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Title: Convergence of adenosine and GABA signaling for synapse stabilization during development

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Abstract: During development, neural circuit formation requires the stabilization of active GABAergic synapses and the elimination of inactive ones. Here we demonstrate that, while the activation of postsynaptic GABA_A receptors (GABA_AR) and adenosine A_{2A} receptors (A_{2A}Rs) stabilize GABAergic synapses, only A_{2A}R activation is sufficient. Both GABA_AR- and A_{2A}R- dependent signaling pathways act synergistically to produce cAMP through recruitment of the Calcium (Ca²⁺)- Calmodulin (CaM)-Adenylyl Cyclase (AC) pathway. Protein kinase A (PKA) thus activated, phosphorylates gephyrin on Ser303, which is required for GABA_AR stabilization. Finally, the stabilization of pre- and post- synaptic GABAergic elements involves the interaction between gephyrin and the synaptogenic membrane protein Slitrk3. We propose that A_{2A}Rs act as detectors of active GABAergic synapses releasing GABA and ATP/adenosine, to regulate their fate towards stabilization or elimination.

One-Sentence Summary: A_{2A} receptors are key controllers of the stabilization/elimination of GABAergic synapses during synaptogenesis.

Introduction

During development, brain circuits go through different phases of synapse formation, stabilization and elimination, which involve numerous molecular mechanisms, in particular at GABAergic synapses. Synaptic cell adhesion molecules are essentials for synapse formation and maturation, including neuroligins and leucine-rich repeat transmembrane proteins (LRRTMs) that interact with presynaptic neurexins, Slit- and Trk-like family proteins (Slitrks), which bind to presynaptic protein tyrosine phosphatases (PTPs), immunoglobulin superfamily proteins (IgSFs), cadherin family proteins, and transmembrane tyrosine kinase receptors (1–4). The γ -aminobutyric acid (GABA) neurotransmitter itself regulates the maturation and innervation patterns of GABAergic synapses, as well as their elimination and pruning (4–8). GABA operates via the activation of type A GABA receptors (GABA_ARs) and elevation in intra-neuronal Ca²⁺ level following the activation of voltage-dependent Ca²⁺ channels (9, 10). In addition, the adenosine signaling pathway emerges as an important actor in development: extracellular adenosine builds-up with synaptic activity (11, 12), and adenosine A_{2A} receptors (A_{2A}Rs) control the migration speed of GABAergic neurons (13), axonal elongation and dendritic branching (14), and synapse stabilization/elimination at the neuromuscular junction (15). Since GABA could be co-released with ATP/adenosine in a synaptic-activity manner (16, 17), we tested the role of adenosine signaling on GABAergic synaptogenesis in the brain.

Results

Transient expression of A_{2A}Rs at GABAergic synapses

In the adult hippocampus, there is a low density of A_{2A}Rs, which are essentially presynaptic (18). During the peak of synaptogenesis, between postnatal days P5 and P16, we found a transient increased density of A_{2A}Rs in mouse hippocampi, in particular in purified synaptic contacts (fig.

S1). Electron microscopy showed their postsynaptic localization on symmetric, presumably GABAergic synapses (fig. S1). We also confirmed the presence of A_{2A}Rs in primary cultures of hippocampal neurons. A_{2A}Rs were clustered at synapses containing glutamic acid decarboxylase 67 kD (GAD67, a GABA synthesizing enzymes), and clustering increased during synaptogenesis between 7 and 14 days *in vitro* (DIV) (fig. S1). A_{2A}Rs were not present at all GABAergic synapses but they accumulated at a subset (39.1 ± 3.7 %, n= 34 cells, 3 independent experiments) of synapses. DNA-PAINT imaging further confirmed that A_{2A}Rs were located near the GABAergic post-synapse identified by the presence of the scaffolding molecule gephyrin at DIV 10 (fig. S1). We then asked whether such enrichment of post/peri-synaptic A_{2A}Rs at GABAergic synapses was accompanied by an increase in activity-dependent release of their ligand adenosine.

Increased release of ATP/adenosine during synaptogenesis

Adenosine can originate from its direct activity-dependent release by presynaptic terminals or neighboring glial cells, and/or from the conversion of ATP released by neurons or glial cells via the ecto-5'-nucleotidase CD73 (11, 12). The evoked release of adenosine was larger from P7 than P60 (adult) hippocampal synaptosomes (fig. S2), and blocking CD73 with adenosine 5'-(α,β -methylene)diphosphate (AMPCP, 100 μ M) decreased extracellular adenosine by 25% (fig. S2). Accordingly, we found a large density of CD73 in synapses during the peak of synaptogenesis (fig. S2), in keeping with the tight association of the enzyme with A_{2A}Rs (19). Therefore, one fraction of extracellular adenosine comes from local extracellular ATP metabolism and most of the adenosine is likely released as such through non-concentrative nucleoside transporters upon its intracellular formation as a by-product of metabolic activity sustaining synaptic activity (11, 12, 20). The activity-dependent secretion of ATP and adenosine was also more important at P7 than at P60 (fig. S2). Thus, the activation of synaptic terminals at early stage of synaptogenesis bolsters

the release of adenosine (via yet unclear mechanisms) and of vesicular ATP, which is efficiently converted into extracellular adenosine by CD73. Given the presence of A_{2A}Rs at inhibitory synapses and the activity-dependent production of its ligand adenosine during the period of synaptogenesis, we hypothesized a role for this pathway in GABA synapse formation/elimination. Since GABA controls the number of GABAergic synapses during development (4–8), we tested the relative contribution of GABA_AR and A_{2A}R pathways.

