

Circadian glucocorticoid oscillations promote learning-dependent synapse formation and maintenance

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Excessive glucocorticoid exposure during chronic stress causes synapse loss and learning impairment. Under normal physiological conditions, glucocorticoid activity oscillates in synchrony with the circadian rhythm. Whether and how endogenous glucocorticoid oscillations modulate synaptic plasticity and learning is unknown. Here we show that circadian glucocorticoid peaks promote postsynaptic dendritic spine formation in the mouse cortex after motor skill learning, whereas troughs are required for stabilizing newly formed spines that are important for long-term memory retention. Conversely, chronic and excessive exposure to glucocorticoids eliminates learning-associated new spines and disrupts previously acquired memories. Furthermore, we show that glucocorticoids promote rapid spine formation through a non-transcriptional mechanism by means of the LIM kinase–cofilin pathway and increase spine elimination through transcriptional mechanisms involving mineralocorticoid receptor activation. Together, these findings indicate that tightly regulated circadian glucocorticoid oscillations are important for learning-dependent synaptic formation and maintenance. They also delineate a new signaling mechanism underlying these effects.

Glucocorticoids (CORT) are hormones that respond to stress by coordinating diverse physiological processes aimed at maintaining homeostasis^{1,2}. Many lines of evidence indicate that prolonged secretion of glucocorticoids during chronic stress disrupts learning and memory^{3–5}, but transient release at the time of learning may promote it^{5–7}. Notably, under normal physiological conditions, glucocorticoid activity oscillates in synchrony with circadian rhythms: diurnal peaks and troughs are closely coupled with the active and inactive phases of the circadian cycle^{1,2}. Whether and how circadian glucocorticoid oscillations contribute to learning and memory processes is unknown.

Many lines of evidence suggest that learning-dependent remodeling of synaptic connections is important in learning and memory^{8–12}. For example, motor learning induces the formation of persistent postsynaptic dendritic spines, and the survival of these spines is strongly correlated with behavioral performance after learning¹⁰. Recent studies indicate that glucocorticoids have rapid effects on dendritic spine development and plasticity in the mouse somatosensory cortex¹³. Together, these studies raise the possibility that circadian glucocorticoid oscillations may affect learning and memory by regulating synaptic formation and maintenance.

Previous studies have shown that prolonged exposure to glucocorticoids causes postsynaptic dendritic spine loss and branch atrophy in diverse cortical and subcortical areas^{14–18}. Classically, these effects were presumed to be mediated by transcriptional mechanisms, which typically act relatively slowly by regulating gene expression^{19–21}. Recent reports indicate that rapid, non-transcriptional mechanisms may also contribute. For example, glucocorticoids influence synaptic glutamate release and receptor trafficking through non-transcriptional mechanisms^{22–25} and rapidly modulate the function of inhibitory

interneurons in the prefrontal cortex through non-transcriptional regulation of endocannabinoid signaling^{25–27}. However, it remains unclear whether non-transcriptional mechanisms mediate glucocorticoid effects on learning-related synaptic plasticity.

In this study, we examined the effects of oscillating glucocorticoid activity, glucocorticoid deprivation and prolonged glucocorticoid exposure on learning-associated dendritic spine remodeling using transcranial two-photon microscopy. We found that circadian glucocorticoid oscillations were important for the formation and maintenance of new spines after learning, acting in part through a newly identified, non-transcriptional signaling pathway. Our findings indicate that oscillating levels of glucocorticoid activity balance spine formation, pruning and maintenance, whereas states of prolonged glucocorticoid exposure disrupt these processes.

