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Ovarian hormones organize the maturation of inhibitory neurotransmission in the frontal cortex at puberty onset in female mice

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Summary

The frontal cortex matures late in development, showing dramatic changes after puberty onset, yet few experiments have directly tested the role of pubertal hormones in cortical maturation. One mechanism thought to play a primary role in regulating the maturation of the neocortex is an increase in inhibitory neurotransmission, which alters the balance of excitation and inhibition. We hypothesized that pubertal hormones could regulate maturation of frontal cortex by this mechanism. Here, we report that manipulations of gonadal hormones do significantly alter the maturation of inhibitory neurotransmission in the cingulate region of the mouse medial frontal cortex, an associative region that matures during the pubertal transition and is implicated in decision making, learning, and psychopathology. We find that inhibitory neurotransmission, but not excitatory neurotransmission, increases onto cingulate pyramidal neurons during peripubertal development and that this increase can be blocked by pre-pubertal but not post-pubertal gonadectomy. We next used pre-pubertal hormone treatment to model early puberty onset, a phenomenon increasingly observed in girls living in developed nations. We find that pre-pubertal hormone treatment drives an early increase in inhibitory neurotransmission in the frontal cortex, but not somatosensory cortex, suggesting that earlier puberty can advance cortical maturation in a regionally specific manner. Pre-pubertal hormone treatment also accelerates maturation of tonic inhibition and performance in a frontal cortex-dependent reversal learning task. These data provide rare evidence of enduring, organizational effects of ovarian hormones at puberty and provide a potential mechanism by which gonadal hormones could regulate the maturation of associative neocortex.

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eTOC blurb

Piekarski et al., find that pubertal hormones play a critical role in the adolescent maturation of inhibitory neurotransmission in the frontal cortex. These data provide a putative mechanism by which the timing of puberty onset, independent from age, may play a role in the development of the frontal lobes and their associated functions.

Introduction

The onset of adolescence, initiated by the onset of puberty, is increasingly recognized as an inflection point for the development of associative regions of the neocortex [1]. Across mammals, adolescence is characterized by changes to cognitive and executive functions that coincide with large-scale reorganization of associative cortical regions, including the frontal cortex [2–4]. This period of development is also associated with declining plasticity in language-related circuits, declining capacity for recovery from cortical damage[5–11], and increased risk of psychiatric disease[3, 12]. While it remains unclear if these developmental changes are caused by the pubertal rise in gonadal hormones or are simply coincident [1], it is clear that early puberty onset exacerbates risk of psychiatric illnesses connected to frontal functions[13–15], suggesting a possible causal link. Gonadal steroid receptors are present across the neocortex [16–18], and a number of anatomical changes in human cortex correlate with changes in hormone levels during puberty [19–23]. In rodent experiments that manipulate hormones, frontal cortex neuron density is sensitive to gonadectomy in females but not males [24], but it is still unknown if puberty onset impacts functional measures of frontal cortex circuit development, such as inhibitory and excitatory neurotransmission.

Nuclear estrogen receptors in the associative cortices are expressed almost exclusively in fast spiking interneurons[17, 25], suggesting that the pubertal rise in gonadal hormones may directly impact inhibitory neurotransmission. Fast spiking interneurons are implicated in regulation of cognition, plasticity, and neuropsychiatric illness [26–29] and are thought to play an important regulatory role in brain development by adjusting the balance of excitation and inhibition (E/I) onto cortical pyramidal neurons. This shift in E/I balance is likely a key mechanism regulating sensitive period plasticity in primary sensory cortices [30–32]. We have previously demonstrated a striking rise in the strength of inhibitory neurotransmission in deep layers of the frontal cortex during early adolescence[33], which led us to develop a working model in which gonadal steroids drive frontal cortex maturation by increasing the strength of local inhibitory neurotransmission[1].

In the present report, we manipulated exposure to gonadal steroids in mice at peripubertal ages and measured the strength of excitatory and inhibitory inputs onto layer 2/3 (L2/3) pyramidal cells in the anterior cingulate cortex, a subregion of frontal association cortex implicated in goal-directed behavior, decision-making, and multiple forms of psychiatric disease. We found that intact gonads are necessary for the peripubertal increase in phasic inhibitory neurotransmission onto L2/3 pyramidal neurons, but that after this increase occurs, the gonads are not necessary for maintaining inhibitory neurotransmission at postpubertal levels. Because of the potential health consequences of early puberty onset in girls, we next developed a mouse model of early puberty in which we advanced the age of

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puberty onset by treating mice with gonadal hormones. We found that inducing early puberty advanced the maturation of both phasic and tonic inhibitory neurotransmission in the frontal cortex and altered performance in a frontal cortex-dependent task.

These data demonstrate that pubertal hormones play a critical role in the maturation of inhibitory neurotransmission in the frontal cortex and support a causal role for puberty, independent from age, in adolescent development of the frontal cortex. Extrapolating from rodents to humans, our findings may enhance concerns about the risks of increasingly earlier puberty onset in modern society.

Results

Inhibitory neurotransmission onto L2/3 pyramidal neurons in the cingulate cortex increases during puberty

Because the strength of inhibition onto pyramidal neurons is thought to regulate sensitive period maturation of the visual and auditory cortices, we first investigated if inhibitory neurotransmission increases in the anterior cingulate cortex during development. To this end, we measured miniature inhibitory and excitatory post synaptic currents (mIPSCs and mEPSCs) onto visually identified L2/3 pyramidal cells of prepubertal (P24) and postpubertal mice (P40–45; Figure 1A). We focused on female mice in this study due to public health concerns surrounding early-onset puberty specifically in girls [13–15].

We found that mIPSC total charge transfer increased between P24 and P40 (553.86 ± 72.04 [6 brains, 19 cells] vs 901.36 ± 60.91 [7 brains, 21 cells] pA/s; $p<0.001$; Figure 1B), and this change was driven by a significant increase in mIPSC frequency (3.60 ± 0.38 vs 5.10 ± 0.26 Hz; $p=0.009$; Figure 1C) and amplitude (23.99 ± 1.04 vs 27.45 ± 0.50 pA; $p=0.0015$; Figure 1D). In a subset of cells, mEPSC parameters were also collected. No changes in mEPSC total charge transfer (163.68 ± 38.31 [6 brains, 12 cells] vs 118.55 ± 19.99 [6 brains, 11 cells] pA/s; $p=0.29$; Figure 1E), frequency (3.76 ± 0.88 vs 2.78 ± 0.50 Hz; $p=0.32$; Figure 1F), or amplitude (14.99 ± 1.34 vs 15.26 ± 0.77 pA; $p=0.69$; Figure 1G) were observed. These data suggest there was a change in the balance of excitation and inhibition onto L2/3 pyramidal neurons from P24 to P40 driven by changes in inhibitory neurotransmission.