A_{2A}Rs and GABA_ARs control GABAergic synapse fate

Incubating neurons with tetanus toxin (TeNT, 1–40 nM) *in vitro*, which abolishes vesicular release of neurotransmitters (21), resulted in the disappearance of 62% of GABAergic synaptic terminals labeled for the vesicular GABA Transporter (VGAT) and of 43% of post-synaptic GABA_AR γ 2 subunit clusters, indicating synapse dismantling (fig. S3). Synaptic loss was fully prevented by the activation of A_{2A}Rs with CGS21680 (30 nM) or GABA_ARs with muscimol (10 μ M) for 30 min (fig. S3). The rescue of GABAergic synapses occurred within 30 min of A_{2A}R or GABA_AR activation ruling out *de novo* synapse formation, which requires hours (22). Together, these results show that in the absence of synaptic transmission, the activation of either A_{2A}Rs or GABA_ARs by their respective ligands is sufficient to maintain GABAergic synapses, which would have disappeared otherwise. This led us to hypothesize that both adenosine and GABA signaling pathways play a role in activity-dependent synapse stabilization, and that when presynaptic sites are inactive, such synapses are eliminated. Since the activation of either receptor could rescue GABAergic synapses to an extent similar to that observed when both receptors were simultaneously activated (fig. S3), we further hypothesized a convergence of both A_{2A}R and GABA_AR signaling pathways.

A_{2A}Rs are sufficient to stabilize GABAergic synapses

To test the interdependence of the two A_{2A}R and GABA_AR pathways, we blocked one while activating the other, and we investigated the fate of GABAergic synapses *in vitro*. We knocked-down with specific shRNAs the GABA_AR γ 2 subunit, which leads to synaptic loss (23), or A_{2A}Rs (24) and we activated A_{2A}Rs or GABA_ARs with their selective agonists CGS21680 (30 nM) or muscimol (10 μ M), respectively. Neurons transfected at DIV 6-7 with shRNAs against GABA_AR γ 2 (sh γ 2) or A_{2A}Rs (shA_{2A}R) showed respectively a 43 % and 25 % reduction of GABAergic synapses (Fig. 1, A and B). Since <10 out of 100,000 cells were transfected and since we did not find any evidence of synapse destabilization in the non-transfected cells surrounding the transfected ones, we conclude that the destabilization of GABAergic synapses occurs due to the loss of postsynaptic GABA_ARs or A_{2A}Rs. The activation of A_{2A}Rs with CGS21680 rescued GABAergic synapses in neurons lacking GABA_AR γ 2 (Fig. 1A). In contrast, activation of GABA_ARs by muscimol failed to rescue GABAergic synapses in shA_{2A}R-expressing neurons (Fig. 1B). Thus, A_{2A}R activation appears to be necessary and sufficient for GABAergic synapse stabilization, whereas GABA_AR activation is not necessary as long as A_{2A}Rs remain activated. According to this scheme, blocking A_{2A}Rs should result in synapse loss.

A_{2A}R blockade triggers GABAergic synapse loss

A 30 min application of the selective A_{2A}R antagonist SCH58261 (100 nM) *in vitro* decreased the number of clusters of the presynaptic protein VGAT as well as of postsynaptic GABA_AR γ 2 and scaffolding protein gephyrin (fig. S4), demonstrating a destabilization of GABAergic synapses at both pre- and post-synaptic sites. Acute treatment with SCH58261 induced a loss of GABAergic synapses expressing the γ 2 subunit but also of synapses containing the α 1 or α 2 subunits (fig. S4), suggesting that several types of GABAergic synapses are controlled by A_{2A}Rs. Not all synapses

disappeared as ~30% of GABAergic synapses were destabilized (fig. S4), in keeping with the finding that not all GABAergic synapses are equipped with A_{2A}Rs at any given developmental time (fig. S1). The fact that the loss of GABAergic synapses induced by A_{2A}R blockade was similar to that found in shA_{2A}R-expressing neurons (Fig. 1A) suggests that synaptic loss was not due to an indirect effect on network activity. In keeping with the reduced synaptic clustering of GABA_ARγ2, quantum-dot based single-particle tracking of GABA_ARγ2 in DIV 8 hippocampal neurons revealed that the membrane diffusion of GABA_ARs increased within 10 min after bath application of SCH58261 (100 nM) (fig. S5). Finally, this adenosine-dependent mechanism seems specific to A_{2A}Rs since blocking adenosine A₁ receptors had no effect on GABAergic synapses (fig. S6). We then set to confirm these results *ex vivo*.

Immunohistochemistry revealed that blocking A_{2A}Rs for 30 min with SCH58261 (100 nM) in P6 hippocampal slices triggered a 47% loss of VGAT-containing synaptic terminals (Fig. 1C). Single-cell recordings of miniature inhibitory postsynaptic currents (mIPSCs) in hippocampal CA1 pyramidal cells at P6 showed that a 30 min application of SCH58261 (100 nM) decreased the frequency of mIPSCs by ~40% (Fig. 1D). The amplitude of mIPSCs was decreased by ~30% (Fig. 1D). We found the same effect on mIPSCs recorded in CA1 interneurons (fig. S7) and in layer V pyramidal cells of V1 visual cortex (fig. S7). Since the effects of SCH58261 on GABAergic currents could be an indirect consequence of the modulation of glutamatergic neurotransmission by A_{2A}Rs, we verified that the effect was still present in the presence of glutamate receptor blockers (fig. S8). Together, these results show that A_{2A}Rs control the stability of GABAergic synapses in different cell types and brain regions at early stages of development. We then assessed whether this role of A_{2A}Rs was developmentally regulated and studied the minimum time of A_{2A}R inactivity necessary to trigger synapse removal.

