

We conducted our behavioural tests during adulthood, after the critical window during which dopamine axon growth occurs, so as to observe the enduring behavioral consequences of this misrouting. This experimental approach is designed for the shRNA Netrin-1 virus to be expressed in cells in the dorsopeduncular cortex when the dopamine axons are growing, during adolescence.

References:

Capolicchio T, Hernandez, G., Dube, E., Estrada, K., Giroux, M., Flores, C. (2023) Divergent outcomes of delta 9 - tetrahydrocannabinol in adolescence on dopamine and cognitive development in male and female mice. Society for Neuroscience, Washington, DC, United States [abstract].

Reynolds LM, Yetnikoff L, Pokinko M, Wodzinski M, Epelbaum JG, Lambert LC, Cossette M-P, Arvanitogiannis A, Flores C. 2018b. Early Adolescence is a Critical Period for the Maturation of Inhibitory Behavior. *Cerebral cortex* 29:3676–3686. doi:10.1093/cercor/bhy247

(5) *In Figure 1N-Q, silencing Netrin-1 results in less DA axons targeting to infralimbic cortex, but why the Netrin-1 knocking down mice revealed the improved behavior?*

This is indeed an intriguing finding, and we have now added a mention of it to our manuscript. We have demonstrated that misrouting dopamine axons away from the medial prefrontal cortex during adolescence alters behaviour, but why this improves their action impulsivity ability is something currently unknown to us. One potential answer is that the dopamine axons are misrouted to a different brain region that is also involved in controlling impulsive behaviour, perhaps the dorsal striatum (Kim and Im, 2019) or the orbital prefrontal cortex (Jonker et al., 2015).

We would also like to note that we are finding that other manipulations that appear to reroute dopamine axons to unintended targets can lead to reduced action impulsivity as measured using the Go No Go task. As we mentioned above, current experiments in the lab, which are part of a different line of research, are showing that male mice exposed to tetrahydrocannabinol in adolescence show reduced dopamine innervation in the medial prefrontal cortex in adulthood, but increased dopamine input in the orbitofrontal cortex. In addition, these mice show increased action impulsivity in the Go/No-Go task in adulthood (Capolicchio et al., Society for Neuroscience 2023 Abstracts)

References

Capolicchio T, Hernandez, G., Dube, E., Estrada, K., Giroux, M., Flores, C. (2023) Divergent outcomes of delta 9 - tetrahydrocannabinol in adolescence on dopamine and cognitive development in male and female mice. Society for Neuroscience, Washington, DC, United States [abstract].

Jonker FA, Jonker C, Scheltens P, Scherder EJA. 2015. The role of the orbitofrontal cortex in cognition and behavior. *Rev Neurosci* 26:1–11. doi:10.1515/revneuro2014-0043 Kim B, Im H. 2019. The role of the dorsal striatum in choice impulsivity. *Ann N York Acad Sci* 1451:92–111. doi:10.1111/nyas.13961

(6) What is the effect of knocking down UNC5C on dopamine axons guidance to the cortex?

We have found that mice that are heterozygous for a nonsense Unc5c mutation, and as a result have reduced levels of UNC5c protein, show reduced amphetamine-induced locomotion and stereotypy (Auger et al., 2013). In the same manuscript we show that this effect only emerges during adolescence, in concert with the growth of dopamine axons to the prefrontal cortex. This is indirect but strong evidence that UNC5c receptors are necessary for correct adolescent dopamine axon development.

References

Auger ML, Schmidt ERE, Manitt C, Dal-Bo G, Pasterkamp RJ, Flores C. 2013. unc5c haploinsufficient phenotype: striking similarities with the dcc haploinsufficiency model. European Journal of Neuroscience 38:2853–2863. doi:10.1111/ejn.12270

(7) In Figures 2-4, the authors only showed the amount of DA axons and UNC5C in NAcc. However, it remains unclear whether these experiments also impact the projections of dopaminergic axons to other brain regions, critical for the behavioral phenotypes. What about other brain regions such as prefrontal cortex? Do the projection of DA axons and UNC5c level in cortex have similar pattern to those in NAcc?

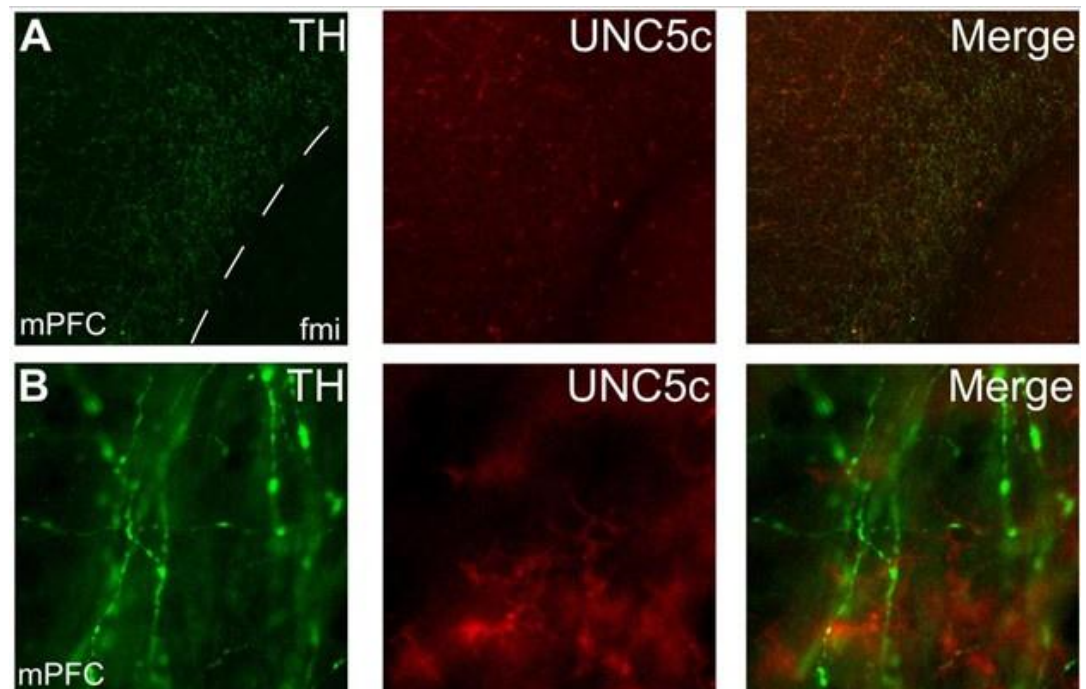
UNC5c receptors are expressed throughout development and are involved in many developmental processes (Kim and Ackerman, 2011; Murcia-Belmonte et al., 2019; Srivatsa et al., 2014). We cannot say whether the pattern we observe here is unique to the nucleus accumbens, but it is certainly not universal throughout the brain.