Intact gonads are necessary for peripubertal maturation of mIPSC frequency in cingulate cortex

To test if intact gonads are necessary for the observed rise in mIPSC frequency, we performed ovariectomy (OVX) at P24 to prevent puberty onset and then collected mIPSC data at P40–45 (4 brains, 16 cells; Figure 2A). A control group underwent sham OVX (6 brains, 19 cells), while a third group was ovariectomized at P24, then injected with estradiol and progesterone during adolescence (5 brains, 15 cells) in a replacement regimen that is sufficient to induce puberty in intact mice (see Figure 3B–E). The three groups differed significantly in total inhibitory charge transfer (sham OVX, 912.44 ± 86.31; OVX, 593.81 ± 57.30; OVX + Hormone, 992.72 ± 89.83; pA/s; $p=0.036$; Figure 2B) and mIPSC frequency (sham OVX, 5.68 ± 0.51; OVX, 3.98 ± 0.28; OVX + Hormone, 5.60 ± 0.36 Hz; $p=0.039$; Figure 2C). Using planned comparisons, we found that Sham OVX and OVX +

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Hormone groups both had significantly higher mIPSC frequency than the OVX group ($p=0.015$; $p=0.008$, respectively), suggesting that gonadal hormones are necessary and sufficient to increase mIPSC frequency. mIPSC amplitude did not vary between groups ($p=0.49$; Figure 2D). Prepubertal OVX did not affect mEPSC charge transfer, frequency, or amplitude (Table S1).

These data demonstrate that intact gonads are necessary for the peripubertal increase in inhibitory neurotransmission onto cingulate cortex L2/3 pyramidal cells, establishing a causal role for pubertal hormones in the maturation of frontal inhibitory neurotransmission.

Gonadal hormone effects on mIPSCs are organizational

The effects of gonadal hormones can be classified as "organizational" or "activational." If pubertal hormones organize the increase in frontal cortex inhibition at puberty, then postpubertal withdrawal of gonadal hormones should not affect inhibition onto these cells. To test this hypothesis, mice were allowed to progress through normal pubertal development until P40, at which point mice underwent either OVX (6 brains, 16 cells) or sham surgeries (6 brains, 10 cells; Figure 2E).

P40 OVX did not affect mIPSC charge transfer (Sham OVX, 848.66 ± 63.72; OVX, 896.79 ± 77.09; $p=0.75$; Figure 2F), frequency (Sham OVX, 4.98 ± 0.28; OVX 4.99 ± 0.34; $p=0.81$; Figure 2G), or amplitude (Sham OVX, 24.90 ± 0.80; OVX 24.61 ± 0.64; $p=0.76$; Figure 2H) two weeks after OVX. These data suggest that peripubertal exposure to gonadal steroids induces long-lasting changes to frontal cortex inhibition (an organizational effect), which does not require concurrent availability of circulating gonadal steroids (not activational).

A mouse model of earlier puberty shows early increases in mIPSC frequency and altered probability of GABA release in the cingulate cortex

The age of puberty onset is advancing in girls [35–39], and early puberty is associated with increased risk for adolescent-onset psychopathology [13–15] and may reduce plasticity [1, 5, 9]. To understand how earlier puberty affects the developing cortex, we developed a mouse model of earlier puberty. This model advances first peripubertal exposure to gonadal steroids by injecting estradiol and progesterone at P24 and P26, respectively (Figure 3A). Control sibling cage-mates were injected with oil vehicle on both days (Figure 3A). This treatment significantly advanced vaginal opening (P26.13 $\pm$ 0.13 vs P29.13 $\pm$ 0.85; n=8 per group; $t_{14}=3.47$, $p<0.01$; Figure 3B, C), which is the first external indicator of puberty onset in female mice. Hormones also advanced first estrus (P27.7 $\pm$ 0.18 vs P33.88 $\pm$ 1.63; $t_{13}=3.50$, $p<0.01$; Figure 3D, E), which agrees with previous literature showing that gonadal hormone injections disinhibit the hypothalamic-pituitary-gonadal axis to induce endogenous puberty [40, 41]. This treatment therefore yields an approximately 3–4 day time period during which age-matched, co-housed siblings vary only with respect to hormone exposure, allowing us to causally separate age- from hormone-dependent maturation of brain and behavior.

To determine if a rise in gonadal steroids can induce frontal cortex inhibitory neurotransmission development, we compared hormone treated (9 brains, 17 cells) and vehicle treated (8 brains, 17 cells) mice at P27–28, when cage mates are age-matched but differ with respect to hormone exposure. The hormone treatment group showed significant

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increases in inhibitory charge transfer (854.84 ± 80.52 vs 521.77 ± 73.04 pA/s; p=0.021; Figure 3F), and mIPSC frequency (5.78 ± 0.37 vs 3.93 ± 0.39 Hz; p=0.018; Figure 3G) but not mIPSC amplitude (21.46 ± 0.94 vs 20.99 ± 0.93 pA; p=0.71; Figure 3H). There were no differences in mEPSC parameters (Table S1).

A change in mIPSC frequency could result from an increase in the number of synapses and/or enhanced probability of GABAergic vesicle release. To test this latter possibility, we conducted a paired pulse ratio experiment in hormone (4 brains, 7 cells) and vehicle (6 brains, 11 cells) treated animals at P27 with interstimulus intervals of 50, 100 and 500ms. There was a main effect of hormone treatment (p<0.001; Figure 3I), and interstimulus interval (p=0.006), and an interaction (p=.001). Simple comparisons revealed that hormone treated mice had significantly more depressing inhibitory synapses than vehicle treated mice at 50ms (0.64 ± 0.06 vs. 0.87 ± 0.35; p<0.001) and 100ms (0.68 ± 0.05 vs. 0.97 ± 0.023; p<0.001). The groups did not differ at the 500ms interstimulus interval after adjusting for multiple comparisons (0.83 ± 0.02 vs. 0.90 ± 0.03; p=0.044 unadjusted). These data suggest that hormones enhance release probability of GABAergic vesicles.

Earlier puberty does not alter later adolescent mIPSC frequency and amplitude

It has been proposed that sensitivity to hormone exposure declines with age [42] such that earlier puberty may cause larger magnitude changes or qualitatively different effects. To test if earlier puberty alters inhibitory neurotransmission later in development, we treated intact prepubertal mice with either hormone or vehicle (as above at P24 and P26; Figure 3A) and collected data at P31–33, P36–38, and P40–45, ages at which unmanipulated mice have begun puberty. A linear mixed model with hormone treatment and age group as between subjects factors revealed no differences in inhibitory charge transfer (Figure 3J) between hormone treatment groups (p=0.81), or age groups (p=0.70), and no interaction (p=0.17). There were also no significant differences in mIPSC frequency (Figure 3K) between hormone treatment groups (p=0.86), or age groups (p=0.98) and no interaction effect (p=0.47) at these post pubertal ages.