Time-course of A_{2A}R-mediated stabilization of GABAergic synapses

In keeping with the transient increased density of A_{2A}Rs during the first two postnatal weeks (fig. S1), we found that the control of GABAergic synapses by A_{2A}Rs *ex vivo* was restricted to the period of synaptogenesis (between P4 and P12), with a maximum effect at P6 (fig. S9). A morphological analysis performed in cultures confirmed that the action of A_{2A}Rs was also developmentally regulated (fig. S10).

We then assessed whether there is a critical time window beyond which A_{2A}R inactivity triggers an irreversible synapse destabilization. Varying the time of application of SCH58261, we found that the effect on mIPSCs was reversible if A_{2A}R blockade did not exceed 10 min (fig. S9). Beyond 20 min, the effect was irreversible (fig. S9). Similarly, there was no loss of GABAergic synapses 10 min following A_{2A}R blockade in cultures, while it occurred after 30 min treatment (fig. S11). Thus, the A_{2A}R-dependent control of GABAergic synapse density is developmentally regulated and a period of at least 20 min of inactivity of A_{2A}Rs is sufficient to trigger the removal of GABAergic synapses.

Together, our results show that A_{2A}Rs satisfy the three requirements of a system able to control the fate of synapses towards stabilization or elimination during development. It is time-dependent, its activation maintains synapses and absence of activation results in synapse elimination. We then investigated the molecular mechanisms upstream and downstream of A_{2A}R activation.

Adenosine is required for synapse stabilization

Application of the CD73 inhibitor AMPCP (100 μ M) together with adenosine deaminase (ADA, 4-20 U/mL), which hydrolyses adenosine into inosine, decreased mIPSC amplitude (30%) and frequency (74%) *ex vivo* (Fig. 2A). The same treatment reduced the time GABA_AR γ 2 spent at inhibitory synapses *in vitro*, an effect that was prevented by the direct activation of A_{2A}Rs with

their agonist CGS21680 (fig. S6). This increased escape of GABA_AR γ 2 from inhibitory synapses upon removal of ambient adenosine was accompanied by a rapid disappearance of active inhibitory synaptic boutons (labelled by pre-loading of a VGAT-Oyster antibody into synaptic vesicles during multiple vesicular exocytosis/endocytosis cycles), and a rapid mobility of VGAT packets along axons (fig. S11). This rapid and dynamic dismantling of GABAergic pre- and post-synaptic compartments induced by the withdrawal of ambient adenosine with ADA + AMPCP resulted in a net loss of the number of GABAergic synapses that could be prevented by A_{2A}R activation *in vitro* (Fig. 2B).

To test whether glial cells are necessary to produce adenosine required to activate A_{2A}Rs, we pretreated cultures with the gliotoxin cytosine arabinoside (araC), resulting in the complete loss of GFAP-stained glial cells (fig. S12) and application of ADA + AMPCP still induced synapse loss, which was prevented by CGS21680 (fig. S12). This indicates that glial cells are not the main source of adenosine and that neurons can produce adenosine (directly or indirectly as a byproduct of its phosphorylated forms) in the absence of glial cells in a sufficient quantity to activate A_{2A}Rs.

Altogether, these results show that direct adenosine activation of A_{2A}Rs is necessary and sufficient to maintain synapse integrity. We then looked at the downstream effects of A_{2A}R activation, starting with the possible cooperativity between GABAergic and A_{2A}R signaling pathways as suggested above (fig. S3).

Convergence of A_{2A}R and GABA_AR signaling pathways

Involvement of the AC/cAMP pathway

A_{2A}Rs are G protein-coupled receptors and their prime transducing system is the activation of adenylyl cyclase (AC), which generates cyclic adenosine monophosphate (cAMP). If synapse stabilization is G protein-dependent, blocking G protein activity should destabilize synapses.

Recording CA1 pyramidal cells with an intracellular solution containing guanosine diphosphate- β -S to block G protein activity directly led to a decrease of mIPSC frequency and amplitude *ex vivo* (15% and 55% respectively; fig. S13), reproducing the effect of A_{2A}R blockade. This result is consistent with a postsynaptic action.

5 Then, we tested the involvement of the AC/cAMP signaling cascade, which is downstream of A_{2A}R activation. Bath application of IBMX (100 μ M, a non-selective inhibitor of phosphodiesterases that metabolize cAMP) and forskolin (10 μ M, an activator of AC) prevented the SCH58261-induced decrease of mIPSC frequency and amplitude in CA1 pyramidal cells *ex vivo* (fig. S13). Similarly, IBMX and forskolin prevented the loss of GABAergic synapses induced
10 by SCH58261 in hippocampal neuron cultures (fig. S13). Therefore, the A_{2A}R-dependent control of GABAergic synapses occurs via the activation of the AC/cAMP signaling cascade.

A_{2A}Rs and GABA_ARs elevate intracellular cAMP levels

At what level do the A_{2A}R and GABA_AR signaling pathways converge to regulate synaptogenesis?

15 A_{2A}Rs and GABA_ARs are linked to AC/cAMP and Ca²⁺ signaling, respectively. During development, the activation of GABA_ARs leads to a rise in intracellular Ca²⁺ (9, 10). Since hippocampal neurons express type 1 and type 8 Ca²⁺-calmodulin (CaM) sensitive ACs (25), we hypothesized that GABA signaling converge on A_{2A}R signaling by tuning CaM sensitive ACs and their downstream effectors.