The brain region we focus on in our manuscript, in addition to the nucleus accumbens, is the medial prefrontal cortex. Close and thorough examination of the prefrontal cortices of adult mice revealed practically no UNC5c expression by dopamine axons. However, we did observe very rare cases of dopamine axons expressing UNC5c. It is not clear whether these rare cases are present before or during adolescence.

Below is a representative set of images of this observation, which is now also included as Supplementary Figure 4:

Author response image 5.

Expression of UNC5c protein in the medial prefrontal cortex of an adult male mouse. Low (A) and high (B) magnification images demonstrate that there is little UNC5c expression in dopamine axons in the medial prefrontal cortex. Here we identify dopamine axons by immunofluorescent staining for tyrosine hydroxylase (TH, see our response to comment #9 regarding the specificity of the TH antibody for dopamine axons in the prefrontal cortex). This figure is also included as Supplementary Figure 4 in the manuscript. Abbreviations: fmi: forceps minor of the corpus callosum, mPFC: medial prefrontal cortex.



References:

- Kim D, Ackerman SL. 2011. The UNC5C Netrin Receptor Regulates Dorsal Guidance of Mouse Hindbrain Axons. *J Neurosci* 31:2167–2179. doi:10.1523/jneurosci.5254-10.2011
- Murcia-Belmonte V, Coca Y, Vegar C, Negueruela S, Romero C de J, Valiño AJ, Sala S, DaSilva R, Kania A, Borrell V, Martinez LM, Erskine L, Herrera E. 2019. A Retino-retinal Projection Guided by Unc5c Emerged in Species with Retinal Waves. *Current Biology* 29:1149–1160.e4. doi:10.1016/j.cub.2019.02.052
- Srivatsa S, Parthasarathy S, Britanova O, Bormuth I, Donahoo A-L, Ackerman SL, Richards LJ, Tarabykin V. 2014. Unc5C and DCC act downstream of Ctip2 and Satb2 and contribute to corpus callosum formation. *Nat Commun* 5:3708. doi:10.1038/ncomms4708

(8) Can overexpression of UNC5c or Netrin-1 in male winter hamsters mimic the observations in summer hamsters? Or overexpression of UNC5c in female summer hamsters to mimic the winter hamster? This would be helpful to confirm the causal role of UNC5C in guiding DA axons during adolescence.

This is an excellent question. We are very interested in both increasing and decreasing UNC5c expression in hamster dopamine axons to see if we can directly manipulate summer hamsters into winter hamsters and vice versa. We are currently exploring virus-based approaches to design these experiments and are excited for results in this area.

(9) The entire study relied on using tyrosine hydroxylase (TH) as a marker for dopaminergic axons. However, the expression of TH (either by IHC or IF) can be influenced by other environmental factors, that could alter the expression of TH at the cellular level.

This is an excellent point that we now carefully address in our methods by adding the following:

In this study we pay great attention to the morphology and localization of the fibres from which we quantify varicosities to avoid counting any fibres stained with TH antibodies that are not dopamine fibres. The fibres that we examine and that are labelled by the TH antibody show features indistinguishable from the classic features of cortical dopamine axons in rodents (Berger et al., 1974; 1983; Van Eden et al., 1987; Manitt et al., 2011), namely they are thin fibres with irregularly-spaced varicosities, are densely packed in the nucleus accumbens, sparsely present only in the deep layers of the prefrontal cortex, and are not regularly oriented in relation to the pial surface. This is in contrast to rodent norepinephrine fibres, which are smooth or beaded in appearance, relatively thick with regularly spaced varicosities, increase in density towards the shallow cortical layers, and are in large part oriented either parallel or perpendicular to the pial surface (Berger et al., 1974; Levitt and Moore, 1979; Berger et al., 1983; Miner et al., 2003). Furthermore, previous studies in rodents have noted that only norepinephrine cell bodies are detectable using immunofluorescence for TH, not norepinephrine processes (Pickel et al., 1975; Verney et al., 1982; Miner et al., 2003), and we did not observe any norepinephrine-like fibres.

Furthermore, we are not aware of any other processes in the forebrain that are known to be immunopositive for TH under any environmental conditions.

To reduce confusion, we have replaced the abbreviation for dopamine – DA – with TH in the relevant panels in Figures 1, 2, 3, and 4 to clarify exactly what is represented in these images. As can be seen in these images, fluorescent green labelling is present only in axons, which is to be expected of dopamine labelling in these forebrain regions.

References:

- Berger B, Tassin JP, Blanc G, Moyne MA, Thierry AM (1974) Histochemical confirmation for dopaminergic innervation of the rat cerebral cortex after destruction of the noradrenergic ascending pathways. *Brain Res* 81:332–337.
- Berger B, Verney C, Gay M, Vigny A (1983) Immunocytochemical Characterization of the Dopaminergic and Noradrenergic Innervation of the Rat Neocortex During Early Ontogeny. In: *Proceedings of the 9th Meeting of the International Neurobiology Society*, pp 263–267 Progress in Brain Research. Elsevier.
- Levitt P, Moore RY (1979) Development of the noradrenergic innervation of neocortex. *Brain Res* 162:243–259.
- Manitt C, Mimee A, Eng C, Pokinko M, Stroh T, Cooper HM, Kolb B, Flores C (2011) The Netrin Receptor DCC Is Required in the Pubertal Organization of Mesocortical Dopamine Circuitry. *J Neurosci* 31:8381–8394.
- Miner LH, Schroeter S, Blakely RD, Sesack SR (2003) Ultrastructural localization of the norepinephrine transporter in superficial and deep layers of the rat prelimbic prefrontal cortex and its spatial relationship to probable dopamine terminals. *J Comp Neurol* 466:478–494.
- Pickel VM, Joh TH, Field PM, Becker CG, Reis DJ (1975) Cellular localization of tyrosine hydroxylase by immunohistochemistry. *J Histochem Cytochem* 23:1–12.
- Van Eden CG, Hoorneman EM, Buijs RM, Matthijssen MA, Geffard M, Uylings HBM (1987) Immunocytochemical localization of dopamine in the prefrontal cortex of the rat at the light and electron microscopical level. *Neurosci* 22:849–862.
- Verney C, Berger B, Adrien J, Vigny A, Gay M (1982) Development of the dopaminergic innervation of the rat cerebral cortex. A light microscopic immunocytochemical study using anti-tyrosine hydroxylase antibodies. *Dev Brain Res* 5:41–52.

