Published in final edited form as:

Sci Transl Med. 2016 June 22; 8(344): 344ra85. doi:10.1126/scitranslmed.aaf7151.

Post-anesthesia AMPA receptor potentiation prevents anesthesia-induced learning and synaptic deficits

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Abstract

Accumulating evidence has shown that repeated exposure to general anesthesia during critical stages of brain development results in long-lasting behavioral deficits later in life. To date, there has been no effective treatment to mitigate the neurotoxic effects of anesthesia on brain development. By performing calcium imaging in the mouse motor cortex, we show that ketamine anesthesia causes a marked and prolonged reduction in neuronal activity during the period of post-anesthesia recovery. Administration of the AMPA/kine drug CX546 [1-(1,4-benzodioxan-6-ylcarbonyl)piperidine] to potentiate AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor activity during emergence from anesthesia in mice enhances neuronal activity and prevents long-term motor learning deficits induced by repeated neonatal anesthesia. In addition, we show that CX546 administration also ameliorates various synaptic deficits induced by anesthesia, including reductions in synaptic expression of NMDA (N-methyl-D-aspartate) and AMPA receptor subunits, motor training-evoked neuronal activity, and dendritic spine remodeling associated with motor learning. Together, our results indicate that pharmacologically enhancing neuronal activity during the post-anesthesia recovery period could effectively reduce the adverse effects of early-life anesthesia.

INTRODUCTION

General anesthetics are commonly used to modulate the activity of neuronal networks, producing a reversible loss of sensation and consciousness in surgeries (1–3). Although the vast majority of patients restore their physiological homeostasis soon after anesthesia, some may suffer from long-term adverse effects of anesthesia. A higher incidence of learning disabilities and attention-deficit and hyperactivity disorders has been found in children

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Author contributions: L.H. and G.Y. designed the experiments. L.H. performed and analyzed all the experiments on animal behavior, spine imaging and protein level examination. L.H. and J.C. conducted and analyzed calcium imaging experiments. I.N. helped with the neuronal activity data analysis. G.Y. wrote the article.

Competing interests: The authors declare that they have no competing interests.

repeatedly exposed to procedures requiring general anesthesia (4–8). Animal studies further demonstrated that prolonged exposure to anesthetics and sedatives during critical stages of brain development causes neurodegeneration and behavioral deficits in both rodents and nonhuman primates (9–13). So far, there has been no effective strategy to alleviate the neurotoxic effects of anesthetic drugs.

Ketamine is a dissociative anesthetic, which depresses neuronal activity and reduces calcium influx into neurons, in part through blockade of N-methyl-D-aspartate (NMDA)-type glutamate receptors (2). Recent in vivo imaging studies have shown that multiple exposures to general anesthesia induced by ketamine-xylazine (KX) during early postnatal development impair motor learning and learning-dependent remodeling of postsynaptic dendritic spines in adolescent mice (14). Given the important roles of neuronal activity in shaping the development of neuronal circuits (15, 16), an intriguing possibility is that pharmacologically enhancing neuronal activity after anesthesia could be beneficial in alleviating the adverse effects of neonatal exposure to anesthesia.

Here, we examined the effects of an AMPAkinone drug, 1-(1,4-benzodioxan-6-ylcarbonyl)piperidine (CX546), on KX anesthesia-induced learning and synaptic deficits. AMPAkinones are a class of compounds that increase α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor transmission. These compounds bind to a modulatory site on the AMPA receptor to reduce the kinetics of channel deactivation and desensitization, and they have been documented to enhance learning and memory in rodents and possibly humans (17–19). Using in vivo two-photon imaging, we show that KX causes a substantial and prolonged reduction in neuronal Ca^{2+} activity that persists into the period of post-anesthesia recovery. We found that administration of CX546 after anesthesia in mice restores neuronal Ca^{2+} activity and prevents neonatal anesthesia-induced motor learning deficits in adulthood. Furthermore, CX546 rescues KX anesthesia-induced synaptic deficits, including decreased expression of synaptic protein and impaired synaptic functional and structural plasticity associated with learning. Together, our results indicate that pharmacologically enhancing neuronal activity by the AMPAkinone CX546 during the post-anesthesia recovery period effectively reduces learning and synaptic deficits caused by early exposure to anesthesia.