RESULTS

Circadian peaks promote spine formation after learning

Experience-dependent dendritic spine remodeling involves two distinct processes: first, learning induces spine formation, and subsequently, a fraction of new spines is selectively stabilized and a subset of pre-existing spines is pruned^{10,28}. We began by investigating how circadian glucocorticoid oscillations might affect the process of spine formation in a rotarod motor skill-learning model¹⁰. We assessed how learning-associated spine formation varied with naturally occurring circadian peaks and troughs and with exogenous manipulations of the diurnal glucocorticoid rhythm (Fig. 1a; see Online Methods). Two-day spine formation in layer V pyramidal cells was quantified by imaging the forelimb area of motor cortex 1 d before and immediately after two sessions of motor training in transgenic mice overexpressing yellow fluorescent protein

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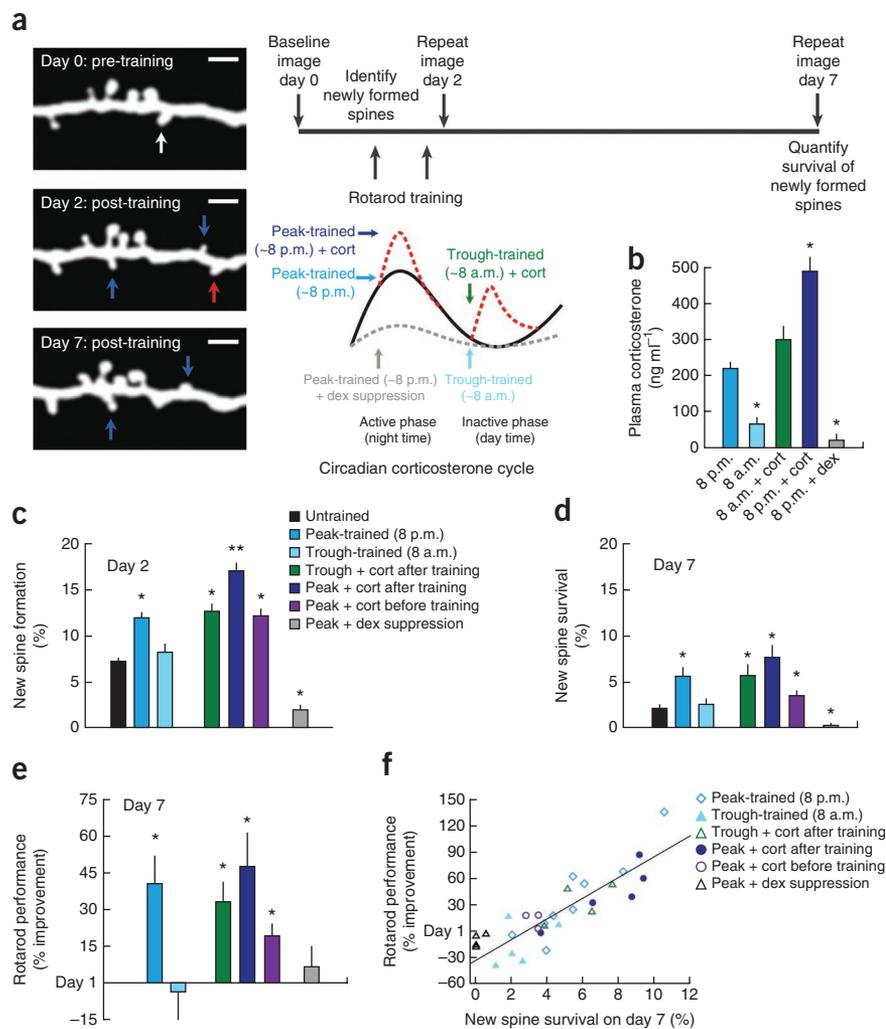
Received 23 January; accepted 28 March; published online 28 April 2013; corrected before print 6 May 2013 (details online); doi:10.1038/nn.3387

Figure 1 Circadian glucocorticoid peaks promote spine formation after learning.