(10) *Are Netrin-1/UNC5C the only signal guiding dopamine axon during adolescence? Are there other neuronal circuits involved in this process?*

Our intention for this study was to examine the role of Netrin-1 and its receptor UNC5C specifically, but we do not suggest that they are the only molecules to play a role. The process of guiding growing dopamine axons during adolescence is likely complex and we expect other guidance mechanisms to also be involved. From our previous work we know that the Netrin-1 receptor DCC is critical in this process (Hoops and Flores, 2017; Reynolds et al., 2023). Several other molecules have been identified in Netrin-1/DCC signaling processes that control corpus callosum development and there is every possibility that the same or similar molecules may be important in guiding dopamine axons (Schlienger et al., 2023).

References:

Hoops D, Flores C. 2017. Making Dopamine Connections in Adolescence. Trends in Neurosciences 1–11. doi:10.1016/j.tins.2017.09.004

Reynolds LM, Hernandez G, MacGowan D, Popescu C, Nouel D, Cuesta S, Burke S, Savell KE, Zhao J, Restrepo-Lozano JM, Giroux M, Israel S, Orsini T, He S, Wodzinski M, Avramescu RG, Pokinko M, Epelbaum JG, Niu Z, Pantoja-Urbán AH, Trudeau L-É, Kolb B, Day JJ, Flores C. 2023. Amphetamine disrupts dopamine axon growth in adolescence by a sex-specific mechanism in mice. Nat Commun 14:4035. doi:10.1038/s41467-023-39665-1

Schlienger S, Yam PT, Balekoglu N, Ducuing H, Michaud J-F, Makihara S, Kramer DK, Chen B, Fasano A, Berardelli A, Hamdan FF, Rouleau GA, Srour M, Charron F. 2023. Genetics of mirror movements identifies a multifunctional complex required for Netrin-1 guidance and lateralization of motor control. Sci Adv 9:eadd5501. doi:10.1126/sciadv.add5501

(11) *Finally, despite the authors' claim that the dopaminergic axon project is sensitive to the duration of daylight in the hamster, they never provided definitive evidence to support this hypothesis.*

By “definitive evidence” we think that the reviewer is requesting a single statistical model including measures from both the summer and winter groups. Such a model would provide a probability estimate of whether dopamine axon growth is sensitive to daylight duration. Therefore, we ran these models, one for male hamsters and one for female hamsters.

In both sexes we find a significant effect of daylength on dopamine innervation, interacting with age. Male age by daylength interaction: $F = 6.383$, $p = 0.00242$. Female age by daylength interaction: $F = 21.872$, $p = 1.97 \times 10^{-9}$. The full statistical analysis is available as a supplement to this letter (Response_Letter_Stats_Details.docx).

Reviewer 3

(1) *Fig 1 A and B don't appear to be the same section level.*

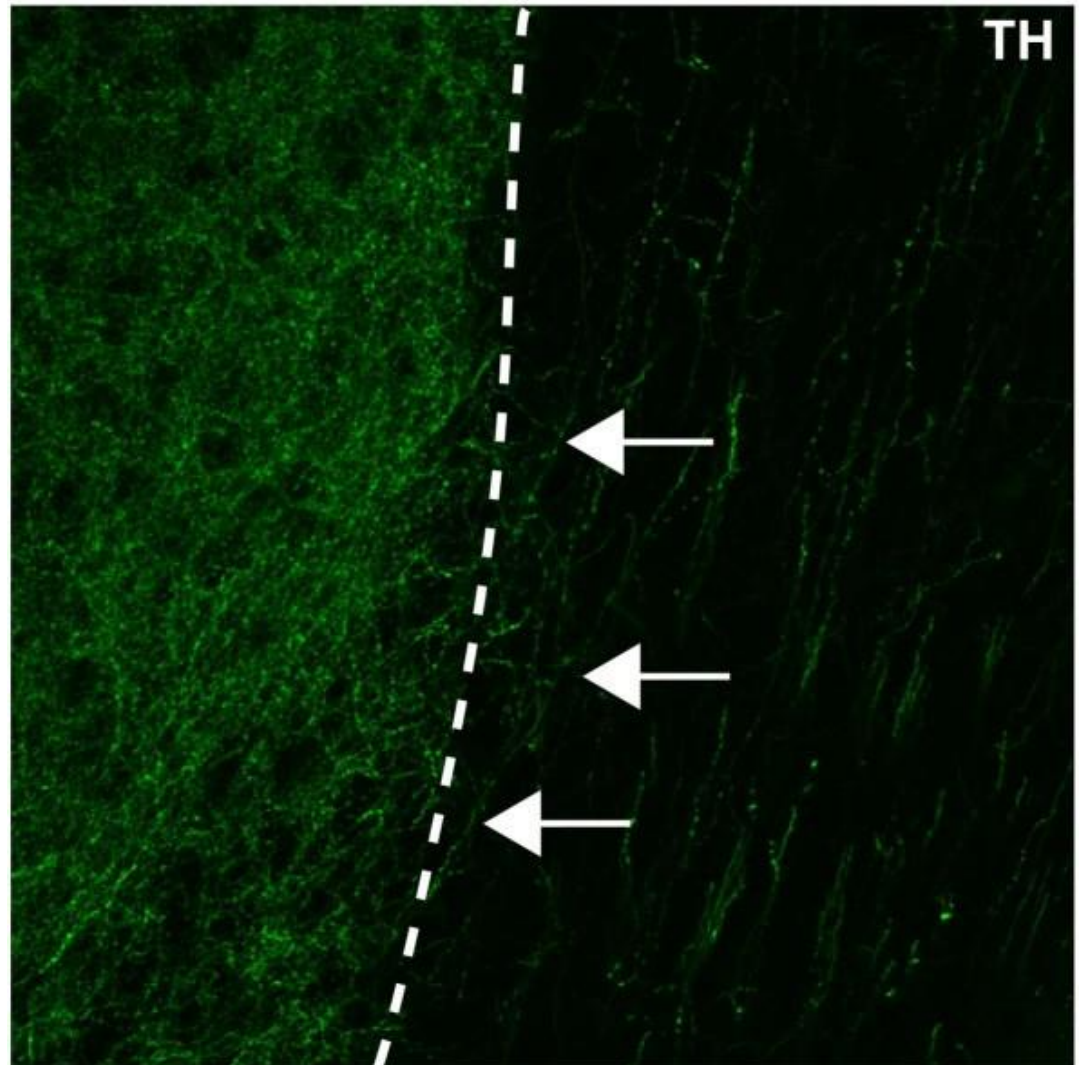
The reviewer is correct that Fig 1B is anterior to Fig 1A. We have changed Figure 1A to match the section level of Figure 1B.

(2) *Fig 1C. It is not clear that these axons are crossing from the shell of the NAC.*

We have added a dashed line to Figure 1C to highlight the boundary of the nucleus accumbens, which hopefully emphasizes that there are fibres crossing the boundary. We also include here an enlarged image of this panel:

Author response image 6.