RESULTS

CX546 enhances neuronal activity during the post-anesthesia period

To determine the effect of neonatal KX anesthesia on neuronal activity, we performed in vivo calcium imaging of layer V (L5) pyramidal neurons in the motor cortex of 2-week-old mice with or without anesthesia (Fig. 1A). We used transgenic mice expressing the genetically encoded calcium indicator GCaMP specifically in L5 pyramidal neurons to monitor neuronal activity (Fig. 1B) (20, 21). We found that the level of somatic Ca^{2+} transients was greatly reduced after an intraperitoneal injection of KX [ketamine (20 mg/kg) and xylazine (3 mg/kg)] when compared to that under awake conditions (Fig. 1, B and C; total integrated F/F_0 : $7.9 \pm 0.7\%$ versus $32.7 \pm 1.6\%$, $P < 0.0001$). MK801, an antagonist of the NMDA receptor, mimicked the effect of KX on somatic Ca^{2+} transients (Fig. 1C), suggesting that the reduction in neuronal activity during KX anesthesia is mainly mediated

by NMDA receptor blockade. Although mice started to wake up and exhibited voluntary movements within 1 hour after KX injection as indicated by EMG recording (Fig. 1D), the level of somatic Ca^{2+} transients remained significantly lower as compared to the awake state ($F_{\text{KX-1 h}}/F_{\text{awake}}$: 0.52 ± 0.04 ; $P < 0.0001$; Fig. 1, D and E). The reduction in somatic Ca^{2+} transients persisted for at least 2 hours after the injection of KX ($F_{\text{KX-2 h}}/F_{\text{awake}}$: 0.71 ± 0.05 ; $P = 0.0072$). Thus, KX anesthesia causes a reduction in neuronal activity that persists into the post-anesthesia recovery period.

Previous studies have shown that AMPAkinases are allosteric modulators that directly potentiate AMPA receptor-mediated synaptic transmission (17). To test whether AMPAkinase treatment is effective in restoring neuronal activity in post-anesthesia mice, we treated mice with CX546 1 hour after KX injection (Fig. 1D). We found that a single injection of CX546 caused a dose-dependent increase in neuronal activity (Fig. 1E). Neuronal activity was significantly increased within 15 min after CX546 injection (20 mg/kg, intraperitoneally; $P < 0.0001$). Thirty minutes after CX546 injection (90 min after KX injection), the level of somatic Ca^{2+} transients in cortical pyramidal neurons was comparable to that during the pre-KX awake state (normalized change in Ca^{2+} , 1.14 ± 0.12), indicating that CX546 was effective in restoring neuronal activity during post-anesthesia recovery. In addition to CX546, we found that another AMPAkinase drug, CX516, also increased neuronal activity in the period of post-anesthesia recovery (fig. S1). At the same dosage (20 mg/kg, intraperitoneally), the effect of CX516 on L5 somatic Ca^{2+} activity is ~68% of CX546. Together, these results demonstrate that potentiation of AMPA receptor activity with AMPAkinases helps to restore neuronal activity in post-anesthesia mice.

CX546 rescues motor learning deficits induced by repeated anesthesia

Previous studies have shown that three exposures, but not a single exposure to KX anesthesia during postnatal days 14 to 18 (P14–18) impair the animals' motor learning in adolescence (14). We next determined whether administration of AMPAkinases in post-anesthesia mice may prevent motor learning deficits induced by multiple anesthetic exposures (Fig. 2). Because CX546 is more effective than CX516 in enhancing neuronal activity, we chose CX546 to test our hypothesis. Specifically, mice received one or three injections of KX [ketamine (20 mg/kg) and xylazine (3 mg/kg)] or saline during the second or third postnatal week. In either adolescence (P30) or adulthood (P60), mice were trained to run on an accelerated rotarod (Fig. 2, A and B). In this rotarod running task, the animals learn to change their gait pattern to maintain their balance on an accelerated rotating rod (22, 23). The rotarod performance was measured by the average running speed that mice mastered after training. Consistent with previous studies (14), although a single exposure to KX anesthesia has no apparent effect on the animals' behavior at P30, mice subjected to multiple sessions of anesthesia during P7–11 or P14–18 showed worse rotarod performance after 2-day training at both 1 month (Fig. 2A) and 2 months of age (Fig. 2B). Because the rotarod performance on the first training day was not significantly different among all the groups, this finding indicates impaired motor skill learning after repeated anesthesia exposure. This deficit in the rotarod task was not observed in groups of mice that received an intraperitoneal injection of CX546 (20 mg/kg) 1 hour after each KX injection (Fig. 2, A and

B). Administration of CX546 alone had no significant effects on the animals' rotarod performance ($P > 0.5$, Tukey's *post hoc* test).

In addition to the rotarod task, we observed motor learning deficits in another motor learning paradigm, treadmill running, where mice learned to change their gait patterns progressively (Fig. 2, C and D). In this treadmill running task, the animals' gait patterns were classified as drag, wobble, sweep, and steady run (24). Mice displayed large proportions of untrained gait features (drag, wobble, and sweep) when they first ran on the treadmill, whereas the percentage of trained gait feature (steady run) increased after training. In both adolescent and adult mice, when treadmill performance was assessed 5 hours after an initial 1-hour training, we found significant increases in trained gait feature in saline-treated controls (post-training versus pre-training: $P = 0.0005$ and $P < 0.0001$, paired *t* test), whereas mice with repeated KX exposure failed to show training-related performance improvement (Fig. 2, C and D). Mice that were given CX546 after KX anesthesia showed similar increases in treadmill performance as the control group (Fig. 2, C and D). Together, these observations indicate that long-lasting motor learning deficits caused by repeated KX anesthesia during early development could be rescued by administration of CX546 during the post-anesthesia recovery period.