Our analysis did reveal a significant increase in mIPSC amplitude with age (p=0.012; Figure 3L), but there was no effect of hormone treatment (p=0.81) and no interaction (p=0.19).

These data suggest that early exposure to gonadal hormones does not lead to abnormal inhibitory neurotransmission later in development.

Pubertal hormones do not strengthen inhibitory neurotransmission in barrel cortex

To test if hormone-driven increases in inhibitory neurotransmission are specific to the frontal cortex, we treated mice with either hormone (7 brains, 15 cells) or vehicle (4 brains, 10 cells) before puberty (Figure 4A) and collected data at p27–28 from somatosensory barrel cortex. Hormone treatment did not affect mIPSC inhibitory charge transfer (790.93 ± 119.41 hormone vs 1044.63 ± 281.66 vehicle, pA/s; p=0.33; Figure 4B) or frequency (4.31 ± 0.53 hormone vs 4.71 ± 1.00 vehicle, Hz; p=0.69; Figure 4C). However, hormone treated mice had significantly smaller mIPSC amplitudes (23.15 ± 0.88) compared to vehicle controls (26.09 ± 1.35 pA; p=0.046; Figure 4D). These data suggest hormonal effects on the maturation of inhibition during adolescence are regionally specific.

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Tonic inhibition is sensitive to gonadal hormones

While the above experiments focused on phasic inhibition at GABAergic synapses, tonic inhibition mediated by non-synaptic GABA receptors also affects neuronal excitability and changes dramatically at puberty onset in the hippocampus [43]. In the cortex, a major contributor to tonic inhibition are receptors expressing the δ subunit [44]. These receptors are directly modulated by neurosteroids [45, 46] and are implicated in pubertal changes in synaptic plasticity and function of the mouse hippocampus [47].

To measure tonic inhibition (Figure 5A), L2/3 pyramidal cells in the cingulate cortex were patched and excitatory neurotransmission blocked. δ subunit containing GABA receptors were opened by bath application of the superagonist THIP (1 μM), and then closed by the $GABA_A$ antagonist bicuculline (20μM; Figure 5B). The shift in holding current was used to infer tonic inhibition.

Tonic inhibition was measured at P27 in vehicle (4 brains, 6 cells; −26.55 ± 8.08 pA) and hormone (4 brains, 9 cells; −7.93 ± 2.88 pA) treated mice (the mouse model of earlier puberty, as above), and in untreated postpubertal mice at ~P40 (3 brains; 6 cells; 1.53 ± 3.2 pA) (Figure 5A). There was a significant difference in tonic inhibition among groups ($p<0.001$; Figure 5C). Post-hoc comparisons revealed differences between hormone and vehicle treated mice ($p=0.005$) and vehicle treated and adolescent mice ($p<0.001$), but no difference between hormone treated P27 mice and P40 mice ($p=0.12$).

These data suggest that the potential size of the tonic inhibitory current declines in L2/3 cingulate pyramids from P27 to P40, and early hormone treatment can advance this decline.

Exposure to gonadal hormones alters performance in a cognitive task sensitive to the integrity of the dorsomedial frontal cortex

After documenting changes in inhibitory neurotransmission that occur in the cingulate after hormonal exposure, our next goal was to determine if pubertal hormones also alter behavior dependent on this brain region. We previously established that excitotoxic lesions centered on the cingulate disrupt performance in an odor-based 4-choice reversal task in both juveniles and adults [48]. Performance in this task also changes significantly with development such that mice at P26 show more efficient reversal learning compared to mice P40 or older [1, 48]. We predicted that early hormone exposure would prematurely induce adult-like performance in the reversal phase of the task, indicated by an increase in trials to criterion.

Mice were treated at P24 and P26 with either hormone or vehicle (as above) and tested on the 4-choice discrimination and reversal task at P27–28 (Figure 6A). As predicted, hormone treated mice required more trials to reach criterion during the reversal phase than littermate vehicle controls (controlling for cage; $F_{1,10}=5.50$; $p=0.041$; Figure 6B). Hormone and vehicle pairs did not differ in the discrimination phase ($F_{1,10}=1.33$; $p=0.28$; Figure 6B), suggesting more basic aspects of task performance were comparable.

In order to determine if hormones were necessary for the developmental increase in trials to criterion in this task, we also performed OVX or sham OVX at P24 and tested mice under

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the same paradigm between P40 and P45 (Figure 6C). OVX and sham groups did not differ in performance in the discrimination ($F_{1,9}$=0.27; p=0.62; Figure 6C) or reversal phase ($F_{1,9}$=0.09; p>0.77; Figure 6C).

These data suggest hormones can prematurely induce adult-like reversal learning performance, but that maturation of reversal learning can still occur in the absence of intact gonads.

Discussion

In the present report, we provide evidence that the rise in gonadal steroids at puberty is necessary for a developmental increase in phasic inhibitory neurotransmission in anterior cingulate cortex during adolescence. These data demonstrate that puberty itself, not just age, plays a role in frontal cortex maturation, supporting a broad literature of correlational studies that suggest both puberty and its timing play an important role in the development of associative neocortex function, plasticity, and associated pathology.

The changes we observe in phasic inhibitory neurotransmission at puberty become independent of hormones/gonads at postpubertal ages, a hallmark of an organizational effect of gonadal steroids. Organizational effects are frequently observed in sexually dimorphic subcortical regions [49–52], are often regulated by androgens, and may vary in their intensity based on timing of hormonal exposure[42, 53]. In females, there is evidence that ingestive [54] and parental behavior [55] may be organized by ovarian hormones at puberty, but to our knowledge, the present data are the first to demonstrate changes in neurotransmission organized by ovarian hormones at puberty.

Our current study included only females, however, we previously found that inhibitory neurotransmission matures similarly between P25 and P40 in layer 5 in males and females [33]. Androgen and estrogen receptor expression is also comparable between sexes in rats [17], and aromatase can convert androgens to estrogens, enabling signaling via estrogen receptors in either sex. Future studies in males will be needed to determine if the present results replicate in males.

Understanding the full cascade of pubertal changes—from gonad to cortical circuit—will require continued investigation

Changes to mIPSC frequency likely reflect changes to the presynaptic cell, while changes to mIPSC amplitude are thought to reflect the number of GABA receptors on the postsynaptic membrane. Because we primarily observe an effect of gonadal steroids on mIPSC frequency, our data suggest that gonadal steroids may primarily affect presynaptic inhibitory cells. An increase in mIPSC frequency could be caused by a larger number of synapses, higher probability of vesicle release, or an increase in the number of docked synaptic vesicles per bouton. We found that early hormone exposure caused inhibitory synapses to become more depressing, suggesting that the release probability in GABAergic terminals was increased, which would enhance measures of mIPSC frequency and total inhibitory charge transfer. However, increased number of synapses or docked vesicles could still contribute.