(a) Experimental procedure. Blue arrows: formed on day 2, persisting on day 7. Red arrows: formed on day 2, eliminated on day 7. White arrows: eliminated on day 2. Scale bar, 2 μ m. (b) ELISA quantification of plasma corticosterone (cort) ($F_{4,34} = 41.0$, $P < 0.001$). * $P < 0.05$ versus circadian peak-trained group after Holm-Bonferroni correction. (c) Effects of circadian cort on training-induced spine formation ($F_{6,33} = 42.2$, $P < 0.001$). Spine formation rates represent the number of spines formed during the 2-d training period, as a percentage of the total number quantified at day 0 baseline. * $P < 0.05$ (corrected) versus untrained control. ** $P < 0.001$ versus untrained control and peak-trained group. (d) Effects of circadian cort on spine survival on day 7 ($F_{6,28} = 9.40$, $P < 0.001$). Spine survival represents the number of spines formed during the 2-d training period and persisting on day 7, as a percentage of the total number quantified at baseline. * $P < 0.05$ (corrected) versus untrained control. (e) Corresponding cort effects on rotarod performance on day 7 ($F_{5,55} = 2.84$, $P = 0.02$). Day 7 performance is expressed as percent change in each subject's baseline performance on day 1. * $P < 0.05$ (corrected) versus day 1 baseline. (f) Survival of spines that formed during the training period predicted memory retention on day 7 ($r = 0.86$, $P < 0.001$). Error bars, s.e.m. See **Supplementary Table 1** for statistics and details.

in cortical layer V pyramidal cells (YFP-H line; see Online Methods). To minimize the potential effects of training on diurnal corticosterone rhythms, mice were first habituated to a constant, low-speed (15 r.p.m.) rotating rod for 3 d and then trained on the accelerating rod for 2 d. We validated manipulations of circulating glucocorticoids by ELISA quantification of plasma corticosterone in blood obtained from a separate cohort of mice approximately 45 min after training under identical conditions (Fig. 1b; see also **Supplementary Fig. 1** for more data on effects of training and habituation).

Training increased spine formation when it coincided with the circadian glucocorticoid peak. In contrast, training during the circadian trough had no significant effect on spine formation in habituated mice (Fig. 1c; for effects on filopodia, see **Supplementary Fig. 2**). Four lines of evidence indicate that elevated glucocorticoid exposure immediately after training was important for learning-related spine formation (Fig. 1c). First, a corticosterone injection (2.5 mg per kilogram body weight) immediately after training during the trough increased spine formation to a value comparable to that observed in mice that were trained during the circadian peak. Second, administration of the same corticosterone injection immediately after training during the peak further increased spine formation. Third, corticosterone specifically enhanced learning-induced spine formation and not merely formation in general: 2-d formation was significantly greater when corticosterone was administered immediately after training than when an identical dose was applied 8 h before each training session. And fourth, these effects were not merely an artifact of exogenous corticosterone treatment: suppression of endogenous glucocorticoid activity by dexamethasone (see Online Methods) was sufficient to block the formation of new spines.



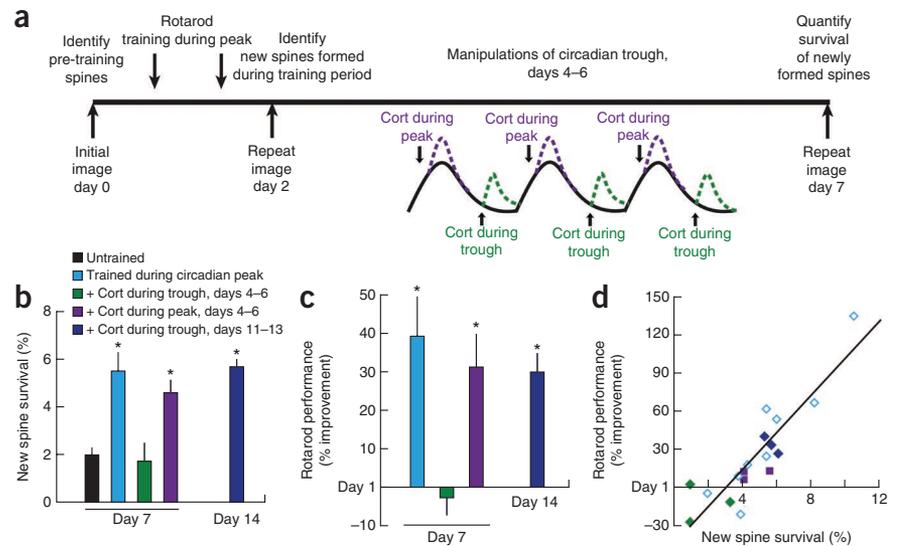
Together, these findings suggest that the circadian glucocorticoid peak is important physiologically for generating new spines after learning.