CX546 treatment prevents the reduction of synaptic protein expression after repeated anesthesia

The results above indicate that pharmacologically enhancing neuronal activity with CX546 during post-anesthesia recovery effectively prevents motor learning deficits induced by early exposure to anesthesia. To explore the mechanisms underlying anesthesia-induced learning deficits and CX546-mediated protection, we examined the amounts of various proteins in the cortex after repeated KX exposure, focusing on those involved in synaptic activity and function. Synaptosome and whole cell preparations were generated from the cortex of P30 and P60 mice that had received three KX injections during P7–11, and protein concentrations were determined by Western blot analysis (Fig. 3A). We found that in both adolescent and adult mice, postsynaptic glutamate NMDA receptor subunits (GluN1 and GluN2A) and the glutamate AMPA receptor subunits (GluA1 and GluA2) were significantly decreased in synaptosomes from KX-treated mice as compared to saline-treated controls (P30: $P = 0.0068$, 0.0344 , 0.0317 , and 0.0400 ; P60: $P = 0.0083$, 0.05 , 0.0104 , and 0.0306 , Tukey's *post hoc* test; Fig. 3, B to D). The amounts of GluN1, GluN2A, GluA1, and GluA2 in the whole-brain fraction, however, remained unaltered (Fig. 3, E to G). These results demonstrate that repeated anesthesia at the neonatal stage causes long-lasting reduction of glutamate receptors at synapses. Mice that received both KX and CX546 exhibited normal synaptic expression of NMDA and AMPA receptor subunits during adolescence and adulthood ($P > 0.2$ versus saline; Fig. 3, B to D), suggesting that potentiating neuronal activity with CX546 during the post-anesthesia period prevents synaptic deficits later in life.

CX546 treatment rescues training-evoked neuronal activity after repeated anesthesia

Ionotropic glutamate receptors are responsible for the glutamate-mediated postsynaptic excitation of neurons (Fig. 1C) (25, 26). The rescue of anesthesia-induced reduction of NMDA and AMPA receptors at synapses by CX546 suggests that deficits of neuronal

function may also be rescued. To test this, we examined the neuronal activity in the primary motor cortex by performing in vivo Ca^{2+} imaging of L5 pyramidal neurons while mice performed a motor skill task. In this experiment, mice received three KX or saline injections at P7–11. At either P30 or P60, mice were head-restrained and trained to run forward on a custom-built, free-floating treadmill under a two-photon microscope (Fig. 4A) (24). L5 pyramidal neurons of the motor cortex were imaged across three two-photon imaging planes, which span the cortical column (Fig. 4B). Here, we were able to record Ca^{2+} activity in apical tuft dendrites (10 to 50 μm from pial surface), in apical trunk dendrites (200 to 250 μm from pial surface), and at the level of the L5 soma (500 to 600 μm from pial surface) during the quiet resting state and forward running (Fig. 4C; total recording time: 2.5 min for resting and 2.5 min for treadmill running). We observed a significant reduction in the number of Ca^{2+} transients generated in the apical tuft/trunk dendrites and soma of L5 neurons when 1-month-old mice with neonatal KX exposure were running on the treadmill as compared with saline-injected controls (Fig. 4, D and E; tuft: $P=0.0016$; trunk: $P=0.0453$; L5 soma: $P=0.0472$; Tukey's *post hoc* test). The peak amplitudes ($\Delta F/F_0$) of Ca^{2+} transients in the apical tuft, apical trunk, and L5 soma were not significantly different in KX-treated mice as compared to saline-injected controls (Fig. 4F). CX546 treatment after repeated KX at P7–11 rescued training-evoked Ca^{2+} transients generated in the apical tuft, trunk, and L5 soma to the same level as the saline controls (Fig. 4, D and E). CX546 treatment did not alter the peak amplitude of Ca^{2+} transients generated during running as compared to KX-treated mice (Fig. 4F). Similar to what was observed in 1-month-old animals, we found that at P60, the level of motor training-evoked Ca^{2+} activity in L5 neurons remained significantly lower in KX-treated mice relative to saline-treated controls (tuft, $P<0.0001$; trunk, $P=0.0008$; L5 soma, $P<0.0001$), but not in mice with CX546 treatment (Fig. 4G). Together, these results show that mice with repeated KX anesthesia during early development have decreased motor training-evoked neuronal activity and that CX546 reverses these changes in neuronal activity.