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Because all inhibitory inputs onto patched pyramidal cells were recorded, it remains unknown what interneuron subtypes mediate the increase in phasic inhibition, but a strong candidate cell type is the parvalbumin positive fast-spiking interneuron. In the rodent frontal cortex, estrogen receptor beta is expressed primarily in these interneurons [17, 25], providing a mechanism for pubertal estrogens to act specifically on these cells. This mechanism, however, still needs to be tested.

While hormone exposure increased phasic inhibitory neurotransmission, it reduced tonic inhibitory currents in the same population of cells. We speculate that changes in tonic inhibition may maintain homeostatic balance in the face of changing E/I balance. A decrease in the ratio of tonic to phasic inhibitory current would potentially alter information processing and circuit plasticity [26, 30], providing a basis for some of the many functional changes observed in the frontal cortex during adolescence [1].

Increases in inhibition have been implicated in regulating sensitive periods in neocortex

Studies in sensory cortices have identified numerous sensitive periods that vary by cortical region and input [56–58]. A common mechanism linking many of these identified sensitive periods is a change in E/I balance, driven largely by increased inhibitory neurotransmission. For example, in the literature on ocular dominance in the visual cortex, pharmacologically increasing inhibition can prematurely open [59] and close [60] sensitive period plasticity, while reducing inhibition in adulthood can reopen sensitive period plasticity [61]. Changes in mIPSCs in the visual cortex also track shifts in ocular dominance plasticity, such that an increase in mIPSC frequency onto L2/3 pyramidal neurons occurs with age (from week 3 to week 5) and is blocked by dark rearing, an environmental manipulation that delays sensitive period onset [62].

The present data demonstrate that pubertal hormones can be causally linked to an increase in phasic inhibition in the frontal cortex, providing a potential mechanism by which puberty could regulate E/I balance and putative sensitive periods for plasticity in this region. Importantly, hormone treatment did not strengthen inhibitory neurotransmission in the somatosensory cortex, demonstrating regional specificity of the effect. It remains unclear how many regions of cortex are affected by pubertal hormones, but it is possible that associative cortex may be particularly sensitive to puberty. If pubertal hormones regulate sensitive periods of plasticity and learning in frontal cortex in the human brain, the timing of educational and psychiatric interventions in relation to puberty may be critically important for their effectiveness [9, 63].

Early maturation of a frontal cortex dependent behavior can be elicited by hormonal treatment, but maturation was not blocked by ovariectomy

It is impossible to investigate human language learning and many education relevant variables in mice, but we can probe simpler functions of the frontal cortex in mouse models. We have previously established that performance in an odor-based reversal learning task in mice is dependent upon the integrity of the cingulate and surrounding frontal regions [48] and matures between P26 and P40 [1, 48]. Here, our mouse model of earlier puberty showed more adult-like performance at P27, but prepubertal ovariectomy did not prevent adult-like

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performance at P40. We speculate that capacity for learning or plasticity surrounding the time of puberty onset may be altered, but puberty-independent and/or compensatory processes may be sufficient to eventually produce adult-form behavioral performance in ovariectomized mice.

Puberty onset is advancing to earlier ages, and early puberty is associated with disadvantage and greater disease burden

Adolescence is a paradoxical life stage in which physical health is strong but there are increasing risks for wide ranges of psychiatric disorders and other negative behavioral outcomes [3, 12, 14, 15]. Earlier than normal puberty onset is associated with increased risk of psychiatric disease and lower educational achievement [14, 65–73]. In recent decades, the age at puberty onset has significantly advanced, particularly in girls [35–39], with the youngest ages observed in urban poor, African American, and Hispanic populations [37]. It is difficult to determine if negative psychiatric and educational outcomes associated with early puberty are mediated by direct effects of pubertal hormones on the brain, indirect effects of psychosocial difficulties associated with early puberty, or complex interactions between pubertal timing and psychosocial factors [15]. However, the frontal cortex is implicated in many of these psychiatric and behavioral outcomes, and our data demonstrate that maturation of this region is directly impacted by gonadal hormones. Interestingly, we find that early exposure to gonadal hormones advances the timing of the maturation of inhibition but does not alter the levels that are reached in young adulthood. This result is in contrast to data in which earlier rather than later exposure to testosterone was associated with lasting differences in behavior in hamsters [42, 53]. As we increase our knowledge, we speculate that we will find that some developing systems are sensitive to pubertal hormones while others are not, and the timing of puberty onset may alter the phase-relationship between a number of intricately interconnected neural systems, resulting in different neural, emotional, and behavioral outcomes mediated by age at pubertal onset.

Conclusion

Our data suggest that pubertal hormones are critical for the maturation of inhibitory neurotransmission in the frontal cortex. Further work is required to understand the mechanisms by which gonadal maturation impacts neuronal development and how inhibitory neurotransmission alters plasticity of frontal cortical networks. However, our data provide novel evidence of enduring, organizational effects of pubertal ovarian steroids and provide further evidence that puberty directly impacts cerebral cortex maturation. Given that inhibitory neurotransmission regulates sensitive periods in other cortical regions and is implicated in psychopathology, we conclude that pubertal timing likely plays a powerful role in frontal cortex development. To fully understand normative and pathological development of brain and behavior, measures of puberty onset and changes in circulating gonadal hormones are important variables to consider in future investigations of adolescent brain maturation.

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests should be directed to and will be fulfilled by the Lead Contact, Linda Wilbrecht (Wilbrecht@berkeley.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All experiments were conducted in female C57BL/6J mice from the Thy-1-YFP-H line bred in our animal facility. All mice were weaned at P21 and randomly separated into same-sex groups of 2 or 3 littermates. Mice varied in age from P24 to P55, depending on the experiment. All were housed on a 12:12 reverse light:dark cycle (lights on at 10PM). All procedures were approved by the Animal Care and Use Committee of the University of California, Berkeley and conformed to principles enunciated in the NIH guide for the use and care for laboratory animals.

METHOD DETAILS

Puberty Onset

To collect normative data from our colony on the age at pubertal milestones, we determined the age at vaginal opening and first estrus. Age at vaginal opening was determined by visual examination of the vagina beginning on P24. To determine age at first estrus, starting on the day of vaginal opening, vaginal lavages were collected by placing a small drop of distilled water at the vaginal opening to collect vaginal cells. The first day in which cornified epithelials represent a majority of cells was recorded as first estrus.

The mice used for vaginal lavages were not used for collection of any other data (i.e. behavior or electrophysiology) in order to avoid potential confounds related to the additional handling required for vaginal lavage. Visual assessment of vaginal opening was performed to confirm pubertal status of mice used in electrophysiology and behavior studies, but this one-time visual assessment (approximately 2 seconds) did not require any additional handling beyond what would otherwise be required to transfer mice to a behavior apparatus or anesthetize mice for perfusion/electrophysiology.