An enlarged image of Figure1c in the manuscript. The nucleus accumbens (left of the dotted line) is densely packed with TH+ axons (in green). Some of these TH+ axons can be observed extending from the nucleus accumbens medially towards a region containing dorsally oriented TH+ fibres (white arrows).



(3) Fig 1. Measuring width of the bundle is an odd way to measure DA axon numbers. First the width could be changing during adult for various reasons including change in brain size. Second, I wouldn't consider these axons in a traditional bundle. Third, could DA axon counts be provided, rather than these proxy measures.

With regards to potential changes in brain size, we agree that this could have potentially explained the increased width of the dopamine axon pathway. That is why it was important for us to use stereology to measure the density of dopamine axons within the pathway. If the width increased but no new axons grew along the pathway, we would have seen a decrease in axon density from adolescence to adulthood. Instead, our results show that the density of axons remained constant.

We agree with the reviewer that the dopamine axons do not form a traditional “bundle”. Therefore, throughout the manuscript we now avoid using the term bundle.

Although we cannot count every single axon, an accurate estimate of this number can be obtained using stereology, an unbiased method for efficiently quantifying large, irregularly distributed objects. We used stereology to count TH+ axons in an unbiased subset of the total area occupied by these axons. Unbiased stereology is the gold-standard technique for estimating populations of anatomical objects, such as axons, that are so numerous that it would be impractical or impossible to measure every single one. Here and elsewhere we generally provide results as densities and areas of occupancy (Reynolds et al., 2022). To avoid confusion, we now clarify that we are counting the width of the area that dopamine axons occupy (rather than the dopamine axon “bundle”).

References:

Reynolds LM, Pantoja-Urbán AH, MacGowan D, Manitt C, Nouel D, Flores C. 2022. Dopaminergic System Function and Dysfunction: Experimental Approaches. *Neuromethods* 31–63. doi:10.1007/978-1-0716-2799-0_2

(4) TH in the cortex could also be of noradrenergic origin. This needs to be ruled out to score DA axons

This is the same comment as Reviewer 1 #9. Please see our response below, which we have also added to our methods:

In this study we pay great attention to the morphology and localization of the fibres from which we quantify varicosities to avoid counting any fibres stained with TH antibodies that are not dopamine fibres. The fibres that we examine and that are labelled by the TH antibody show features indistinguishable from the classic features of cortical dopamine axons in rodents (Berger et al., 1974; 1983; Van Eden et al., 1987; Manitt et al., 2011), namely they are thin fibres with irregularly-spaced varicosities, are densely packed in the nucleus accumbens, sparsely present only in the deep layers of the prefrontal cortex, and are not regularly oriented in relation to the pial surface. This is in contrast to rodent norepinephrine fibres, which are smooth or beaded in appearance, relatively thick with regularly spaced varicosities, increase in density towards the shallow cortical layers, and are in large part oriented either parallel or perpendicular to the pial surface (Berger et al., 1974; Levitt and Moore, 1979; Berger et al., 1983; Miner et al., 2003). Furthermore, previous studies in rodents have noted that only norepinephrine cell bodies are detectable using immunofluorescence for TH, not norepinephrine processes (Pickel et al., 1975; Verney et al., 1982; Miner et al., 2003), and we did not observe any norepinephrine-like fibres.

References:

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Van Eden CG, Hoorneman EM, Buijs RM, Matthijssen MA, Geffard M, Uylings HBM (1987) Immunocytochemical localization of dopamine in the prefrontal cortex of the rat at the light and electron microscopical level. *Neurosci* 22:849–862.

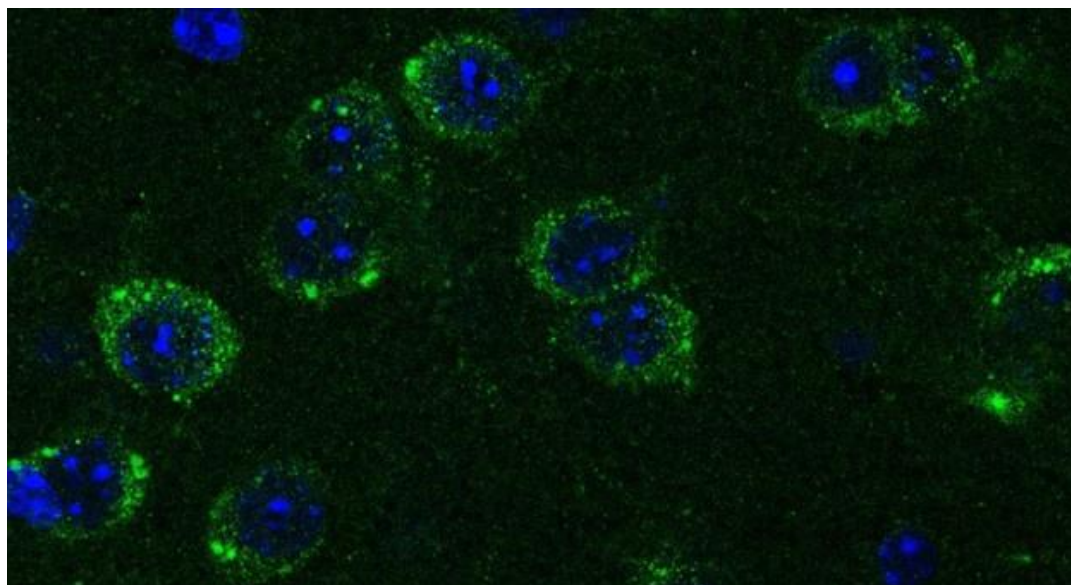
Verney C, Berger B, Adrien J, Vigny A, Gay M (1982) Development of the dopaminergic innervation of the rat cerebral cortex. A light microscopic immunocytochemical study using anti-tyrosine hydroxylase antibodies. *Dev Brain Res* 5:41–52.

(5) *Netrin staining should be provided with NeuN + DAPI; its not clear these are all cell bodies. An in situ of Netrin would help as well.*

A similar comment was raised by Reviewer 1 in point #1. Please see below the immunofluorescent and RNA scope images showing expression of Netrin-1 protein and mRNA in the forebrain.