20 Acute activation of GABA_ARs with 10 μ M muscimol significantly increased [Ca²⁺]_i in DIV8 hippocampal neurons, while decreasing it in DIV14 neurons (fig. S14), in keeping with the developmentally regulated effect of GABA_ARs on intracellular Ca²⁺. In contrast, an acute application of 30 nM CGS21680 did not change [Ca²⁺]_i in DIV8 or DIV14 neurons (fig. S14). Buffering intracellular Ca²⁺ with BAPTA-AM (20 μ M) lead to the destabilization of inhibitory

synapses, an effect that could be prevented by A_{2A}R activation with CGS21680 (Fig. 3A). Thus, activation of A_{2A}Rs is sufficient to maintain synapse integrity in the absence of intracellular Ca²⁺ signaling.

Since GABA but not adenosine signaling can elevate [Ca²⁺]_i and since A_{2A}R-mediated activation of ACs can be boosted by CaM (10), we tested the convergence of the GABA_AR and A_{2A}R pathways on cAMP. To address this question, we performed cAMP imaging in DIV8 hippocampal neurons infected with recombinant Sindbis virus encoding EPAC-SH150, a sensor with increased sensitivity for cAMP (26). As expected, A_{2A}R activation with CGS21680 increased intracellular cAMP levels (fig. S15). GABA_AR activation with muscimol also increased cAMP levels within a similar range as CGS21680 (fig. S15). Application of both CGS21680 and muscimol strongly elevated intracellular cAMP levels (fig. S15), demonstrating that the activation of GABA_ARs and A_{2A}Rs converge with additive effects on cAMP production.

Stabilization of GABAergic synapses requires cAMP production

We then tested the implication of intracellular Ca²⁺ and CaM in GABAergic synapse stabilization. The loss of GABAergic synapses induced by expressing shRNA against GABA_AR γ2 subunit (shγ2) in DIV10 neurons was prevented by the activation of CaM with the cell-permeable activator CALP3 (100 μM) during 30 min (Fig. 3B). Thus, CaM is involved in the stabilization of GABAergic synapses. Application of the AC inhibitor SQ22536 (20 μM) prevented the CALP3-mediated rescue of GABAergic synapses (Fig. 3B), demonstrating that CaM stabilizes synapses through activation of ACs.

Hippocampal neurons express Ca²⁺ dependent and independent ACs targeted respectively to lipid raft and non-raft plasma membranes (27). To investigate the involvement of Ca²⁺ dependent and independent ACs, we used genetically encoded 'cAMP sponges' targeted to (Lyn-cAMP sponge)

or outside (Kras-cAMP sponge) lipid rafts, enabling local perturbation of cAMP downstream signaling (28). A variant of Lyn-cAMP sponge (Lyn-mut-cAMP sponge) unable to bind and buffer cAMP was used as a control. In basal conditions, none of cAMP sponges altered the density of GABAergic synapses (fig. S16). However, buffering cAMP near lipid rafts blocked the CALP3-mediated rescue of GABAergic synapses in neurons expressing *shy2* (Fig. 3C). This effect was specific of sponges targeted to lipid rafts since the density of synapses in neurons expressing Lyn-mut-cAMP or Kras-cAMP sponges were not distinguishable from CALP3-treated neurons lacking GABA_AR γ 2 (Fig. 3C). Thus, the convergence of GABA_AR and A_{2A}R pathways on the stabilization of GABAergic synapses relies on cAMP production by Ca²⁺-dependent CaM-activated ACs. We then investigated the mechanism downstream of cAMP production.

Adenosine stabilizes synapses through PKA-phospho-regulation of gephyrin

Protein kinase A (PKA) is a downstream effector of cAMP following A_{2A}R activation. As expected, intracellular inhibition of PKA with the protein kinase inhibitor peptide (PKI) decreased mIPSC amplitude and frequency *ex vivo* (26% and 50%, respectively; fig. S17), to the same extent as that found with SCH58261 (100 nM). Since PKA-mediated phosphorylation of gephyrin is required for its postsynaptic stabilization and the anchoring of GABA_ARs at synapses (29), we hypothesized that PKA activation via the A_{2A}R-AC/cAMP cascade insures the phosphorylation of gephyrin required to maintain GABA_ARs at the synapse.

Gephyrin is a direct substrate of PKA, hence we tested whether gephyrin phosphorylation at the Ser303 PKA-sensitive site was increased after A_{2A}R activation by CGS21680. For this, we used HEK293 cells and measured gephyrin phosphorylation using S303 site specific gephyrin phospho-antibody (30). Removal of ambient adenosine with ADA + AMPCP and simultaneous activation of A_{2A}Rs with CGS21680 significantly increased gephyrin phosphorylation at the Ser303 site (Fig.

4A). These effects of ADA + AMPCP or ADA + AMPCP + CGS21680 were not observed in cells that expressed a gephyrin phospho-null mutant (gephyrin-S303A) (Fig. 4A). This demonstrates that gephyrin can be phosphorylated on its PKA site upon A_{2A}R activation.