Hormone Manipulations

To accelerate puberty, female mice were injected with a regimen of steroid hormones dissolved in oil. The regimen consisted of a single subcutaneous (s.c.) 0.01 μg/g 17β-estradiol benzoate injection followed 48 hours later by a single subcutaneous progesterone (0.2 mg/g) injection.

To eliminate circulating gonadal steroids, ovariectomy or a control sham surgery was performed. In both cases, mice were pretreated with s.c. injection of the analgesics buprenorphine (0.05mg/kg) and meloxicam (10mg/kg) and anesthetized with isoflurane vapors (Baxter Healthcare, Deerfield, IL). Mice were then ovariectomized through a midline incision in the abdomen. Each ovary was ligated with absorbable suture and excised. Incisions were closed with sterile sutures and wound clips (Mikron Auto Clip 9 mm, Becton Dickinson, Franklin Lakes, NJ, USA). Follow up doses of meloxicam and buprenorphine

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were administered 12–24 hours after surgery (10mg/kg). Sham surgeries were also conducted in which a midline abdominal incision was made and the ovaries were simply visualized by the surgeon, rather than being ligated and excised. Sutures, post-op care, and analgesia were identical between procedures.

Slice Electrophysiology

Mice were deeply anesthetized with an overdose of ketamine/xylazine solution and perfused transcardially with ice-cold cutting solution containing (in mM): 110 choline-Cl, 2.5 KCl, 7 MgCl2, 0.5 CaCl2, 25 NaHCO3, 11.6 Na-ascorbate, 3 Na-pyruvate, 1.25 NaH2PO4, and 25 D-glucose, and bubbled in 95% O2/5% CO2. 300 μm thick coronal sections were cut in ice-cold cutting solution before being transferred to ACSF containing (in mM): 120 NaCl, 2.5 KCl, 1.3 MgCl2, 2.5 CaCl2, 26.2 NaHCO3, 1 NaH2PO4 and 11 Glucose. Slices were bubbled with 95% O2/5% CO2 in a 37°C bath for 30 min, and allowed to recover for 60 min at room temperature before recording.

All recordings were made using a Multiclamp 700 B amplifier and were not corrected for liquid junction potential. Data were digitized at 10 kHz and filtered at 1 or 3 kHz using a Digidata 1440 A system with PClamp 10.2 software (Molecular Devices, Sunnyvale, CA, USA). Only cells with access resistance of <25 MΩ were retained for analysis. Access resistance was not corrected. Cells were discarded if parameters changed more than 20%. Data were analyzed using MiniAnalysis (Synaptosoft, INC, GA), PClamp, or R (R 3.3.1; R Foundation for Statistical Computing, Vienna, AT).

Miniature inhibitory and excitatory post synaptic currents (mIPSCs and mEPSCs, respectively) were recorded in L2/3 pyramidal cells in the anterior cingulate cortex. Whole cell mEPSCs were recorded at −70mV and mIPSCs at +10mV. The bath contained 32°C aCSF with 0.5 μM tetrodotoxin to block action potentials. Recording pipettes had 2–5 MΩ resistances and were filled with internal solution (in mM): 115 Cs-methanesulfonate, 10 HEPES, 10 BAPTA, 10 Na2-phosphocreatine, 5 NaCl, 2 MgCl2, 4 Na-ATP, 0.3 Na-GTP. An integrated measure of the total inhibitory charge transfer was calculated using the equation: $Q = f \times Q_{mPSCs}$, where f is the frequency (Hz), and Q_{mPSCs} is the average charge transfer for each mPSC [74].

To calculate paired pulse ratio, layer 2/3 pyramidal cells in the anterior cingulate cortex (ACC) were whole-cell patch clamped, and inhibitory currents were isolated using a high chloride internal solution (in mM): 115 CsCl, 10 HEPES, 10 Na2-phosphocreatine, 5 NaCl, 2 MgCl2, 4 Na-ATP, 0.3 Na-GTP, 0.02 EGTA and 15 KCl. The bath solution contained 5 μM NBQX and 25 μm APV to block AMPA/Kainate and NMDA currents, respectively. A bipolar stimulating electrode was placed ~300 uM from the cell body, and stimulation intensity ranged between 1 and 2 μA.

Paired pulse ratio was calculated by injecting 0.1μS stimulations at either 50 ms, 100 ms or 500 ms inter stimulus intervals and dividing the second pulse by the first pulse. With short inter-stimulus intervals, the second pulse overlapped with the decay phase of the first pulse; thus, all traces were baseline subtracted.

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In experiments to measure tonic inhibition, inhibitory currents were isolated using a high chloride internal solution (in mM): 115 CsCl, 10 HEPES, 10 Na2-phosphocreatine, 5 NaCl, 2 MgCl2, 4 Na-ATP, 0.3 Na-GTP, 0.02 EGTA and 15 KCl. The bath solution contained 0.5 μM tetrodotoxin (Tocris), 5 μM NBQX (Tocris) and 25 μm D-AP5 (Tocris) to block action potentials, AMPA/Kainate, and NMDA currents, respectively.

A cell was patched and voltage clamped at −70mV. After achieving a stable baseline, 1μM 4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridine-3-ol (THIP) (Tocris) was washed on for three minutes to open all GABA receptors containing the δ subunit, which provide extrasynaptic (tonic) inhibition in these cells. Finally, 20 μM bicuculline (Tocris) was added to the aCSF. Tonic inhibition was calculated by subtracting the holding current during THIP administration from the holding current after bicuculline administration, after spontaneous IPSCs had been reduced.

In order to avoid oversampling from individual mice, a maximum of 4 cells per mouse were included for analysis across all electrophysiology experiments.

Behavior

For a full description of procedures, see [48, 77]. Briefly, mice were trained in a 4-choice discrimination and reversal task. Prior to training, mice were food restricted to 85% of *ad libitum* body weight, adjusting for expected weight gain with growth in young animals. On day 1, mice were habituated to the testing arena, on day 2 were taught to dig for cheerio rewards in a pot filled with wood shavings, and on day 3 underwent a 4-choice odor discrimination and reversal task.

During the discrimination phase, the mouse learned to discriminate among four different pots differentiated only by the scent of the wood shavings, to learn that a cheerio was hidden beneath only the anise scented shavings. The stimulus presentation was pseudo-randomized so that an odor only repeated in a quadrant once per 4 trials in order to prevent mice from using a spatial strategy to solve the task. Each trial was considered complete when the mouse indicated a “decision” by digging in a pot. If the chosen pot contained a reward, the mouse was allowed to finish eating the cheerio, but if the pot did not contain a cheerio, the mouse was immediately placed back in the starting cylinder to prevent multiple digging choices. If a mouse did not dig in a pot within 3 minutes of the start of the trial, the mouse was placed back in the starting cylinder and the trial was scored an omission. In the unusual circumstance of 3 consecutive omissions, a pot with unscented shavings was placed in the start cylinder with the mouse in order to reinitiate digging. The discrimination phase ended after the mouse had successfully chosen the correct pot on eight out of the last ten choices.