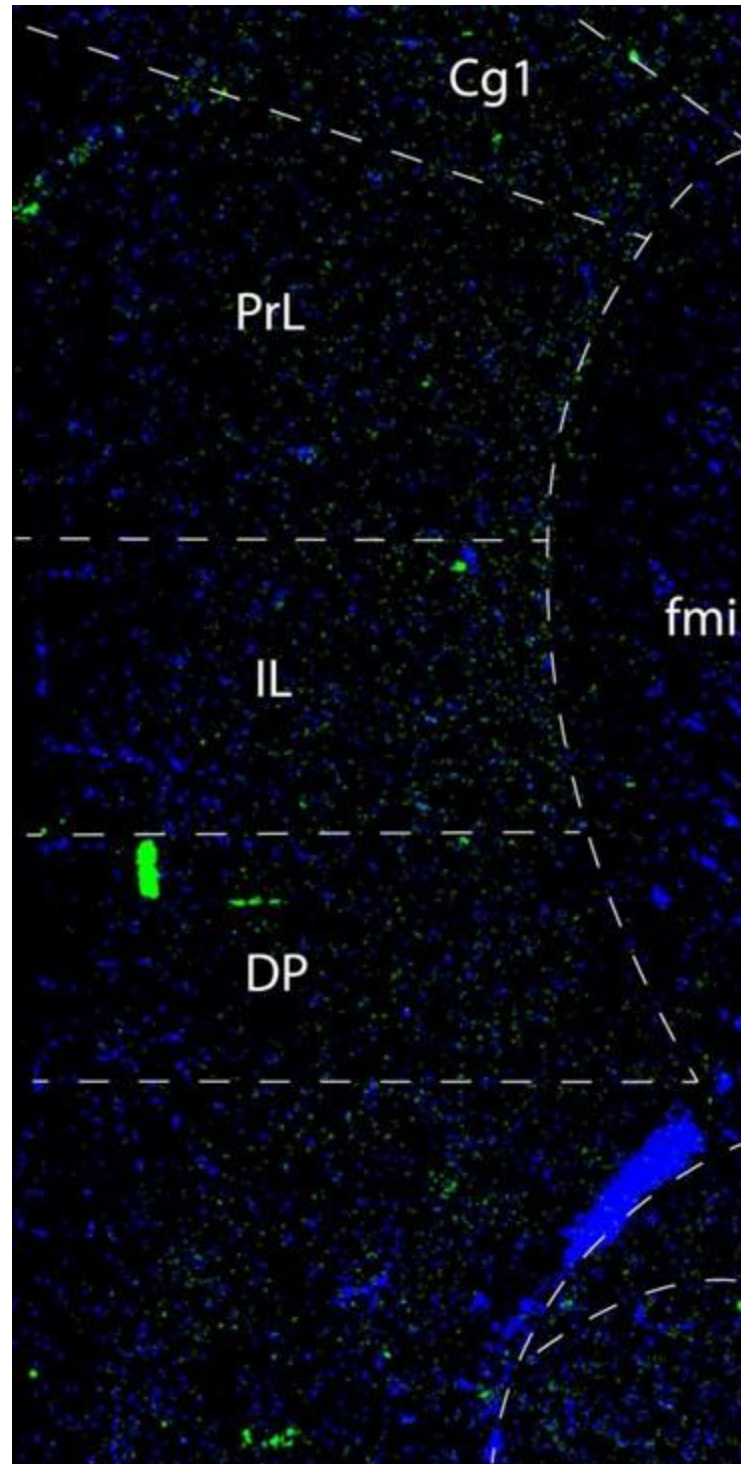
Author response image 7.

This confocal microscope image shows immunofluorescent staining for Netrin-1 (green) localized around cell nuclei (stained by DAPI in blue). This image was taken from a coronal section of the lateral septum of an adult male mouse. Scale bar = 20µm



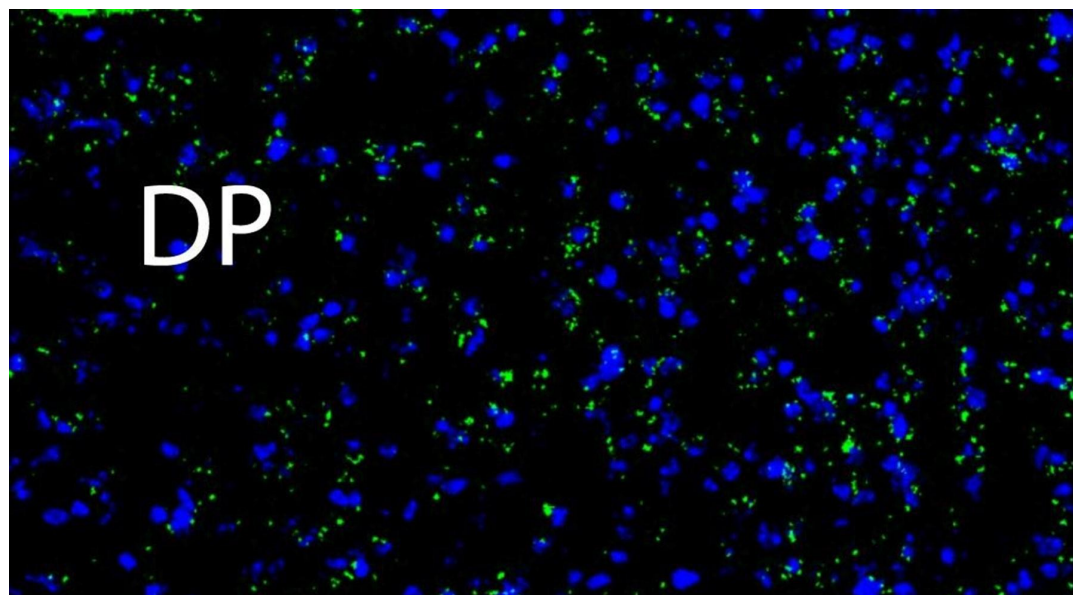
Author response image 8.

This confocal microscope image of a coronal brain section of the medial prefrontal cortex of an adult male mouse shows Netrin-1 mRNA expression (green) and cell nuclei (DAPI, blue). RNAscope was used to generate this image. Brain regions are as follows: Cg1: Anterior cingulate cortex 1, DP: dorsopeduncular cortex, IL: Infralimbic Cortex, PrL: Prelimbic Cortex, fmi: forceps minor of the corpus callosum



Author response image 9.

A higher resolution image from the same sample as in Figure 2 shows Netrin-1 mRNA (green) and cell nuclei (DAPI; blue). DP = dorsopeduncular cortex



(6) *The Netrin knockdown needs validation. How strong was the knockdown etc?*

This comment was also raised by Reviewer 1 #1.

We have previously established the efficacy of the shRNA Netrin-1 knockdown virus used in this experiment for reducing the expression of Netrin-1 (Cuesta et al., 2020). The shRNA reduces Netrin-1 levels in vitro and in vivo.