CX546 treatment partially rescues motor learning-induced dendritic spine formation after repeated anesthesia

The formation and elimination of synaptic connections are thought to play an important role in learning and memory formation (23, 27–32). Our previous studies have shown that repeated exposure to KX in mice at P14–18 results in the reduction of motor learning-dependent dendritic spine formation (14). To examine whether CX546 rescues deficits in motor learning-induced spine plasticity, we first examined the dynamics of postsynaptic dendritic spines of L5 pyramidal neurons in the motor cortex of *Thy1-YFP* (yellow fluorescent protein) mice that received a single or repeated KX exposure at P7–11 (Fig. 5A). Under baseline conditions (no motor training), we found that spine formation and elimination over 2 days in 1-month-old mice that received a single or repeated KX exposure at P7–11 were comparable to those in saline-treated controls (Fig. 5, B and C). These results indicate that early exposure to KX does not alter the baseline dynamics of dendritic spines in the motor cortex in adolescence.

Next, we examined whether motor learning-induced spine remodeling is altered after repeated KX exposure (Fig. 5D). We found significant decreases in motor learning-induced

spine formation ($7.8 \pm 0.4\%$, 742 spines, $n = 5$ mice; $P < 0.0001$, Bonferroni's *post hoc* test) and spine elimination ($5.1 \pm 0.2\%$; $P = 0.0125$) in 1-month-old mice that have received three KX injections at P7–11 as compared with saline-treated controls (formation, $13.4 \pm 0.8\%$; elimination, $7.5 \pm 0.5\%$; 687 spines, $n = 5$ mice) (Fig. 5, D to F). A single injection of KX at P7 had no effects on motor learning–induced spine remodeling. Mice with consecutive administrations of KX and CX546 exhibited significant increases in motor learning–induced spine formation ($10.6 \pm 0.5\%$, 676 spines, $n = 5$ mice; $P = 0.0198$) and spine elimination ($7.7 \pm 0.6\%$; $P = 0.0071$) as compared to mice with KX injections only. Similar results were observed in adult mice at 2 months of age: Motor learning–induced spine formation was significantly lower in mice with three KX injections at P7–11 ($6.1 \pm 0.6\%$; $P < 0.0001$), but not in mice with CX546 treatment after anesthesia ($8.8 \pm 0.3\%$), as compared with saline-treated controls ($10.4 \pm 0.3\%$) (Fig. 5G). We did not find significant differences in motor learning–induced spine elimination among the three groups of mice at P60 (Fig. 5H). These results show that CX546 treatment rescues motor learning–induced dendritic spine plasticity in the motor cortex of mice subjected to repeated neonatal anesthesia.

DISCUSSION

With an increasing number of infants and young children exposed to anesthetic agents each year, there is a growing concern about the safety of general anesthesia for the developing brains. Growing preclinical evidence indicates that early-life anesthesia could impair the development of neuronal circuits and cause long-lasting learning deficits (9–13). Here, we show that KX anesthesia causes a prolonged reduction of neuronal activity in the motor cortex. The reduction in neuronal activity extends well into the post-anesthesia recovery period and can be rescued rapidly after administration of an AMPA drug, CX546. Adult mice with repeated KX anesthesia during the second postnatal week display deficits in motor skill learning and learning-induced synaptic plasticity. Administration of CX546 during post-anesthesia recovery prevents anesthesia-induced motor learning and synaptic impairments. Together, our studies indicate that pharmacologically enhancing neuronal activity during the post-anesthesia recovery period could be an important strategy for reducing adverse effects caused by early exposure to anesthesia.

Ketamine is a dissociative anesthetic that primarily blocks NMDA receptors (2, 33). In rodents, ketamine is frequently used together with the $\alpha 2$ -adrenoreceptor agonist xylazine to decrease the ketamine dose for anesthesia induction. Although a single injection of ketamine (20 mg/kg) combined with xylazine (3 mg/kg) produces a light surgical level of anesthesia in young mice for less than 1 hour (14), we found that neuronal activity in the cortex remains decreased for at least 2 hours, suggesting a prolonged aftereffect of KX on brain activity. Blockade of NMDA receptors with MK801 produced a similar effect on neuronal Ca^{2+} dynamics as KX, suggesting that the effect of KX on neuronal activity is largely caused by the blockade of NMDA receptors. Although the KX combination is not used clinically, ketamine is widely used in pediatric patients for either anesthetic or sedative purposes. It is possible that pediatric patients undergoing ketamine anesthesia or sedation may suffer from reduced neuronal activity as we observed in the mouse brain. Furthermore, other anesthetics used in pediatric medicine could also cause prolonged reduction in neuronal activity during emergence from anesthesia and may impair brain development.

