A Critical Role for α4βδ GABA<sub>A</sub> Receptors in Shaping Learning Deficits at Puberty in Mice

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The onset of puberty defines a developmental stage when some learning processes are diminished, but the mechanism for this deficit remains unknown. We found that, at puberty, expression of inhibitory α4βδ γ-aminobutyric acid type A (GABA<sub>A</sub>) receptors (GABAR) increases perisynaptic to excitatory synapses in CA1 hippocampus. Shunting inhibition via these receptors reduced N-methyl-d-aspartate (NMDA) receptor activation, impairing induction of long-term potentiation (LTP). Pubertal mice also failed to learn a hippocampal, LTP-dependent spatial task that was easily acquired by δ−/− mice. However, the stress steroid THP (3βOH-5α[Δ4]-pregnan-20-one), which reduces tonic inhibition at puberty, facilitated learning. Thus, the emergence of α4βδ GABARs at puberty impairs learning, an effect that can be reversed by a stress steroid.

Certain learning and cognitive processes decline at the onset of puberty (1–3). The pubertal process that shapes this developmental decline is unknown but is likely to involve the hippocampus, which is widely regarded as the site for learning (4–6). In addition to excitatory input, the inhibitory GABAergic (GABA, γ-aminobutyric acid) system plays a pivotal role in shaping developmental plasticity, as in the visual cortex (7), where drugs that target the γ-aminobutyric acid type A (GABA<sub>A</sub>) receptor (GABAR) alter the timing of the critical period. The GABAR mediates most central nervous system inhibition and consists of diverse subtypes with distinct properties. Of these, α4βδ GABARs increase at pubertal onset in the mouse hippocampus (8), suggesting that they may shape plasticity here.

We employed immunocytochemical, electron microscopic techniques (9) to localize and quantify α4 and δ GABAR subunits on CA1 hippocampal pyramidal cells across the pubertal state of female mice, because females exhibit greater deficits in learning at puberty than males (10, 11). We detected immunostaining of both subunits perisynaptic to asymmetric synapses on the plasma membrane of spines of the apical dendrite, which increased up to 700% at puberty (Fig. 1, A to C, and fig. S1; α4, P = 0.0048; δ, P = 0.00091) (9). In contrast, α4 and δ immunoreactivity on the dendritic shaft increased by less than 100% at puberty (fig. S2). Functional expression of δ-containing GABAR at puberty was demonstrated by robust responses of pyramidal cells at puberty to 100 nM gaboxadol, which, at this concentration, is selective for this receptor (Fig. 1, D and E) (12). Gaboxadol had no effect before puberty and only a modest effect in the adult hippocampus (Fig. 1, D and E), where α4 and δ expression is lower than at puberty (fig. S3).

Fig. 1. α4 and δ GABA<sub>A</sub> receptor subunit expression increases on dendritic spine membranes of CA1 hippocampal pyramidal cells at puberty. (A) α4 and (B) δ silver-intensified immunogold labeling (SIG) occurs along the plasma membrane of spines forming excitatory synapses. Arterisks, postsynaptic density; t1 to t3, presynaptic axon terminals; a, nonsynaptic axons. In (B), the neck connects the labeled shaft with the spine. Scale bar, 500 nm. Arrowheads indicate SIG immunolabeling of the indicated GABAR subunits. (C) Proportion of labeled synapses (upper panel: α4, *P < 0.018; δ, **P < 0.002) and immunoreactivity per spine (lower panel: α4, *P < 0.005, δ, **P = 0.00091) increase at puberty (Pub) relative to prepuberty (Pre-pub) (9). Error bars indicate SE of the mean. (D) Representative pyramidal-cell currents in response to the GABA agonist gaboxadol (applied continuously, arrows) and the GABAR antagonist SR95531 (applied continuously, arrows). Scale, 50 pA, 50 s. (E) Averaged data. *P = 0.0002, **P = 0.02, Pub versus Pre-pub.
while reducing outward current (8) by polarity-dependent desensitization (8, 16). Therefore, THP should facilitate NMDA EPSCs in CA1 at puberty, when GABAAergic current is outward (8), but not before puberty, when it is inward (fig. S6).

30 nM THP reduced the threshold and increased amplitudes of NMDA EPSCs and EPSPs at puberty (Fig. 2, A, C, and D, and fig. S4; P = 0.05). In contrast, THP modestly reduced NMDA currents in the prepubertal and adult hippocampus (Fig. 2, A and B), where THP is inhibitory (fig. S7) (8). Importantly, THP had no effect on the NMDA/AMPA ratio under total GABAR blockade (Fig. 2, E and F) or in the pubertal δ−/− hippocampus (Fig. 2, A and B). The paired pulse ratio was unchanged by THP at puberty (Fig. 2, G and H), indicating that THP was not altering glutamate release.