Previous studies have shown that long-term retention of improvements in motor performance correlates with the survival of learning-related spines¹⁰. To evaluate the long-term impact of glucocorticoids on spine formation and memory retention, we tested and reimaged the same mice on day 7, after a 5-d period without further rotarod training (Fig. 1a). Spines that formed during the 2-d training period persisted in greater numbers in the corticosterone-exposed groups (Fig. 1d), with correspondingly enhanced retention of the learned motor skill (Fig. 1e). The persistence of these training-related spines strongly predicted performance on day 7 ($r = 0.86$, $P < 0.001$; Fig. 1f and **Supplementary Figs. 3** and **4**). Notably, mice that were trained during the circadian trough failed to retain the learned motor skill. This lack of improvement may have been due in part to prior habituation²⁹, as modest but significant increases in performance ($P = 0.05$) and spine formation ($P = 0.03$) were observed in a group of trough-trained mice that were not habituated (**Supplementary Fig. 5**). Together, these experiments show that elevated glucocorticoid secretion during the circadian peak facilitates the formation of enduring new spines after learning and enhances long-term memory retention.

Circadian glucocorticoid troughs stabilize new spines

Although the results above highlight the importance of the circadian glucocorticoid peak in experience-dependent spine formation, it is

Figure 2 Circadian glucocorticoid troughs preserve newly formed spines. **(a)** Schematic of experimental procedure. **(b)** Disruption of the circadian trough reduced the survival of spines that formed during the training period ($F_{4,18} = 5.02$, $P = 0.007$). Corticosterone (cort) that was administered during the second week (days 11–13) had no effect on spine survival, indicating that new spines require circadian troughs only during a critical period after their formation. New spine survival represents the number of spines formed during the 2-d training period and persisting on day 7, as a percentage of the total number of spines at baseline. $*P < 0.05$ (corrected) versus untrained control. **(c)** Manipulations of the circadian trough had corresponding effects on motor skill memory retention ($F_{3,35} = 4.24$, $P = 0.01$). Rotarod performance is depicted as a percent change in each group's baseline performance on day 1. $*P < 0.05$ (corrected) versus day 1 baseline. **(d)** Survival of spines that formed during the 2-d training period predicted retention of the learned skill on day 7 ($r = 0.89$, $P < 0.001$). Error bars, s.e.m. See **Supplementary Table 2** for statistics and details.



not known whether the circadian glucocorticoid oscillation influences spine maintenance. Learning-related new spines are initially highly unstable: most new spines will be pruned within days after their formation, but a subset will be selectively stabilized over time¹⁰, and most of those that survive will contain functional synapses^{30,31}. To investigate whether glucocorticoid oscillations affect this stabilization process in the days after learning, we trained mice during the circadian peak for 2 d, administered corticosterone injections (2.5 mg kg⁻¹ intraperitoneally (i.p.) once daily) to disrupt the circadian trough for 3 d after training and then examined spine remodeling and performance on day 7 (**Fig. 2a**). Disruption of the diurnal trough reduced the survival of learning-related new spines on day 7 (**Fig. 2b**) and impaired the rotarod performance (**Fig. 2c,d**). By contrast, corticosterone administered during the circadian peak did not affect spine retention or performance, indicating a specific function of the circadian trough beyond merely reducing cumulative exposure. We also found that the circadian trough was critical only during the first few days after training: disruption of the trough in the second week after learning did not affect the survival of new spines or performance on day 14 (**Fig. 2b–d**). These results show that a subset of newly formed spines is progressively stabilized through a process that requires intact circadian troughs. They also indicate that the impact of glucocorticoids on learning-related remodeling evolves over time: whereas elevated glucocorticoid secretion enhances spine formation immediately after training, the stabilization of these newly formed spines depends on reduced glucocorticoid secretion in the days that follow.