We then directly tested the contribution of PKA-dependent phosphorylation of gephyrin in synapse stabilization *in vitro* by expressing constructs harboring mutations of the PKA Ser303 site to aspartate (S303D) to mimic the gephyrin phosphorylated state (30). Decreasing extracellular adenosine with AMPCP + ADA led to synapse destabilization in neurons expressing wild type gephyrin (Fig. 4B), whereas the expression of the gephyrin mutant prevented synapse loss upon removal of extracellular adenosine (Fig. 4B). Thus, the PKA Ser303 phospho-site of gephyrin is required for the A_{2A}R-mediated synapse stabilization. This effect was specific of this phospho-site since decreasing extracellular adenosine led to synapse destabilization in neurons expressing the glycogen synthase kinase 3 β (GSK3 β) phospho-null gephyrin mutant (S270A) (fig. S18). We conclude that activation of A_{2A}Rs leads to GABAergic synapse stabilization through CaM-AC-cAMP-PKA-mediated phosphorylation of gephyrin at position Ser303. This mechanism accounts for the disappearance of the postsynaptic site when A_{2A}Rs are not activated by adenosine for more than 10 min (fig. S11).

Gephyrin phosphorylated on Ser303 interacts with Slitrk3

How can we explain the concurrent loss of the presynaptic element? Overexpression of the PKA-phosphorylation site gephyrin mutant S303D was sufficient to rescue the loss of VGAT-containing terminals on neurons transfected with sh γ 2 (Fig. 4C). This demonstrates that gephyrin is sufficient to stabilize synapses. We thus hypothesized that gephyrin phosphorylated at Ser303 interacts with the post-synaptic Neuroligin-2 (31, 32) or Slitrk3 (33) transmembrane proteins that bind in the synaptic cleft to presynaptic Neurexins or PTP σ to organize inhibitory synapses. The knock-down

of post-synaptic Neuroligin-2 with a specific shRNA or the antibody-induced blockade of pre-synaptic Neurexin led, as expected (34–36), to a loss of GABAergic synapses, which could be rescued upon A_{2A}R activation with CGS21680 (fig. S19). Thus, A_{2A}R-dependent synapse stabilization does not depend upon the Neurexin-Neuroligin-2 trans-synaptic complex. In contrast, GABA_AR activation did not rescue the synapse loss induced by the expression of a shRNA against Neuroligin-2 (fig. S19).

A_{2A}R or GABA_AR activation did not prevent synaptic loss induced by a shRNA against post-synaptic Slitrk3 (33) (Fig. 5A), supporting the involvement of Slitrk3 in A_{2A}R- and GABA_AR-dependent synapse stabilization. We then overexpressed the Myc-Slitrk3-Y969A mutant, since this site is critical for the formation of GABAergic synapses (37) (Fig. 5B). A_{2A}R activation was not able to restore synapses in cells overexpressing Myc-Slitrk3-Y969A (Fig. 5B). Therefore, deletion of Slitrk3 or a single amino acid mutation of Slitrk3 was enough to prevent A_{2A}R-dependent stabilization of GABAergic synapses. Finally, using co-immunoprecipitation experiments, we found that gephyrin-WT and gephyrin-S303D interact directly with Slitrk3 (Fig. 5C). These results suggest that A_{2A}R signaling stabilizes the pre- and post-synaptic GABAergic elements via gephyrin phosphorylation and Slitrk3 recruitment.

Transient blockade of A_{2A}Rs induces cognitive deficits

All previous experiments were performed *in vitro* and *ex vivo* to unravel the molecular mechanism. We next tested whether the role of A_{2A}Rs could also be evidenced *in vivo* during the postnatal synaptogenesis period. We could not use A_{2A}R knockout animals, because a delayed migration of GABAergic neurons occurs in these animals (13), which would result in a decreased number of GABAergic synapses. This would prevent us to distinguish between effects due to a delayed migration from a direct effect on the stability of GABAergic synapses. We thus injected shRNAs

against A_{2A}Rs *in vivo* in the hippocampus at P3. This led to the loss of GABAergic synapses evaluated at P16 (fig. S20), confirming the physiological role of A_{2A}Rs in the stabilization of GABAergic synapses. Additionally, we treated pups during the peak of synaptogenesis between P3 and P16 with SCH58261 (0.1 mg/kg). Although we cannot rule out non-specific effects, the treatment produced a loss of GABAergic synapses at P16 in the hippocampus (fig S20) similar to that found *in vitro* (fig. S4) and *ex vivo* (Fig. 1C). Given such synaptic loss, we predicted detrimental functional consequences, in particular for hippocampus-dependent spatial memory. We thus tested P70 animals, which had been treated between P3 and P16 with SCH58261. As predicted, we found deficits in the novel object location task (fig. S20). In contrast, open field, anxiety and novel object recognition tests were not modified (fig. S20). These results indicate that removal of GABAergic synapses during synaptogenesis has long-term deleterious consequences.

Discussion

This A_{2A}R-dependent mechanism adds to other molecules and signaling pathways known to control hippocampal GABAergic synapse during development (1, 4, 38), including GABA (4–8) and GABA_AR-induced elevation of intra-neuronal Ca²⁺ level following the activation of voltage-dependent Ca²⁺ channels (9, 10). We propose that this in turn activates Ca²⁺-Calmodulin (CaM)-sensitive ACs leading to a rise in intracellular cAMP in hippocampal neurons. Thus, one key outcome of GABA_AR activation in immature neurons is to elevate intracellular cAMP levels. A hypothesis is that adenosine/ATP is co-released with GABA (16, 17) during development, which would provide A_{2A}Rs with a direct information that the presynaptic terminal is active. Once activated, Gs-coupled A_{2A}Rs will trigger a rise in intracellular cAMP levels, leading in turn to PKA activation and stabilization of GABAergic synapses (fig. S21).