References:

Cuesta S, Nouel D, Reynolds LM, Morgunova A, Torres-Berrío A, White A, Hernandez G, Cooper HM, Flores C. 2020. Dopamine Axon Targeting in the Nucleus Accumbens in Adolescence Requires Netrin-1. *Frontiers Cell Dev Biology* 8:487. doi:10.3389/fcell.2020.00487

(7) *If the conclusion that knocking down Netrin in cortex decreases DA innervation of the IL, how can that be reconciled with Netrin-Unc repulsion.*

This is an intriguing question and one that we are in the planning stages of addressing with new experiments.

Although we do not have a mechanistic answer for how a repulsive receptor helps guide these axons, we would like to note that previous indirect evidence from a study by our group also suggests that reducing UNC5c signaling in dopamine axons in adolescence increases dopamine innervation to the prefrontal cortex (Auger et al, 2013).

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Auger ML, Schmidt ERE, Manitt C, Dal-Bo G, Pasterkamp RJ, Flores C. 2013. unc5c haploinsufficient phenotype: striking similarities with the dcc haploinsufficiency model. *European Journal of Neuroscience* 38:2853–2863. doi:10.1111/ejn.12270

(8) *The behavioral phenotype in Fig 1 is interesting, but its not clear if its related to DA axons/signaling. IN general, no evidence in this paper is provided for the role of DA in the adolescent behaviors described.*

We agree with the reviewer that the behaviours we describe in adult mice are complex and are likely to involve several neurotransmitter systems. However, there is ample evidence for the role of dopamine signaling in cognitive control behaviours (Bari and Robbins, 2013; Eagle et al., 2008; Ott et al., 2023) and our published work has shown that alterations in the growth of dopamine axons to the prefrontal cortex leads to changes in impulse control as measured via the Go/No-Go task in adulthood (Reynolds et al., 2023, 2018a; Vassilev et al., 2021).

The other adolescent behaviour we examined was risk-like taking behaviour in male and female hamsters (Figures 4 and 5), as a means of characterizing maturation in this behavior over time. We decided not to use the Go/No-Go task because as far as we know, this has never been employed in Siberian Hamsters and it will be difficult to implement. Instead, we chose the light/dark box paradigm, which requires no training and is ideal for charting behavioural changes over short time periods. Indeed, risk-like taking behavior in rodents and in humans changes from adolescence to adulthood paralleling changes in prefrontal cortex development, including the gradual input of dopamine axons to this region.

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Bari A, Robbins TW. 2013. Inhibition and impulsivity: Behavioral and neural basis of response control. *Progress in neurobiology* 108:44–79. doi:10.1016/j.pneurobio.2013.06.005

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Ott T, Stein AM, Nieder A. 2023. Dopamine receptor activation regulates reward expectancy signals during cognitive control in primate prefrontal neurons. *Nat Commun* 14:7537. doi:10.1038/s41467-023-43271-6

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Reynolds LM, Pokinko M, Torres-Berrío A, Cuesta S, Lambert LC, Pellitero EDC, Wodzinski M, Manitt C, Krimpenfort P, Kolb B, Flores C. 2018a. DCC Receptors Drive Prefrontal Cortex Maturation by Determining Dopamine Axon Targeting in Adolescence. *Biological psychiatry* 83:181–192. doi:10.1016/j.biopsych.2017.06.009

Vassilev P, Pantoja-Urbán AH, Giroux M, Nouel D, Hernandez G, Orsini T, Flores C. 2021. Unique effects of social defeat stress in adolescent male mice on the Netrin-1/DCC pathway, prefrontal cortex dopamine and cognition (Social stress in adolescent vs. adult male mice). *Eneuro* ENEURO.0045-21.2021. doi:10.1523/eneuro.0045-21.2021

(9) *Fig2 - boxes should be drawn on the NAc diagram to indicate sampled regions. Some quantification of Unc5c would be useful. Also, some validation of the Unc5c antibody would be nice.*

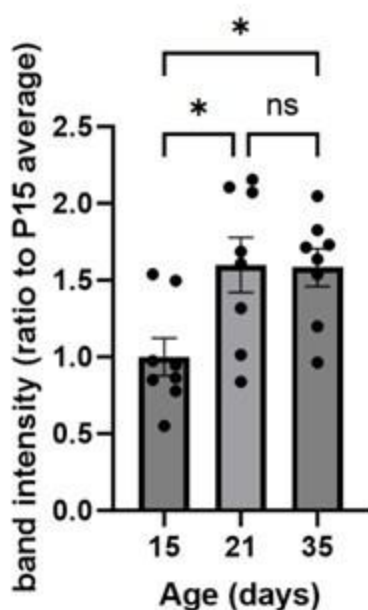
The images presented were taken medial to the anterior commissure and we have edited Figure 2 to show this. However, we did not notice any intra-accumbens variation, including

between the core and the shell. Therefore, the images are representative of what was observed throughout the entire nucleus accumbens.

To quantify UNC5c in the accumbens we conducted a Western blot experiment in male mice at different ages. A one-way ANOVA analyzing band intensity (relative to the 15-day-old average band intensity) as the response variable and age as the predictor variable showed a significant effect of age ($F=5.615$, $p=0.01$). Posthoc analysis revealed that 15-day-old mice have less UNC5c in the nucleus accumbens compared to 21- and 35-day-old mice.

Author response image 10.

The graph depicts the results of a Western blot experiment of UNC5c protein levels in the nucleus accumbens of male mice at postnatal days 15, 21 or 35 and reveals a significant increase in protein levels at the onset adolescence.



Our methods for this Western blot were as follows: Samples were prepared as previously (Torres-Berrío et al., 2017). Briefly, mice were sacrificed by live decapitation and brains were flash frozen in heptane on dry ice for 10 seconds. Frozen brains were mounted in a cryomicrotome and two 500um sections were collected for the nucleus accumbens, corresponding to plates 14 and 18 of the Paxinos mouse brain atlas. Two tissue core samples were collected per section, one for each side of the brain, using a 15-gauge tissue corer (Fine surgical tools Cat no. NC9128328) and ejected in a microtube on dry ice. The tissue samples were homogenized in 100ul of standard radioimmunoprecipitation assay buffer using a handheld electric tissue homogenizer. The samples were clarified by centrifugation at 4C at a speed of 15000g for 30 minutes. Protein concentration was quantified using a bicinchoninic acid assay kit (Pierce BCA protein assay kit, Cat no.PI23225) and denatured with standard Laemmli buffer for 5 minutes at 70C. 10ug of protein per sample was loaded and run by SDS-PAGE gel electrophoresis in a Mini-PROTEAN system (Bio-Rad) on an 8% acrylamide gel by stacking for 30 minutes at 60V and resolving for 1.5 hours at 130V. The proteins were transferred to a nitrocellulose membrane for 1 hour at 100V in standard transfer buffer on ice. The membranes were blocked using 5% bovine serum albumin dissolved in tris-buffered saline with Tween 20 and probed with primary (UNC5c, Abcam Cat. no ab302924) and HRP-conjugated secondary antibodies for 1 hour. a-tubulin was probed and used as loading

control. The probed membranes were resolved using SuperSignal West Pico PLUS chemiluminescent substrate (ThermoFisher Cat no.34579) in a ChemiDoc MP Imaging system (Bio-Rad). Band intensity was quantified using the ChemiDoc software and all ages were normalized to the P15 age group average.