Upon reaching criterion, the reversal phase was immediately started. All shavings were replaced with new shavings, and the thyme scented pot was replaced with a new pot filled with eucalyptus scented shavings. During this phase, the cheerio was now located in the clove scented pot. The reversal phase was considered complete once the mouse reached criterion of eight out of the last ten choices correct. Animals typically completed both discrimination and reversal phases within 3 hours.

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QUANTIFICATION AND STATISTICAL ANALYSIS

All data was analyzed blind to the experimental group. All statistical analyses were conducted in R (R 3.3.1; R Foundation for Statistical Computing, Vienna, AT) and statistical details are located in the results and figure legends. Because electrophysiology measures included multiple cells from the same brain, these data were analyzed by fitting a linear mixed model using residual maximum likelihood in the *lme4* package (version 1.1.12; [75]). Residuals were verified to be normally distributed. P-values for these analyses were determined using the *car* package (version 2.1.3; [76]). Group assignment was included as the fixed effect and subject as the random effect. Student's t tests or analysis of variance (ANOVA) were used to analyze pubertal milestones and behavior, respectively. When required, Tukey's HSD test was used to control for error-rate in post-hoc analyses. All results are reported as the mean ± standard error of the mean (S.E.M). Differences were considered significant if $p < 0.05$.

DATA AND SOFTWARE AVAILABILITY

NA

ADDITIONAL RESOURCES

NA

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Inhibitory neurotransmission increases in the frontal cortex after puberty
- Pre-pubertal but not post-pubertal gonadectomy blocks this increase
- Pre-pubertal hormone treatment accelerates maturation of tonic and phasic inhibition
- Hormone treatment, which drives early puberty, impacts behavioral flexibility

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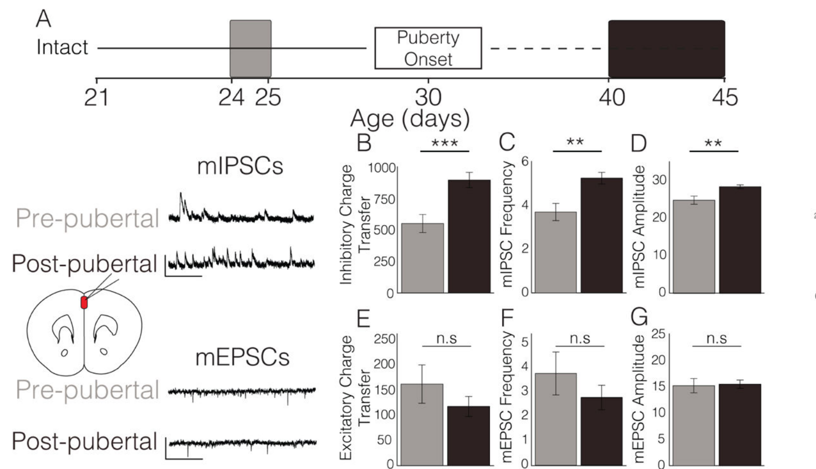

Figure 1. Inhibitory neurotransmission increases in strength during pubertal development in layer 2/3 of the cingulate cortex, but excitatory neurotransmission does not

(A) We made whole cell recordings from L2/3 pyramidal cells in the anterior cingulate cortex before puberty (P24–25) and after puberty (P40–45). mIPSC charge transfer (B) frequency (C) and amplitude (D) were significantly higher in adolescent mice (7 brains, 21 cells) compared to juveniles (6 brains, 19 cells). Neither mEPSC charge transfer (E), frequency (F), nor amplitude (G) was different between juvenile (6 brains, 12 cells) and adolescent (6 brains, 11 cells) age groups. These data suggest a developmental change in the balance of excitation and inhibition onto these cingulate neurons is driven primarily by changes in inhibitory neurotransmission. All graphs are mean ± S.E.M. Hypothesis tests were conducted using linear mixed models. **$p<0.01$, ***$p<0.001$; n.s, not significant. Scale bar: 50pA, 500ms.

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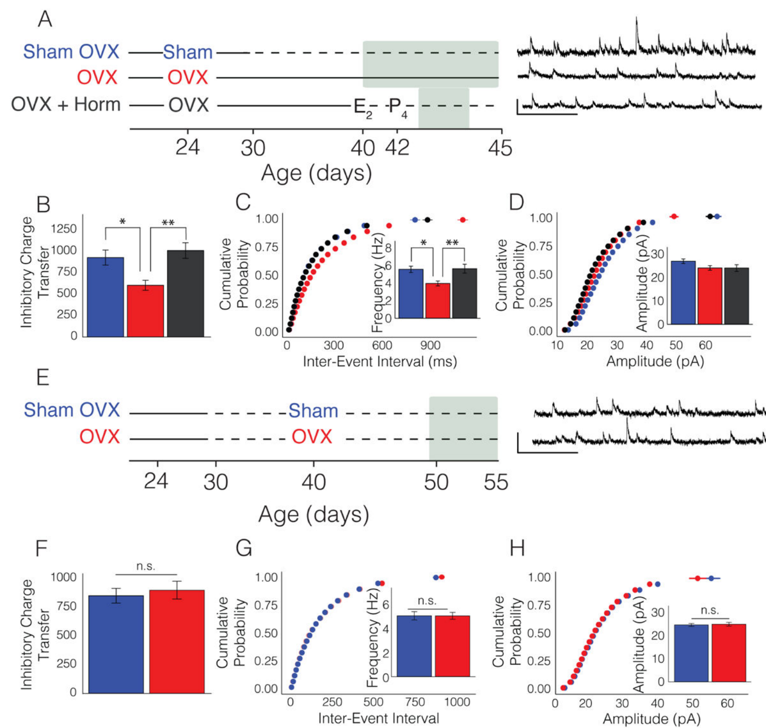

Figure 2. Prepubertal, but not postpubertal, ovariectomy prevents the rise in inhibitory neurotransmission

(A) Mice were either ovariectomized (OVX) or sham ovariectomized (Sham) prior to puberty. One group of OVX mice received hormone replacement during mid-adolescence (OVX + Horm). L2/3 pyramidal cells were patched between P40 and P45 for the OVX and Sham OVX groups, and within two days of progesterone injection in the OVX + Horm group. Pre-pubertal OVX (4 brains, 16 cells) resulted in significantly decreased inhibitory charge transfer (B), and mIPSC frequency (C) but no differences in mIPSC amplitude (D) compared to both sham controls (6 brains, 19 cells) and OVX+ Horm mice (5 brains, 15 cells; planned comparisons). In a follow-up study, mice were either OVX or Sham OVX after puberty (P40) and L2/3 pyramidal cells were patched 10–15 days later (E). There were no differences between sham OVX (6 brains, 10 cells) and OVX (6 brains, 16 cells) mice in inhibitory charge transfer (F), mIPSC frequency (G) or mIPSC amplitude (H). All graphs are mean ± S.E.M. Hypothesis tests conducted using linear mixed models. *$p<0.05$; **$p<0.01$; n.s, not significant. Scale bar: 50pA, 500ms. See also Table S1.