Given the important role of neuronal activities in neural development, we reasoned that under the circumstance when anesthesia is not optional, swift restoration of neuronal activity and calcium influx into neurons during the recovery phase of general anesthesia could be an effective strategy to reduce its adverse effects. Realizing well-documented neurotoxic effects of some NMDA receptor agonists, we focused our attention on compounds that potentiate AMPA receptor activity. AMPAkinases are a group of small compounds that slow the onset of AMPA receptor desensitization and/or deactivation, thereby increasing fast excitatory transmission (17). These compounds do not have agonistic or antagonistic properties but instead modulate the receptor rate constants for transmitter binding, channel opening, and desensitization. They can freely cross the blood-brain barrier (34) and have been documented to enhance learning and memory in animal and human studies (19). Our studies show that CX546 is effective in restoring neuronal activity during the post-anesthesia recovery period and preventing repetitive KX-induced motor learning deficits.

Although it has become increasingly clear that prolonged exposure to general anesthesia could cause substantial changes in the developing brain (9–12, 35), the precise neuropathology underlying cognitive dysfunction in adulthood is not clear. Here, we found decreases in a number of synaptic proteins (GluN1, GluN2A, GluA1, and GluA2) in the cortex of both adolescent and adult mice with repeated neonatal exposure to KX anesthesia. There were no changes in the abundance of GluN1, GluN2A, GluA1, and GluA2 in the whole-cell fraction. The differences in glutamate receptor subunit expression between synaptosome and whole-brain fraction could be due to the fact that these proteins are not only expressed at synaptic sites, but also present in nonsynaptic sites of neurons, as well as in glial cells. Given the pivotal roles of ionotropic glutamate receptors in fast excitatory synaptic transmission in the brain (25), reduced expression of NMDA and AMPA receptor subunits identified here can have a major impact on the level of neuronal network activity. Using *in vivo* Ca^{2+} imaging, we found that motor training-evoked Ca^{2+} transients in L5 pyramidal neurons of the motor cortex were reduced in mice with repeated ketamine exposure. Together, these findings show that repeated exposure to anesthesia causes long-lasting changes in synapse maturation, which in turn contributes to motor learning deficits observed in our study.

Synaptic structural plasticity in the primary motor cortex is important for motor skill learning (23, 28, 30). Previous studies have demonstrated that rotarod training over 2 days results in a 5 to 7% increase in new spines in the motor cortex (23, 30). A fraction of training-associated new spines persist over weeks, and the amount of persistent new spines strongly correlates with the animals' performance (23, 30). Although baseline spine dynamics remain unaltered in mice with repeated KX exposures, we observed a reduction in learning-induced dendritic spine remodeling after 2-day training. Together with the finding of decreased neuronal activity during training in these mice, these results suggest that after early anesthetic exposure, cortical circuits are less responsive to modulation by learning experience and exhibit reduced synaptic plasticity in adulthood. The reductions in both motor learning-induced neuronal activity and synaptic structural plasticity can be rescued by CX546 treatment, further supporting the therapeutic potential of CX546 in ketamine anesthesia-induced cognitive impairments.

The mechanisms by which CX546 alleviates anesthesia-induced synaptic and learning deficits remain to be determined. The beneficial effect of CX546 could be simply due to the shortening of the post-anesthesia depression of cortical activity as a result of potentiation of AMPA receptor activity by CX546. In addition to restoring cortical activity, CX546 may cause other changes in the brain to offset the deleterious effects of anesthetic exposure on the developing brain. For example, it has been shown that AMPAkinine treatments increase the expression of brain-derived neurotrophic factor (BDNF) (36–39). BDNF is a potent regulator of synaptic plasticity (40) and therefore could have an important role in the rescue of synaptic and behavioral phenotypes associated with early anesthetic exposure. Future studies are needed to determine whether brief AMPAkinine treatments would elicit the positive trophic effects associated with the neurotrophin in the developing brain.

Our experiments with CX546 treatment are proof-of-principle studies, which support the hypothesis that pharmacologically enhancing neuronal activity is an effective strategy for the treatment of long-lasting effects of early exposure to anesthesia. Over the past decade, AMPAkinines have been favored for the treatment of cognitive deficits. Several compounds have generated very promising preclinical results in the treatment of a number of neurological disorders such as Alzheimer's disease, Huntington's disease, schizophrenia, and depression (19, 39, 41). They have also been used to improve cognition in healthy and elderly human volunteers (42). Drugs like CX546 and CX516 appear to be inherently safe because their ability to prolong AMPA receptor activity is kinetically limited. In the future, it would be of great interest and importance to test whether these drugs are effective at minimizing the risk for the development of learning disabilities in children who receive multiple and prolonged anesthesia.

MATERIALS AND METHODS

Study design

The objective of this study was to investigate the effects of potentiating AMPA receptor activity on mitigating learning and synaptic deficits induced by neonatal anesthesia. Mice were randomly assigned to three experimental groups: (i) saline-treated controls, (ii) three episodes of anesthesia, and (iii) three episodes of anesthesia plus AMPAkinine treatment. For all three groups of mice, motor learning performance was examined by rotarod and treadmill running tasks, synapse protein expression was measured by Western blot, training-evoked neuronal activity and synapse structural remodeling were determined by in vivo two-photon imaging. No statistical methods were used to predetermine sample size. Group sample size was chosen on the basis of previous studies using the same methodologies, and variance was similar between groups being statistically compared. The researchers were blind to group assignment, and no data points were excluded from the statistical analysis.