Validation of the UNC5c antibody was performed in the lab of Dr. Liu, from whom it was kindly provided. Briefly, in the validation study the authors showed that the anti-UNC5C antibody can detect endogenous UNC5C expression and the level of UNC5C is dramatically reduced after UNC5C knockdown. The antibody can also detect the tagged-UNC5C protein in several cell lines, which was confirmed by a tag antibody (Purohit et al., 2012; Shao et al., 2017).

References:

Purohit AA, Li W, Qu C, Dwyer T, Shao Q, Guan K-L, Liu G. 2012. Down Syndrome Cell Adhesion Molecule (DSCAM) Associates with Uncoordinated-5C (UNC5C) in Netrin-1-mediated Growth Cone Collapse. *The Journal of biological chemistry* 287:27126–27138. doi:10.1074/jbc.m112.340174

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(10) "In adolescence, dopamine neurons begin to express the repulsive Netrin-1 receptor UNC5C, and reduction in UNC5C expression appears to cause growth of mesolimbic dopamine axons to the prefrontal cortex".....This is confusing. Figure 2 shows a developmental increase in Unc5c not a decrease. So when is the "reduction in Unc5c expression" occurring?

We apologize for the mistake in this sentence. We have corrected the relevant passage in our manuscript as follows:

In adolescence, dopamine neurons begin to express the repulsive Netrin-1 receptor UNC5C, particularly when mesolimbic and mesocortical dopamine projections segregate in the nucleus accumbens (Manitt et al., 2010; Reynolds et al., 2018a). In contrast, dopamine axons in the prefrontal cortex do not express UNC5c except in very rare cases (Supplementary Figure 4). In adult male mice with *Unc5c* haploinsufficiency, there appears to be ectopic growth of mesolimbic dopamine axons to the prefrontal cortex (Auger et al., 2013). This miswiring is associated with alterations in prefrontal cortex-dependent behaviours (Auger et al., 2013).

References:

Auger ML, Schmidt ERE, Manitt C, Dal-Bo G, Pasterkamp RJ, Flores C. 2013. *unc5c* haploinsufficient phenotype: striking similarities with the *dcc* haploinsufficiency model. *European Journal of Neuroscience* 38:2853–2863. doi:10.1111/ejn.12270

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(11) In Fig 3, a statistical comparison should be made between summer male and winter male, to justify the conclusions that the winter males have delayed DA innervation.

This analysis was also suggested by Reviewer 1, #11. Here is our response:

We analyzed the summer and winter data together in ANOVAs separately for males and females. In both sexes we find a significant effect of daylength on dopamine innervation, interacting with age. Male age by daylength interaction: $F = 6.383$, $p = 0.00242$. Female age by daylength interaction: $F = 21.872$, $p = 1.97 \times 10^{-9}$. The full statistical analysis is available as a supplement to this letter (Response_Letter_Stats_Details.docx).

(12) Should axon length also be measured here (Fig 3)? It is not clear why the authors have switched to varicosity density. Also, a box should be drawn in the NAC cartoon to indicate the region that was sampled.

It is untenable to quantify axon length in the prefrontal cortex as we cannot distinguish independent axons. Rather, they are “tangled”; they twist and turn in a multitude of directions as they make contact with various dendrites. Furthermore, they branch extensively. It would therefore be impossible to accurately quantify the number of axons. Using unbiased stereology to quantify varicosities is a valid, well-characterized and straightforward alternative (Reynolds et al., 2022).

References:

Reynolds LM, Pantoja-Urbán AH, MacGowan D, Manitt C, Nouel D, Flores C. 2022. Dopaminergic System Function and Dysfunction: Experimental Approaches. *Neuromethods* 31–63. doi:10.1007/978-1-0716-2799-0_2

(13) In Fig 3, Unc5c should be quantified to bolster the interesting finding that Unc5c expression dynamics are different between summer and winter hamsters. Unc5c mRNA experiments would also be important to see if similar changes are observed at the transcript level.

We agree that it would be very interesting to see how UNC5c mRNA and protein levels change over time in summer and winter hamsters, both in males, as the reviewer suggests here, and in females. We are working on conducting these experiments in hamsters as part of a broader expansion of our research in this area. These experiments will require a lengthy amount of time and at this point we feel that they are beyond the scope of this manuscript.

(14) Fig 4. The peak in exploratory behavior in winter females is counterintuitive and needs to be better discussed. IN general, the light dark behavior seems quite variable.

This is indeed a very interesting finding, which we have expanded upon in our manuscript as follows:

When raised under a winter-mimicking daylength, hamsters of either sex show a protracted peak in risk taking. In males, it is delayed beyond 80 days old, but the delay is substantially less in females. This is a counterintuitive finding considering that dopamine development in winter females appears to be accelerated. Our interpretation of this finding is that the timing of the risk-taking peak in females may reflect a balance between different adolescent developmental processes. The fact that dopamine axon growth is accelerated does not imply that all adolescent maturational processes are accelerated. Some may be delayed, for example those that induce axon pruning in the cortex. The timing of the risk-taking peak in winter female hamsters may therefore reflect the amalgamation of developmental processes that are

advanced with those that are delayed – producing a behavioural effect that is timed somewhere in the middle. Disentangling the effects of different developmental processes on behaviour will require further experiments in hamsters, including the direct manipulation of dopamine activity in the nucleus accumbens and prefrontal cortex.

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Private Comments

Reviewer #1

(12) The language should be improved. Some expression is confusing (line178-179). Also some spelling errors (eg. Figure 1M).

We have removed the word “Already” to make the sentence in lines 178-179 clearer, however we cannot find a spelling error in Figure 1M or its caption. We have further edited the manuscript for clarity and flow.

Reviewer #2

(1) The authors claim to have revealed how the 'timing of adolescence is programmed in the brain'. While their findings certainly shed light on molecular, circuit and behavioral

processes that are unique to adolescence, their claim may be an overstatement. I suggest they refine this statement to discuss more specifically the processes they observed in the brain and animal behavior, rather than adolescence itself.

We agree with the reviewer and have revised the manuscript to specify that we are referring to the timing of specific developmental processes that occur in the adolescent brain, not adolescence overall.