We found additive effects of GABA and adenosine pathways on cAMP levels. Interestingly, ACs can act as coincidence detectors, promoting cellular responses only when convergent regulatory signals occur close in time and space (39). Our results show that the GABA_AR and A_{2A}R systems are spatially close and operate within a similar time frame to control the fate of some GABAergic synapses. This effect of GABA/adenosine co-signaling at GABAergic synapses would occur only during a critical period of development because of the transient expression of A_{2A}Rs and because of the transient depolarizing action of GABA_ARs and their ability to activate Ca²⁺-sensitive ACs. This described A_{2A}R mechanism therefore provides a conceptual framework to understand how some synapses are stabilized or removed during development. The common view is that inactive synapses are eliminated. This requires the existence of a machinery that includes a detector of activity from the presynaptic terminal and a mechanism that removes the synapse when the detector is not activated. The adenosine-operated A_{2A}R can subserve all these functions, in addition to the involvement of GABA itself. The post/peri-synaptic localization of A_{2A}Rs can detect the activity-dependent release of adenosine/ATP (20, 40, 41), which mostly involves a direct release of adenosine as a signal proportional to the metabolic support of synaptic activity as well as a CD73-mediated extracellular formation of vesicular ATP-derived adenosine (11, 12, 20). The non-activation of A_{2A}Rs will prevent the continuous phosphorylation of gephyrin, which is essential for synapse stabilization. Importantly, synapse removal requires a certain period of A_{2A}R inactivity (20 min), which may guarantee unwanted disappearance of synapses. Notably, this A_{2A}R-mediated control of synapse selection seems to be autonomous, since it does not strictly require BDNF-TrkB receptor signaling (fig. S22).

Methods

Electrophysiology: mIPSCs were recorded in coronal CA1 hippocampal slices (350 μ m) from male GIN-mice as in (42).

Stereotaxic hippocampal injection of shA_{2A}R was done at P3 with 0.15 μ L of AAV2.1-U6-shNT-GFP or AAV2.1-U6-shA_{2A}R-GFP (70 nL/min).

Immunohistochemistry: For acute SCH58261 treatment, P6 slices were fixed with 4% paraformaldehyde (PFA), cryoprotected in 20% sucrose, frozen on dry ice, and incubated in rabbit anti-VGAT antibody (1:1000, SYSY). Images were acquired with a Zeiss LSM 510 microscope. For chronic SCH58261 treatment or shA_{2A}R expression, P16 brains were post-fixed in 4% PFA, cryoprotected (30% sucrose) and cut in parasagittal free-floating sections (30 μ m) (43) that were incubated in rabbit anti-VGAT (1:1000, from B. Gasnier). Z-stacks were acquired with a Leica SP5 confocal microscope and quantified using Imaris. For A_{2A}R detection, P3-12 coronal free-floating sections (100 μ m) were incubated in goat anti-A_{2A}R antibody (1:200, Santa Cruz). Images were acquired with a Zeiss Z2 microscope. Ultrastructural analysis of A_{2A}R was performed using the pre-embedding immunogold method (44) with guinea pig anti-A_{2A}R (Frontier Institute, Japan). Images were acquired with Jeol-1010 microscope.

A_{2A}R binding (18) was with 2 nM of ³H-SCH58261 using 140-210 μ g of protein. Release of ATP and adenosine (20) from hippocampal synaptosomes was assessed using the luciferin-luciferase assay (ATP) and HPLC (adenosine) upon K⁺-induced depolarization (20).

Western blotting: A_{2A}R density (18, 45) was studied with goat (Santa Cruz) or mouse (Millipore) anti-A_{2A}R antibodies. Validation of shA_{2A}Rs was done with rabbit anti-A_{2A}R (1:500, Alomone) antibodies in N2a cells infected with AAV2.1-U6-shNT-GFP/AAV2.1-U6-shA_{2A}R-GFP. Gephyrin-Slitrk3 interaction was assessed with rabbit anti-Slitrk3 (1:1000, Sigma) antibodies in HEK293T cells transfected with pCMV3-Myc-Slitrk3 or eGFP-gephyrin-WT (46) or eGFP-

gephyrin-S303D (30). Gephyrin phosphorylation was analyzed with rabbit custom made anti-phospho-gephyrin and mouse anti-gephyrin (3B11, 1:1000, SYSY) antibodies in HEK293T cells transfected with eGFP-gephyrin-WT or eGFP-gephyrin-S303A.

Behavioral testing: Open-field, NOL, NOR, elevated plus maze was done as in (47) at P70 on wild-type males treated with saline or SCH58261 (0.1 mg/kg) between P3-P16.

Cultures of hippocampal neurons were prepared as described (48).

Immunocytochemistry was done using rabbit anti-VGAT (1:500, from B. Gasnier), mouse anti-gephyrin (mAb7a, 1:500, SYSY) and guinea pig anti-GABA_AR γ 2 (1:2000, from J.M. Fristchy), or mouse anti-VGAT (1:500, SYSY) and rabbit anti-GABA_AR α 1 or GABA_AR α 2 (1:500, SYSY), or mouse anti-GAD 67 (1:500, Chemicon) and rabbit anti-A_{2A}R (1:100, Alomone). Image acquisition and cluster analysis was as in (48).

Single particle tracking of GABA_AR γ 2 was as described (49).

DNA-PAINT was performed with rabbit anti-A_{2A}R (1:100, Alomone) and mouse anti-gephyrin (mAb7a, 1:500, SYSY) on an inverted Nikon Eclipse Ti microscope using 561 and 647 nm lasers.