Experimental animals

Thy1-YFP mice (H line) (43) were purchased from The Jackson Laboratory and used for dendritic spine imaging experiments. *Thy1*-GCaMP6slow mice (line 1) and *Thy1*-GCaMP2.2c mice (21) were used for Ca²⁺ imaging experiments. Mice were group-housed in New York University Skirball animal facility. All experiments were performed in accordance

with institutional guidelines. For early anesthesia treatment, mice were given an intraperitoneal injection of KX mixture [ketamine (20 mg/kg) and xylazine (3 mg/kg)] every other day (P7, P9, and P11 or P14, P16, and P18), for a total of three injections. One injection of KX at this dose induces anesthesia and provides immobilization in neonatal mice for ~1 hour and does not cause cell apoptosis (14). Control animals received saline injections. For CX546 treatment, mice received an intraperitoneal injection of CX546 (20 mg/kg) 1 hour after each KX injection. During anesthesia, a heating pad was used to maintain the animal's body temperature at about 37°C.

In vivo Ca^{2+} imaging

In vivo Ca^{2+} imaging was performed in awake, head restrained mice. The surgical procedure for preparing awake animal imaging has been described previously (44). In brief, a head holder composed of two parallel micro-metal bars was attached to the animal's skull to reduce motion-induced artifact during imaging. Surgical anesthesia was achieved with an intraperitoneal injection of ketamine/xylazine [ketamine (100 mg/kg) and xylazine (15 mg/kg)]. A midline incision of the scalp exposed the periosteum, and a small skull region (~0.2 mm in diameter) was located over the right motor cortex based on stereotactic coordinates (0.5 mm posterior from the bregma and 1.5 mm lateral from the midline) and marked with a pencil. A thin layer of cyanoacrylate-based glue was first applied to the top of the entire skull surface and to the metal bars, and the head holder was then further fortified with dental acrylic cement. The dental cement was applied so that a well was formed, leaving the motor cortex with the marked skull region exposed between the two bars. All procedures were performed under a dissection microscope. After the dental cement was completely dry, a cranial window was created over the previously marked region. The procedures for preparing a thinned-skull cranial window for two-photon imaging have been described in detail in previous publications (45). The completed cranial window was covered with silicon elastomer, and mice were given at least 1 day to recover from the surgery-related anesthesia. Later, mice with head mounts were habituated to the imaging apparatus a few times (10 min each time) to minimize potential stress effects of head restraining and imaging.

To image neuronal Ca^{2+} activity in the cortex of awake mice, the head holder was screwed to two metal cubes attached to a solid metal base, and the silicon elastomer was peeled off to expose the thinned skull region and artificial cerebrospinal fluid was added to the well. The head-restrained animal was then placed on the stage of a two-photon microscope. These in vivo Ca^{2+} imaging experiments were performed using an Olympus two-photon system equipped with a DeepSee Ti:Sapphire laser (Spectra Physics). The average laser power on the sample was ~20 to 30 mW. Most experiments were acquired at frame rates of 2 Hz at a resolution of 256×256 pixels using a $25\times$ water immersion objective. Image acquisition was performed using Olympus FV1000 software and analyzed *post hoc* using National Institutes of Health (NIH) ImageJ software. F/F_0 was calculated by $(F-F_0)/F_0$, where F_0 is the baseline fluorescence signal averaged over a 2-s period. In Fig. 4E, Ca^{2+} transients were defined as the events when changes of fluorescence (F/F_0) observed in dendrites or soma were >20% during the 2.5-min imaging sessions.

Motor skill training

Two motor tasks were used in this study. For the rotarod running task, we used an EZRod system with a test chamber (44.5 cm × 14 cm × 51 cm dimensions) to perform the test. Animals were placed on a motorized rod (30 mm in diameter) in the chamber. The rotation speed increased gradually from 0 to 100 rpm over the course of 3 min. The time latency and rotation speed were recorded when the animals were unable to keep up with the increasing speed and fell. Rotarod training/testing was performed in one 30-min session (20 trials) per day. Rotarod performance was measured as the average speed animals achieved during the 20-trial training session. A treadmill running task was introduced to provide a different motor training, as well as to perform two-photon Ca^{2+} imaging and motor training at the same time. A custom built, free-floating treadmill (96 cm × 56 cm × 61 cm dimensions) was used to allow head-fixed mice to move their forelimbs freely to perform motor running tasks. At the onset of a trial, the motor was turned on and the belt speed gradually increased from 0 to 8 cm/s within ~3 s, and the speed of 8 cm/s was maintained for the rest of the trial.