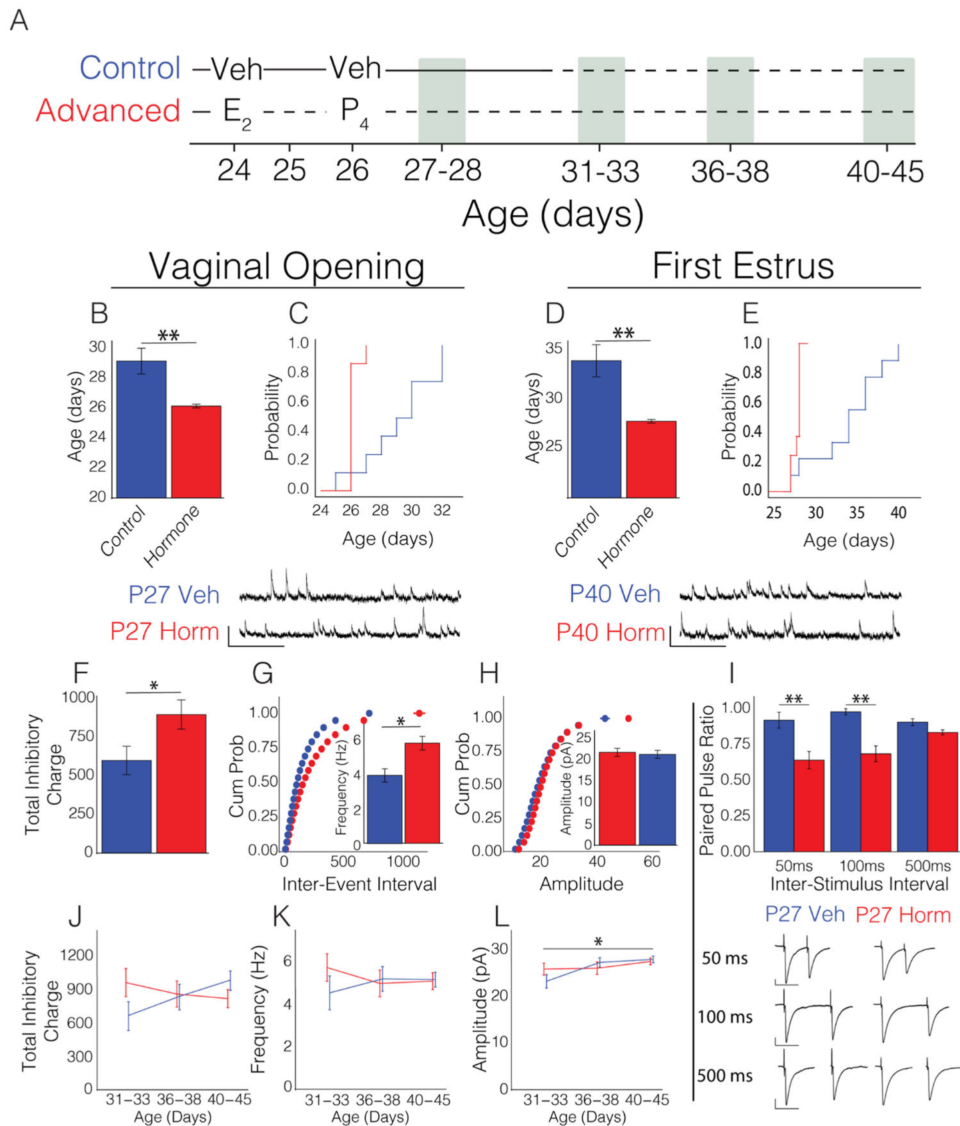

Figure 3. Frontal cortical changes in a mouse model of earlier puberty: Early hormone exposure advances puberty, increases inhibitory neurotransmission, and decreases paired pulse ratio in intact females

(A) To model earlier puberty, intact female mice were injected with estradiol at P24 and progesterone at P26, while controls were injected with vehicle at both ages. This hormone treatment was timed to occur before an expected rise in gonadal steroids and to drive a leftward (earlier) shift in the age at puberty onset (B, C). We confirmed that age at vaginal opening (B, C) and first estrus (D, E) occurred at significantly earlier ages in hormone treated (n=8) compared to vehicle treated (n=8) mice. In a separate cohort of hormone and vehicle treated mice, we recorded mPSCs at P27–P28, when the groups differed with respect to pubertal status but not age. At this time, the hormone treatment group (9 brains, 17 cells) showed higher total inhibitory charge transfer (F) and higher mIPSC frequency (G) compared to controls (8 brains, 17 cells). mIPSC amplitude (H) did not differ between groups. (I) In a follow up experiment we investigated presynaptic function at P27–28 using a

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standard paired pulse inhibition paradigm. Measuring electrically evoked currents in Layer 2/3 pyramids, we found that hormone treated mice (4 brains, 7 cells) exhibited more depressing inhibitory synaptic transmission at 50 and 100 ms inter-stimulus intervals compared to vehicle treated mice (6 brains, 11 cells). (J) We also collected mIPSC data from hormone and vehicle treated groups from post-pubertal timepoints to determine if early hormone exposure induced long-lasting changes to mIPSC measures. Data was collected at three age group: P31–33 (8 cells, 4 brains in hormone group; 9 cells, 3 brains in vehicle group); P36–38 (3 brains, 10 cells in hormone group; 3 brains, 8 cells in vehicle group); and P40–45 (3 brains 10 cells in hormone group and 4 brains, 11 cells in vehicle group). There were no main effects of hormone treatment or age, and no interaction in total charge transfer (J), or frequency (K). A main effect of age was found for mIPSC amplitude (L), without treatment or interaction effects. All plots are mean ± S.E.M. Hypothesis tests on pubertal milestones were conducted with two-sided t tests, and hypothesis tests for electrophysiology data were conducted using linear mixed models. Post hoc tests in paired pulse ratio experiment (I) were conducted using Tukey's HSD. *$p<0.05$; **$p<0.01$. Scale bar: 50 pA, 500 ms for mini traces, 50 pA, 50ms for paired pulse ratio. See also Table S1.