Because NMDA receptors are essential for long-term potentiation (LTP), an in vitro model of learning (6, 20, 21), we examined whether puberty onset impaired LTP induced by theta-burst stimulation (TBS) (figs. S8 and S9) of the Schaffer collaterals (20). TBS induced NMDA receptor–dependent LTP (Fig. 3A and fig. S10) in both the prepubertal and adult hippocampus, with more success before puberty (Fig. 3A; P = 0.00018). However, LTP was not induced at puberty (Fig. 3A; P = 0.0022 versus prepuberty). In contrast, LTP was robustly produced under complete GABAR blockade (Fig. 3C). as well as in the pubertal δ−/− hippocampus (Fig. 3B). In adults, induction of LTP was of similar magnitude in wild-type (WT) and δ−/− mice (Fig. 3, A and D).

Because THP facilitated NMDA receptor activation at puberty, we predicted it would also facilitate LTP. Indeed, 30 nM THP restored LTP at puberty (Fig. 3A), whereas it reduced LTP before puberty. In contrast, its inactive βOH-isomer (8), which blocks THP’s effects (8), prevented LTP induction when administered before THP (fig. S11).

Synaptic GABAR blockade did not reverse the deficit in LTP induction at puberty, nor did it prevent LTP induction by local dendritic application of THP during TBS (Fig. 3D). Application of THP 5 min after LTP induction had no effect (Fig. 3E), verifying that THP was facilitating LTP induction rather than maintenance.

We tested whether spatial learning would be impaired at puberty using a hippocampus-dependent spatial learning task that requires LTP for memory storage (6, 22) and produces minimal stress compared with other tasks (23). Mice were trained across three sessions to avoid a moving zone (0.3 mA; Fig. 4A), which delivered a minimal footshock threshold for stress steroid release (24).

The time to first enter the zone was recorded as a measure of learning.

We found that puberty impaired learning: The time to enter the shock zone decreased by 70% (Fig. 4B; P < 0.05), and fewer animals learned (fig. S12) compared with prepubertal WT and pubertal δ−/− mice (Fig. 4). THP (10 mg/kg intraperitoneally) completely reversed the learning deficit at puberty (Fig. 4, B and C), whereas it impaired learning before puberty. In contrast, the number of shocks per entry was unaltered across groups (fig. S13), indicating that the shock was equally aversive for all animals. In contrast to prepubertal mice, both WT and δ−/− adults learned shock avoidance, but not as well as did prepubertal mice (Fig. 4C).

Although effects of puberty on synaptic plasticity have not been studied previously, the development of LTP in the CA1 hippocampus is maximal at ~3 weeks of age (25–27). In the absence of GABAR blockade, LTP declines around 35 to 45 postnatal days (27), consistent with puberty onset. This developmental time course is also reflected behaviorally (11). Thus, increased...
expression of extrasynaptic α4βδ GABAR at puberty may represent the mechanism for this decline.

LTP induction requires voltage-triggered Mg2+ unblock of the NMDA receptor (28), where local depolarization (29) has a greater effect on LTP induction than back-propagating action potentials. In this context, a GABAR shunting inhibition on spines, where we observe the greatest increase in α4βδ expression, would be more effective at impairing NMDA receptor activation than inhibition on the dendritic shaft. In the visual system, increased activity of fast-spiking basket cells targeting α1 receptors delimits the critical period (7). Taken together, these results suggest that diverse types of GABA inhibition shape plasticity during development.

In the adult, drugs that alter GABAR function also alter plasticity (30–33), probably mediated by dendritic α5-containing GABARs (31, 33), which localize at spines and modify learning (34, 35). α4βδ GABARs did not play a role in adult synaptic plasticity, when their expression is low (36), and learning and LTP induction in δ−/− mice were similar to that in WT animals.

The learning deficit at puberty is acutely reversed by the stress steroid THP via its inhibition of α4βδ GABAR, in contrast to its typical impairment of learning at other ages (30). THP effects are distinguishable from corticosterone, which alters learning after a delay (37, 38) but has no effect acutely (39). Thus, the stress steroid THP provides a novel means for rapid changes in synaptic plasticity at puberty.

Fig. 3. LTP induction is attenuated at puberty: reversal by the stress steroid THP. (A) TBS (dashed line) induced LTP (black) before puberty (Pre-pub, left) and in adult (right), but not in the pubertal (Pub, middle) CA1 hippocampus. THP (red, 30 nM) permitted LTP induction at puberty. (Inset) Representative field EPSPs. TBS, arrow. Scale, 0.5 mV, 50 ms. (B) Pubertal δ−/−. (C) Complete GABAR blockade (Pre-pub, black; Pub, blue). (D) Adult δ−/−. (E) Local application of THP (arrow) to stratum radiatum during TBS under synaptic GABA blockade. (F) THP (arrows) applied before TBS and after TBS (Pub).