Isolation of synaptosome fractions and Western blot

Mice were deeply anesthetized and perfused with 40 ml of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Dulbecco's phosphate buffered saline. The cortex was dissected and homogenized with a dounce homogenizer in buffer A (320 mM sucrose, 1 mM NaHCO_3 , 1 mM MgCl_2 , and 0.5 mM CaCl_2) and cleared of nuclei and insoluble material by centrifugation. The resulting supernatant was centrifuged at 30,000g to pellet membranes, and the pellet was subsequently resuspended in buffer B (320 mM sucrose and 1 mM NaHCO_3). Membranes were fractionated using a discontinuous sucrose gradient consisting of 1.0 and 1.2 M sucrose at 120,000 3 g for 2 hours at 4°C. After centrifugation, the synaptosomal fraction was isolated at the 1.0/1.2 interface, diluted in Buffer B, and pelleted at 120,000g for 45 min at 4°C. The resulting synaptosomes were solubilized in 25 mM tris (pH = 7.4) and 2% SDS. Protein content was determined by bicinchoninic acid assay (Thermo Scientific), and 10 µg of total protein was loaded per lane on a 10% SDS-polyacrylamide gel electrophoresis gel. Separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and blocked for 30 min with 2% bovine serum albumin in tris-buffered saline–Tween 20. Blocked membranes were probed overnight with the following antibodies: GluN1, GluN2A, GluA1, GluA2 (NeuroMab), and actin (Sigma). After washing, membranes were incubated with anti-rabbit or anti-mouse immunoglobulin G–horseradish peroxidase secondary antibodies (Jackson ImmunoResearch), washed, and incubated with enhanced chemiluminescence reagent (GE Life Sciences) before exposure to film. Densitometry analysis was performed by manual scanning and digitalization of film, and quantified using the gel analysis plugin for NIH ImageJ software.

In vivo imaging of dendritic spines and data analysis

The surgical procedure for chronic transcranial two-photon imaging has been described previously (45). While the animal was under deep anesthesia, the skull surface was exposed with a midline scalp incision and a small skull region (~0.2 mm in diameter) was located over the primary motor cortex based on stereotaxic coordinates. A custom-made, stainless steel plate was glued to the skull with a central opening over the cortical region of interest.

To create a cranial window for imaging, the skull surface was immersed in artificial cerebrospinal fluid, and a high-speed drill and a microsurgical blade were used to carefully reduce the skull thickness to about 20 μm under a dissection microscope. The entire surgical procedure usually took less than 30 min, and the two-photon imaging took place immediately after the skull thinning. During imaging, the animal was placed under an Olympus two-photon microscope with the laser tuned to the optimal excitation wavelength for YFP (920 nm). Low laser power (20 to 30 mW at the sample) was used during imaging to minimize phototoxicity. The images were acquired with a 60 $\times$ water-immersion objective (numerical aperture, 1.1) at a zoom of 1.0 to 3.0. A stack of image planes within a depth of 100 μm from the pial surface was collected, yielding a full three-dimensional data set of dendrites in the area of interest. The step size was 2 μm for the initial low magnification image (no zoom) for relocation at later time points and 0.75 μm for all the other experiments ($\times 3.0$ zoom). After imaging, the plate was gently detached from the skull, and the scalp was sutured with 6-0 silk. The animals were returned to their home cages until the next view.

Data analysis was performed with NIH ImageJ software as described previously (23, 46). The same dendritic segments were identified from three-dimensional image stacks taken at both time points. The number and location of dendritic protrusions were identified in each view. Filopodia were identified as long, thin structures without enlarged heads, and the rest of the protrusions were classified as spines. Spines were considered the same between two views based on their spatial relationship to adjacent landmarks and spines. Spines in the second view were considered different if they were more than 0.7 μm away from their expected positions based on the first view. The formation or elimination rates of spines were measured as the number of spines formed or eliminated divided by the number of spines existing in the first view.

Statistics

Prism software (GraphPad 6.0) was used to conduct the statistical analysis. Data were presented as means $\pm$ SEM. Tests for differences between populations were performed using Student's *t* test or a one- or two-way ANOVA followed by Tukey or Bonferroni *post hoc* test as specified in the text. For the *post hoc* multiple comparisons, all the drug treatment groups were compared with the control group. Significant levels were set at $P = 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank W.B. Gan for providing *Thy1*-GCaMP6s mice, G. Feng for providing *Thy1*-GCaMP2.2c mice, and T. Blanck for helpful discussions.

Funding: This study was supported by NIH grants GM107469, AG048410 (to G.Y.), and HD076914 (to I.N.)