Calcium imaging of AAV-GCaMP6-ruby infected neurons was done on a Leica DMI4000 microscope (Yokogawa CS20 spinning Nipkow disk) with a 491 nm laser. Time lapse (0.33 Hz for 600 seconds) of stacks (~21 sections, step 0.3 μ m) were acquired.

cAMP imaging of EPAC-sh150 (26) was done on a two-photon Leica TCS/MP5 microscope with a Ti:sapphire laser (Coherent). Z-stacks (8-10 sections, step 1-2 μ m) were acquired every 15 seconds.

Videomicroscopy of presynaptic terminals (22) stained with rabbit anti-VGAT-Oyster⁵⁵⁰ (1:200, SYSY) was done using an Olympus IX71 microscope. Time-lapse (1 image every 5 min) were acquired.

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Author contributions:

Conceptualization: SL, CB and RAC

15 Immunofluorescence experiments in hippocampal cultures: FGC, MRu and CM, performed most (FGC) and some (MRu and CM) immunofluorescence experiments in hippocampal cultures, and analyzed the data.

MRu performed hippocampal cultures and molecular biology.

20 SZ, CGS and CB performed electrophysiological experiments, and ME performed post hoc morphology.

JCP performed calcium imaging, characterized the shA_{2A}R by western blot, performed the stereotaxic injection of AAVshA_{2A}R in the hippocampus in vivo, chronically treated animals with SCH, performed immunohistochemistry in chronically treated and shA_{2A}R expressing animals and quantified the data.

5 SZ and ME performed and analyzed the immunofluorescence in acute hippocampal slices treated with A_{2A}R antagonists.

RL performed the electron microscopy of A_{2A}R.

SL and MRu performed the single particle tracking experiments and analyzed the data.

GC and CL performed DNA-PAINT experiments and MRe analyzed the data.

10 NG performed cAMP imaging and analyzed the data.

PMC, FQG, SAM, ES, RJR, PA, ART performed and analyzed the biochemical experiments exploring ATP and adenosine release as well as A_{2A}R density during the synaptogenesis period of development in vivo.

15 ST designed the biochemical experiments testing gephyrin phosphorylation and gephyrin-Slitrk3 interaction; MF performed the experiments and analyzed the data.

OT produced Neurexin blocking antibody.

XN produced the cAMP sponges.

FGC, SZ, CB, SL prepared the figures.

Funding acquisition: SL, CB and RAC.

20 Project administration: SL, CB and RAC.

Supervision of the work: SL, CB and RAC.

Writing – original draft: SL, CB and RAC.

Writing – revised manuscript: SL & CB.

Writing – review & editing: SL, CB, RAC, FGC, CL, XN, OT, MRe.

5 **Competing interests:** Authors declare that they have no competing interests.

Data and materials availability: All data are available in the main text or the supplementary materials.

Supplementary Materials

10 Materials and Methods

Figs. S1 to S22

Figure Legends

Fig. 1. A_{2A}R activation stabilizes GABAergic synapses. (A) VGAT staining (left) and quantification (right) in DIV 10-11 neurons transfected with non-target (shNT) or on-target GABA_ARγ2 (shγ2) shRNAs exposed or not to CGS21680 (30 nM) for 30 min. shNT n=41, shγ2 n=35, shγ2 + CGS21680 n=35, 4 cultures. The loss of GABAergic synapses upon suppression of GABA_ARγ2 was rescued by activation of A_{2A}Rs. (B) VGAT staining (left) and quantification (right) in DIV 10-11 neurons transfected with non-target (shNT) or on-target A_{2A}R (shA_{2A}R) shRNAs exposed or not to muscimol (mus, 10 μM) for 30 min. shNT n=56, shA_{2A}R n=60, shA_{2A}R

+ muscimol n=48, 4-5 cultures. The loss of GABAergic synapses upon suppression of A_{2A}R was not rescued by GABA_AR activation. Scale bar, 5 μm. Arrowheads show examples of inhibitory synapses labeled for VGAT. (C) Acute application of SCH58261 (100 nM) during 30 min in hippocampal slices decreased by 47% the density of VGAT-immunoreactive terminals (8 slices, 3 P6 pups) as compared to controls (6 slices, 3 P6 pups). (D) Application of SCH58261 (100 nM) during 30 min reduced the amplitude and frequency of GABA_AR-mediated mIPSCs in CA1 pyramidal cells (8 cells, 8 slices, 6 P6 pups). Top: Left, examples of mIPSCs recorded before and after application of SCH58261. Insets: IPSC amplitude or interevent interval. Values were normalized to the control values (A, B); histograms represent means and s.e.m.; Mann-Whitney

test (A, B, C); Kolmogorov-Smirnov test (D): ns - not significant; * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 2. Synapse stabilization requires extracellular adenosine production. (A) Decreasing extracellular adenosine levels with a 30 min treatment of ADA (4-20 U/mL), AMPCP (100 μ M) or ADA+AMPCP led to a decrease of mIPSCs amplitude and frequency in CA1 pyramidal cells. N= 12 cells, 12 slices, 6 pups. (B) Immunostaining and quantification of VGAT and GABAAR γ 2 in DIV 10 neurons in the absence or presence of the indicated drugs for 30 min. Arrowheads show examples of inhibitory synapses labeled for VGAT and GABAAR γ 2. Scale bar, 5 μ m. Decreasing extracellular adenosine levels leads to synapse destabilization, an effect prevented by the direct activation of A_{2A}Rs with 30 nM CGS21680. N= 26-41 cells, 3 cultures. Histograms represent

means and s.e.m.; Kolmogorov-Smirnov test (A), Mann-Whitney test (B): ns - not significant;
* $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 3. GABAergic synapse stabilization by calmodulin-activated calcium-sensitive ACs. (A)