Fig. 4. Spatial learning is attenuated at puberty: reversal by the stress steroid THP. (A) Spatial learning platform (shock zone, black sector). (B) Times for first entry of the shock zone. Pre-pub mice attained the longest entry times. Pre-pub, black square; δ−/−, open circle; Pub, black triangle; +/+, open triangle (+Pre-pub versus Pre-pub δ−/−, Pub, P < 0.05; *Pub versus Pub +THP, P < 0.05, Tukey’s test). Error bars indicate SE of the mean. (C) Time to reach criterion (120 s) indicated for each group (numbers, best entry time). Vehicle, white bars; THP, hatched bars. *P < 0.05 Pre-pub versus Pub; **P < 0.05 versus vehicle. (D) Longest first entry time for Pub +/+, and δ−/− mice after vehicle or THP (vehicle, white bars; THP, hatched bars). *P < 0.05 versus δ−/− vehicle; **P < 0.05 versus +/+ THP.
**CKAMP44: A Brain-Specific Protein Attenuating Short-Term Synthetic Plasticity in the Dentate Gyrus**

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**MPA receptors (AMPArs) mediate most of the fast excitatory transmission in the vertebrate central nervous system, and their function is regulated by subunit composition, posttranslational modifications, and protein-protein interactions (J). Several AMPAR-interacting proteins such as TARPs (transmembrane AMPAR regulatory proteins), Sol-1, and comichons have been identified that affect the receptors’ subcellular localization, synaptic stabilization, and kinetics (2–5). We searched for previously unknown AMPAR-interacting proteins using immunoprecipitation and mass spectrometry of AMPAR complexes [see Supporting Online Material (SOM)]. This proteomic search suggested an interaction of AMPARs with the gene product of the Mus musculus RIKEN CDNA gene locus 2700045P11Rik. Our reverse transcription polymerase chain reaction (RT-PCR) analysis identified this protein as a type I transmembrane protein, containing an extracellular N-terminal cysteine-rich motif, with eight cysteines highly conserved across vertebrate species. We named the protein according to its predicted molecular weight of 44 kD CKAMP44 (cysteine-knot AMPAR modulating protein) (Fig. 1A). The CKAMP44 gene is located on mouse and human chromosome 16 and contains five translated exons. The CKAMP44 precursor protein of 424 amino acids features an N-terminal signal peptide (23 amino acids) and a single putative transmembrane segment (20 amino acids), the latter separating the N-terminal extracellular region (128 amino acids) from the cytoplasmic segment (253 amino acids), which terminates in a PDZ type II ligand motif (Glu-Val-Thr-Val). Six of the eight cysteine residues in CKAMP44 might stabilize a Cys-knot structure found in δ-conotoxins (Fig. 1A) (6). CKAMP44 might thus operate as an endogenous modulator of the AMPARs.

The gene for CKAMP44 is specifically expressed in the brain, as demonstrated by a tissue-specific Northern blot (Fig. 1B). RT-PCR on RNA from different mouse tissues confirmed the brain-specific expression and revealed two splice variants, CKAMP44a and CKAMP44b, that differ by only 48 bases (Fig. 1B). In situ hybridization on horizontal mouse brain sections with a probe recognizing both splice variants of CKAMP44 indicated neuronal expression in the majority of brain regions, including hippocampus, cerebral cortex, striatum, thalamus, olfactory bulb, and cerebellum (Fig. 1C). CKAMP44 mRNA can be seen in most brain structures during embryonic and postnatal development.

We used a CKAMP44-specific antibody that recognizes both splice variants (see Fig. S1 for antibody specificity) to determine whether the interaction of endogenous CKAMP44 and AMPARs is subunit specific. The antibody immunoprecipitated proteins associated with CKAMP44 from forebrain lysates of wild-type mice and of mice lacking either the AMPAR subunit GluA1, GluA2, or GluA3. The immunoprecipitates from forebrain lysates of wild-type mice and of mice lacking either the AMPAR subunit GluA1, GluA2, or GluA3. The immunoprecipitates from wild-type mice and of mice lacking either the AMPAR subunit GluA1, GluA2, or GluA3. The immunoprecipitates from wild-type mice and of mice lacking either the AMPAR subunit GluA1, GluA2, or GluA3. The immunoprecipitates from wild-type mice and of mice lacking either the AMPAR subunit GluA1, GluA2, or GluA3. The immunoprecipitates from wild-type mice and of mice lacking either the AMPAR subunit GluA1, GluA2, or GluA3.