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Fig. 1. CX546 enhances neuronal activity during the post-anesthesia period

(A) Schematic illustrating the experimental approach for in vivo Ca^{2+} imaging of cortical neurons in awake mice. (B) Two-photon images of L5 neurons expressing GCaMP6s before KX injection (awake), and 15 min and 1 hour after KX injection. Scale bar, 10 μm . (C) Cells show a large decrease in somatic Ca^{2+} 15 min after KX [ketamine (20 mg/kg) and xylazine (3 mg/kg)] or MK801 (0.25 mg/kg) injection (58 to 62 cells from five mice for each group, unpaired t test). (D) Neuronal calcium fluorescence traces and electromyography (EMG) signals before and after KX injection, with and without CX546 treatment. Examples of 25-s traces are shown. i.p., intraperitoneally. (E) Normalized neuronal Ca^{2+} activity over time after KX and CX546 injection ($n = 5$ mice per group, unpaired t test). Data are presented as means $\pm$ SEM.

Fig. 2. CX546 rescues motor learning deficits induced by repeated KX anesthesia

(A–B) Animals received one or three injections of KX at either P7–11 or P14–18 and were tested on the rotarod running task at 1 (A) or 2 (B) months of age. Rotarod performance is expressed as the average speed reached during the first (pre-training) and last (post-training) training session ($n = 7$ to 10 mice per group). Two-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. See table S1 for statistics and details. n.s., not significant. (C–D) Analysis of the animals' gait patterns when running on a treadmill ($n = 8$ to 12 mice per group, individual animal values were listed in table S2). At both P30 (C) and P60 (D), mice with repeated KX exposures at P7–11 displayed higher proportions of untrained gait features such as drag, wobble, and sweep, but less trained gait feature (steady run) after 1-hour training. Treadmill performance is expressed as percent difference in each subject's trained gait feature between post-training and pre-training. Within each treatment group, post-training performance was compared to pre-training baseline using paired *t* test. Comparisons between groups were performed with one-way ANOVA followed by Tukey's *post hoc* test. Data are presented as means $\pm$ SEM.

Fig. 3. CX546 treatment prevents the reduction of synaptic protein expression after repeated KX anesthesia

(A) Experimental design. (B) Synaptosome fractions were generated from the cortex of P30 or P60 mice after various treatments, and probed with indicated antibodies by Western blot. (C–D) Densitometric quantification of Western blots from the synaptic fractions at P30 (C) and P60 (D) ($n = 4$ mice per group, one-way ANOVA followed by Tukey's *post hoc* test). (E) Whole cell lysates were generated from the mouse cortex, and were probed with indicated antibodies by Western blot. (F–G) Western blot quantification from whole cell lysates at P30 (F) and P60 (G) ($n = 4$ mice per group). Each circle represents an individual animal. Data are presented as means $\pm$ SEM. See table S3 for statistics and details.

Fig. 4. CX546 treatment rescues training-evoked neuronal activity after repeated KX anesthesia (A) Experimental design. (B) Two-photon Ca^{2+} imaging in the primary motor cortex of awake, head restrained mice running on a treadmill. (C) Examples of calcium images acquired from tuft, trunk, and soma of the L5 neurons (shown by arrowheads). (D) Calcium fluorescence traces of apical tuft dendrites, apical trunk dendrites, and L5 soma under various treatment conditions. Examples of 2.5-min traces are shown. (E) Fold change in Ca^{2+} transient generation in tuft/trunk dendrites and soma of P30 mice during running compared to rest ($n = 4$ to 8 mice per group; one-way ANOVA followed by Tukey's *post hoc* test). (F) Peak amplitude of Ca^{2+} transients generated during running. (G) Fold change in total integrated Ca^{2+} activity in tuft, trunk, and soma of P60 mice during running compared to rest ($n = 4$ mice per group). Data are presented as means $\pm$ SEM. See table S4 for statistics and details.

Fig. 5. CX546 treatment partially rescues motor learning-induced dendritic spine remodeling after repeated KX anesthesia

(A) Experimental design. (B–C) Percentages of dendritic spines formed (B) and eliminated (C) over 2 days in saline- and KX-treated mice at 1 month of age ($n = 4$ to 5 mice per group). (D) In vivo time-lapse imaging of the same dendritic segments before and 2 days after rotarod motor training in the primary motor cortex of 1-month-old animals that received various treatments. Filled and empty arrowheads indicate dendritic spines that were formed and eliminated between the two views, respectively. Asterisks indicate dendritic filopodia. (E–F) Percentages of dendritic spines formed (E) and eliminated (F) over 2-day motor training in 1-month-old adolescent mice ($n = 4$ to 5 mice per group, one-way ANOVA followed by Bonferroni's *post hoc* test). (G–H) Percentages of dendritic spines formed (G) and eliminated (H) over 2-day motor training in 2-month-old adult mice ($n = 4$ mice for each group). Each circle represents an individual animal. Data are presented as means $\pm$ SEM. See table S5 for statistics and details.