(2) Along the same lines, the authors should also include a more substantive discussion of how they selected their ages for investigation (for both mice and hamsters), For mice, their definition of adolescence (P21) is earlier than some (e.g. Spear L.P., Neurosci. and Beh. Reviews, 2000).

There are certainly differences of opinion between researchers as to the precise definition of adolescence and the period it encompasses. Spear, 2000, provides one excellent discussion of the challenges related to identifying adolescence across species. This work gives specific ages only for rats, not mice (as we use here), and characterizes post-natal days 28-42 as being the conservative age range of “peak” adolescence (page 419, paragraph 1). Immediately thereafter the review states that the full adolescent period is longer than this, and it could encompass post-natal days 20-55 (page 419, paragraph 2).

We have added the following statement to our methods:

There is no universally accepted way to define the precise onset of adolescence. Therefore, there is no clear-cut boundary to define adolescent onset in rodents (Spear, 2000). Puberty can be more sharply defined, and puberty and adolescence overlap in time, but the terms are not interchangeable. Puberty is the onset of sexual maturation, while adolescence is a more diffuse period marked by the gradual transition from a juvenile state to independence. We, and others, suggest that adolescence in rodents spans from weaning (postnatal day 21) until adulthood, which we take to start on postnatal day 60 (Reynolds and Flores, 2021). We refer to “early adolescence” as the first two weeks postweaning (postnatal days 21-34). These ranges encompass discrete DA developmental periods (Kalsbeek et al., 1988; Manitt et al., 2011; Reynolds et al., 2018a), vulnerability to drug effects on DA circuitry (Hammerslag and Gulley, 2014; Reynolds et al., 2018a), and distinct behavioral characteristics (Adriani and Laviola, 2004; Makinodan et al., 2012; Schneider, 2013; Wheeler et al., 2013).

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(3) Figure 1 - the conclusions hinge on the Netrin-1 staining, as shown in panel G, but the cells are difficult to see. It would be helpful to provide clearer, more zoomed images so readers can better assess the staining. Since Netrin-1 expression reduces dramatically after P4 and they had to use antigen retrieval to see signal, it would be helpful to show some images from additional brain regions and ages to see if expression levels follow predicted patterns. For instance, based on the allen brain atlas, it seems that around P21, there should be high levels of Netrin-1 in the cerebellum, but low levels in the cortex. These would be nice controls to demonstrate the specificity and sensitivity of the antibody in older tissue.

We do not study the cerebellum and have never stained this region; doing so now would require generating additional tissue and we're not sure it would add enough to the information provided to be worthwhile. Note that we have stained the forebrain for Netrin-1 previously, providing broad staining of many brain regions (Manitt et al., 2011)

References:

Manitt C, Mimee A, Eng C, Pokinko M, Stroh T, Cooper HM, Kolb B, Flores C. 2011. The Netrin Receptor DCC Is Required in the Pubertal Organization of Mesocortical Dopamine Circuitry. *J Neurosci* 31:8381–8394. doi:10.1523/jneurosci.0606-11.2011

(4) Figure 3 - Because mice tend to avoid brightly-lit spaces, the light/dark box is more commonly used as a measure of anxiety-like behavior than purely exploratory behavior (including in the paper they cited). It is important to address this possibility in their discussion of their findings. To bolster their conclusions about the coincidence of circuit and behavioral changes in adolescent hamsters, it would be useful to add an additional measure of exploratory behaviors (e.g. hole board).

Regarding the light/dark box test, this is an excellent point. We prefer the term “risk taking” to “anxiety-like” and now use the former term in our manuscript. Furthermore, our interest in the behaviour is purely to chart the development of adolescent behaviour across our treatment groups, not to study a particular emotional state. Regardless of the specific emotion or emotions governing the light/dark box behaviour, it is an ideal test for charting adolescent shifts in behaviour as it is well-characterized in this respect, as we discuss in our manuscript.

(5) Supplementary Figure 4,5 The authors defined puberty onset using uterine and testes weights in hamsters. While the weights appear to be different for summer and winter hamsters, there were no statistical comparison. Please add statistical analyses to bolster claims about puberty start times. Also, as many studies use vaginal opening to define puberty onset, it would be helpful to discuss how these measurements typically align and cite relevant literature that described use of uterine weights. Also, Supplementary Figures 4 and 5 were mis-cited as Supp. Fig. 2 in the text (e.g. line 317 and others).

These are great suggestions. We have added statistical analyses to Supplementary Figures 5 and 6 and provided Vaginal Opening data as Supplementary Figure 7. The statistical analyses confirm that all three characters are delayed in winter hamsters compared to summer hamsters.

We have also added the following references to the manuscript:

Darrow JM, Davis FC, Elliott JA, Stetson MH, Turek FW, Menaker M. 1980. Influence of Photoperiod on Reproductive Development in the Golden Hamster. *Biol Reprod* 22:443–450. doi:10.1095/biolreprod22.3.443

Ebling FJP. 1994. Photoperiodic Differences during Development in the Dwarf Hamsters *Phodopus sungorus* and *Phodopus campbelli*. *Gen Comp Endocrinol* 95:475–482. doi:10.1006/gcen.1994.1147

Timonin ME, Place NJ, Wanderi E, Wynne-Edwards KE. 2006. *Phodopus campbelli* detect reduced photoperiod during development but, unlike *Phodopus sungorus*, retain functional reproductive physiology. *Reproduction* 132:661–670. doi:10.1530/rep.1.00019

(6) The font in many figure panels is small and hard to read (e.g. 1A,D,E,H,I,L...). Please increase the size for legibility.

We have increased the font size of our figure text throughout the manuscript.

Reviewer #3

(15) Fig 1 C,D. Clarify the units of the y axis

We have now fixed this.

Full Reference List

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Hoops D, Flores C. 2017. Making Dopamine Connections in Adolescence. *Trends in Neurosciences* 1–11. doi:10.1016/j.tins.2017.09.004

Kalsbeek A, Voorn P, Buijs RM, Pool CW, Uylings HBM. 1988. Development of the Dopaminergic Innervation in the Prefrontal Cortex of the Rat. *The Journal of Comparative Neurology* 269:58–72. doi:10.1002/cne.902690105

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Schneider M. 2013. Adolescence as a vulnerable period to alter rodent behavior. *Cell and tissue research* 354:99–106. doi:10.1007/s00441-013-1581-2

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Wheeler AL, Lerch JP, Chakravarty MM, Friedel M, Sled JG, Fletcher PJ, Josselyn SA, Frankland PW. 2013. Adolescent Cocaine Exposure Causes Enduring Macroscale Changes in Mouse Brain Structure. *J Neurosci* 33:1797–1803. doi:10.1523/jneurosci.3830-12.2013