Previous work has shown that the stabilization of a subset of new spines is associated with the pruning of a corresponding set of pre-existing spines¹⁰. To better understand the impact of glucocorticoid oscillations on learning-induced synaptic pruning, we studied the elimination of spines that were established before training (**Fig. 3a**). As consistent with previous work¹⁰, we found that learning increased spine elimination 7 d but not 2 d after the first training session (**Fig. 3b** and **Supplementary Fig. 6**). Notably, disruption of the trough on days 4, 5 and 6 not only reduced the survival of learning-related spines (**Fig. 2b**) but also interfered with learning-induced pruning of pre-existing spines (**Fig. 3b**). Spine pruning, in turn, was highly correlated with long-term memory retention on day 7 (**Fig. 3c**; $r = 0.83$, $P < 0.001$).

Together, these results show that glucocorticoids selectively stabilize a subset of learning-related spines during the circadian trough while pruning a corresponding set of pre-existing synapses. Disruption of the circadian trough during a critical period after learning interferes with this stabilization and pruning process.

Chronic glucocorticoids cause spine loss and memory deficit

The results above delineate important roles for circadian glucocorticoid peaks and troughs in generating and preserving new spines after learning. On the one hand, transient disruption of the trough during a critical period after learning increased the elimination of new spines (**Fig. 2b**) but not pre-existing ones (**Fig. 3b**). On the other hand, long-term exposure to high levels of glucocorticoids has been linked to widespread spine loss and dendritic retraction in hippocampus and prefrontal cortex, as well as to learning and memory deficits^{3,13–18}. This raises the possibility that chronic glucocorticoid excess may disrupt both learning-related spines and pre-existing synapses, even long after learning occurs. To test this, we trained mice for 2 d and, after a week of rest, administered daily, high-dose (15 mg kg⁻¹) corticosterone injections for 10 d, beginning during the second week after training (**Fig. 4a**). Whereas transient disruption of the circadian trough with low-dose corticosterone (2.5 mg kg⁻¹ i.p.) had no effect on learning-related spines during this period (**Fig. 2b**), prolonged and excessive glucocorticoid exposure eliminated them (**Fig. 4b**) and caused corresponding deficits in memory retention (**Fig. 4c**).

Notably, chronic glucocorticoid excess also increased the elimination of spines that were present before training (**Fig. 4d**). Consequently, chronic glucocorticoid exposure caused a substantial net loss of cortical spines (**Fig. 4e**). In contrast, neither transient alterations in glucocorticoid activity nor learning caused significant, long-term changes in spine number (**Fig. 4e**). And even though new spines formed at varying rates across all other experimental groups in this study, their long-term survival was always balanced by the pruning of a small, highly correlated quantity of pre-existing connections (**Fig. 4f**; $r = 0.91$, $P < 0.001$), thereby mitigating the impact of learning on total spine number. Prolonged glucocorticoid hyperactivity—a model of chronic stress—disrupted this balance, eliminating both recently formed spines and a substantial proportion of spines formed early in development.