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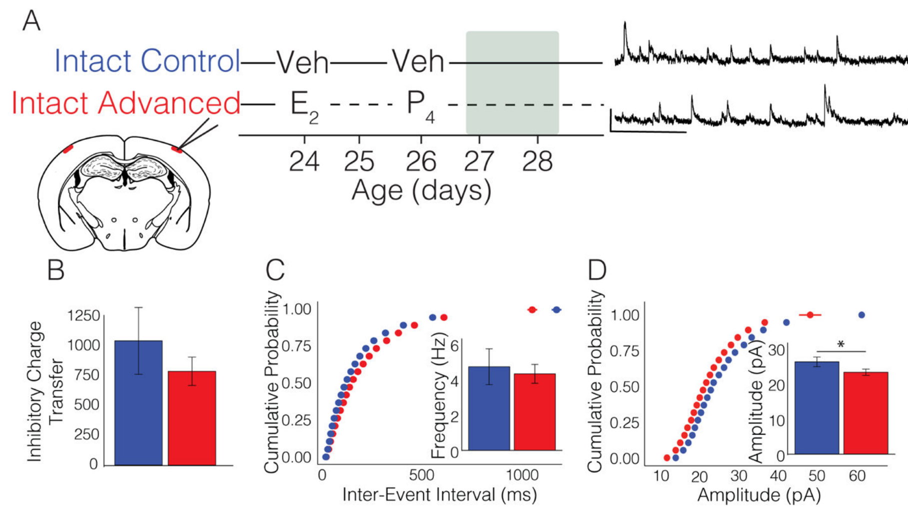

Figure 4. Early exposure to gonadal steroids does not increase inhibitory neurotransmission in somatosensory cortex

(A) Mice were injected with either gonadal steroids (7 brains, 15 cells) or vehicle (4 brains, 10 cells) at P24 and P26. Layer 2/3 pyramidal cells were patched at P27 or P28 in barrel cortex. We observed no significant differences in inhibitory charge transfer (B) or mIPSC frequency (C), but the hormone treatment group did show significantly smaller mIPSC amplitude (D) compared to vehicle. All graphs are mean ± S.E.M. Hypothesis tests conducted using linear mixed models. *p<0.05. Scale bar: 50 pA, 500ms.

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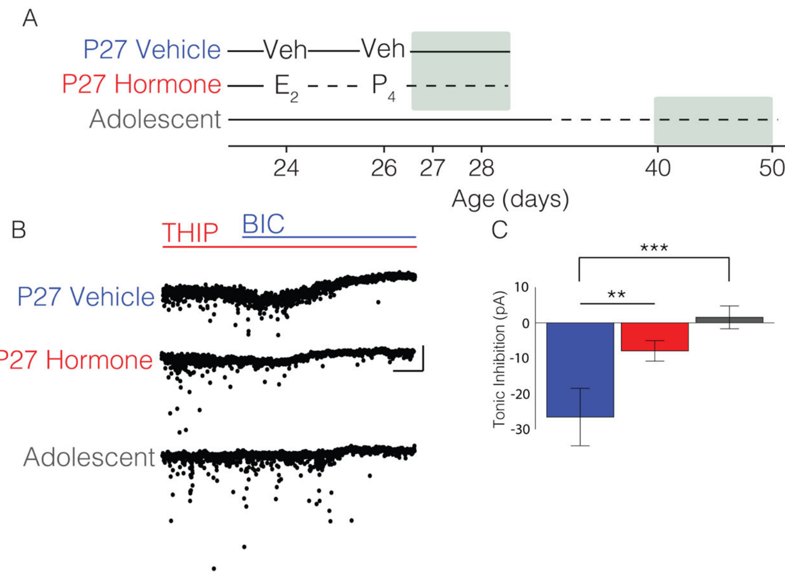

Figure 5. Age and hormone exposure also impacts tonic inhibition in cingulate cortex pyramidal cells

A. Mice were injected with either gonadal hormones or vehicle at P24 and P26, and layer 2/3 cingulate neurons were patched at P27. For comparison, cells in an unmanipulated group were also patched at P40–50 (post puberty). (B) In slice, the $GABA_A$ δ subunit specific agonist THIP was applied in bath, followed by addition of the $GABA_A$ receptor blocker bicuculline (BIC). The difference between holding potential during THIP and THIP + BIC represents the maximal tonic inhibition possible for the patched cell at this dose. (C) Vehicle treated mice at P27 (4 brains, 6 cells) had significantly higher tonic inhibition than either P27 hormone treated (4 brains, 9 cells) or P40 unmanipulated (3 brains; 6 cells) mice. These data suggest the potential size of tonic inhibitory currents is reduced in L2/3 pyramids of the cingulate between P27 and P40 and hormone treatment can speed this reduction. All graphs are mean ± S.E.M. Hypothesis tests conducted using linear mixed models with Tukey's HSD test for post-hoc comparisons. **p<0.01; ***p<0.001. Scale bar: 25pA, 25 seconds.

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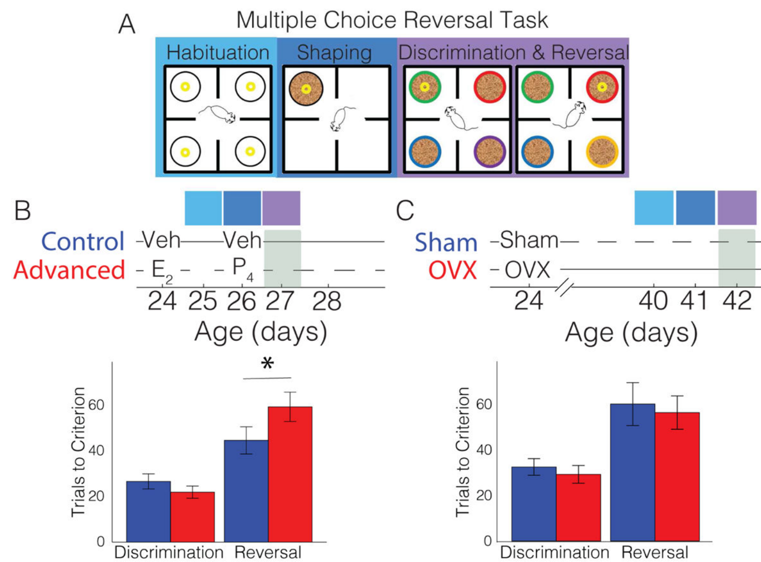

Figure 6. Hormone exposure induces early adult-like performance in a 4-choice odor based reversal learning task

(A) A schematic of the three-day protocol used for the behavior test measuring discrimination learning in which mice learn to choose 1 out of 4 odors, and reversal learning in which they have to update this rule when the reward contingency is changed to a new odor. (B) Littermate mice housed in pairs were either treated with gonadal steroids or vehicle at P24 and P26 (n=11 each group). Mice were habituated to the food reward on P25, shaped to dig in bowls on P26, and run in both the discrimination and reversal phase of the task on P27. There were no differences between groups during the discrimination phase, but hormone treated mice required significantly more trials to reach criterion in the reversal phase. This shift in behavioral performance occurs in a direction consistent with what would be expected to occur with development by P40 [1, 48]. (C) Prepubertal OVX or sham OVX groups did not differ on either discrimination or reversal when tested at P42. Given that maternal effects can impact performance on this this task [77], groups were compared using F-tests controlling for litter/cage. All graphs are mean ± S.E.M. *$p<0.05$.