(A) Immunostaining and quantification of VGAT in DIV 10 neurons in the absence or presence of the indicated drugs for 30 min. Scale bar, 5 μ m. Decreasing intracellular calcium levels with BAPTA-AM (20 μ M) in the absence or presence of ADA (4-20 U/mL) and AMPCP (100 μ M) leads to synapse destabilization, an effect prevented by the direct activation of A_{2A} Rs with

CGS21680 (30 nM). N= 35-38 cells, 3 cultures. **(B)** Calp3 (100 μ M) activation of calmodulin for 30 min rescued the loss of GABAergic synapses in DIV 10-11 neurons expressing GABA_AR γ 2 shRNA (sh γ 2). The effect of calmodulin was blocked by an adenylyl cyclase inhibitor SQ22536 (20 μ M). Scale bar, 5 μ m. shNT n=38, sh γ 2 n=42, sh γ 2 + CALP3 n=43, sh γ 2 + CALP3 + SQ22536 n=30, 3 cultures. **(C)** VGAT staining and quantification of DIV 10-11 neurons transfected with non-target (shNT) or on-target GABA_AR γ 2 (sh γ 2) and/or the different sponges constructs (lyn-cAMP, lyn-mutated cAMP, K-Ras cAMP) exposed to CALP3 for 30 min. Scale bar, 5 μ m. shNT n=40, sh γ 2 n=37, sh γ 2 + CALP3 n=40, sh γ 2 + CALP3 +lyn-cAMP sponge n=23, sh γ 2 + CALP3 +lyn-mutated sponge n=27, sh γ 2 + CALP3 +K-Ras n=31, 4 cultures. Arrowheads show examples of inhibitory synapses labeled for VGAT. In all graphs, histograms represent means and s.e.m.;

Values normalized to their respective controls; Mann-Whitney test: ns - not significant; * $p \leq 0.05$;

** $p < 0.01$; *** $p < 0.001$.

Fig. 4. GABAergic synapse stabilization requires phosphorylation of gephyrin on Ser303. (A)

Western blot and quantification of the gephyrin S303 phosphorylation in control and after 30 min exposure to ADA (4-20 U/mL) + AMPCP (100 μ M) without or with CGS21680 (30 nM) in HEK293 cells transfected with gephyrin-WT or gephyrin-S303A constructs. Note the significant increased phosphorylation of P-S303 of gephyrin-WT but not gephyrin-S303A upon acute A_{2A}R activation with CGS21680. **(B)** Immunostaining (left) and quantification (right) of GABA_Aγ2 and VGAT in the absence or presence of ADA+AMPCP in DIV 10 neurons transfected with 3'UTR gephyrin shRNA and egfp-gephyrin- WT, S303A or S303D constructs. Scale bar, 5 μ m.

Arrowheads show examples of inhibitory synapses triple-stained for VGAT, GABA_AR γ 2 and gephyrin. WT n=53, WT+ADA+AMPCP n=49, S303A n=48, S303A+ADA+AMPCP n=53, S303D n=45, S303D+ADA+AMPCP n=47, 4 cultures. Gephyrin-S303A or S303D block the ADA+AMPCP effect. (C) VGAT staining (left) and quantification (right) of DIV 10-11 neurons transfected with non-target (shNT) or on-target GABA_AR γ 2 (sh γ 2) shRNAs with egfp-gephyrin-WT or egfp-gephyrin-S303D. Scale bar, 5 μ m. Arrowheads show examples of inhibitory synapses labeled for VGAT. shNT n=35, sh γ 2 n=49, sh γ 2 + geph-S303D n=60, 3 cultures. The loss of GABAergic synapses upon suppression of GABA_AR γ 2 can be rescued upon overexpression of gephyrin-S303D. In all graphs, histograms represent means and s.e.m.; values were normalized to

corresponding controls (B, C); ANOVA test (A); Mann-Whitney test (B, C): ns - not significant;
** $p < 0.01$; *** $p < 0.001$.

Fig. 5. A_{2A}Rs stabilize GABAergic synapses via the synaptogenic organizer Slitrk3. (A) The loss of GABAergic synapses following Slitrk3 suppression cannot be rescued by A_{2A}R or GABA_AR activation. VGAT staining (left) and quantification (right) of DIV 10-11 neurons transfected with shMock or shSlitrk3 exposed or not to muscimol (Mus, 10 μ M) or CGS21680 (CGS, 30 nM) for 30 min. Scale bar, 5 μ m. (Muscimol) shMock n=42, shSlitrk3 n=34, shSlitrk3 + muscimol n=41, 3 cultures. (CGS) shNT n=41, shSlitrk3 n=37, shSlitrk3 + CGS n=36, 3 cultures. (B) The loss of GABAergic synapses following overexpression of the Slitrk3-Y969A mutant

cannot be rescued by the activation of A_{2A}Rs. VGAT staining (left) and quantification (right) of DIV 10-11 neurons transfected with Slitrk3-WT or Slitrk3-Y969A exposed or not to CGS21680 for 30 min. Scale bar, 5 μ m. (Muscimol) eGFP n=35, Slitrk3-WT n=38, Slitrk3-Y969A n=39, Slitrk3-Y969A + CGS n=41, 2 cultures. (C) Pull down of gephyrin-WT using the GFP tag followed by a blot for Slitrk3 showing gephyrin-Slitrk3 interaction in HEK293 cells. In all images, arrowheads show examples of inhibitory synapses labeled for VGAT. In all graphs, histograms represent means and s.e.m.; values were normalized to corresponding controls; Mann-Whitney test (A, B): ns - not significant; *p<0.05; ***p<0.001.