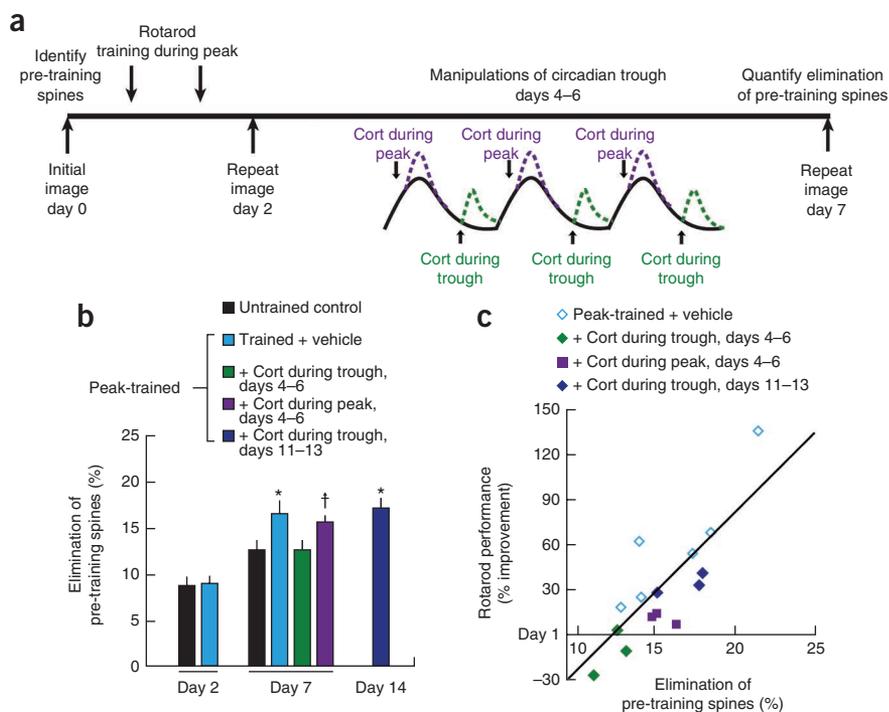


Figure 3 Disruption of glucocorticoid troughs reduces learning-dependent spine pruning.

(a) Schematic of experimental procedure. (b) Learning caused a delayed increase in the elimination of pre-existing spines, which represents the number of spines that were present before training (day 0) and eliminated on day 2 or 7, expressed as a percentage of the total number of spines at baseline. Whereas training had no immediate impact on spine elimination on day 2 ($F_{1,12} = 0.03$, $P = 0.87$), spine elimination was significantly elevated on day 7 ($F_{4,15} = 3.56$, $P = 0.03$). Learning-induced spine pruning required an intact glucocorticoid trough 4–6 d after training. * $P < 0.05$ versus untrained control after Holm-Bonferroni correction. † $P < 0.05$ uncorrected, $P < 0.10$ corrected. (c) Elimination of pre-training spines was correlated with retention of the learned motor skill on day 7 ($r = 0.83$, $P < 0.001$). Cort, corticosterone. Error bars, s.e.m. See **Supplementary Table 3** for statistics and details.

Together, these findings suggest that glucocorticoids promote learning-related spine formation rapidly through a GR-dependent, non-transcriptional pathway.

Distinct mechanisms for formation and pruning

Our findings indicate that circadian oscillations in glucocorticoid activity are important for learning-related spine formation and pruning, but the mechanisms by which glucocorticoids regulate these processes are unclear. Classically, it was thought that glucocorticoids act relatively slowly by regulating gene expression through nuclear corticosteroid receptors, but recent reports indicate that they may also act rapidly through non-transcriptional mechanisms^{23–27}. To better understand how glucocorticoids regulate spine remodeling, we began by investigating the time course of cortical spine formation and elimination hours after corticosterone exposure (15 mg kg⁻¹ i.p.). Corticosterone increased both spine formation (Fig. 5a) and elimination (Fig. 5b), but effects on formation were more rapid. Spine formation was significantly increased by 1 h after injection, whereas effects on elimination were not significant until 5 h and continued to accumulate over the course of 24 h.

The relatively rapid increase in spine formation after corticosterone exposure suggests a non-transcriptional mechanism. To test this, we measured spine turnover after direct application of corticosterone to the cortex (10 μM; see also Online Methods) and after cotreatment with actinomycin D, an inhibitor of transcription. Corticosterone rapidly enhanced spine formation, but not elimination, just 20 min after exposure (Fig. 5c and Supplementary Fig. 7), whereas actinomycin D had no effect. We observed similar effects after treatment with a membrane-impermeant glucocorticoid (corticosterone-bovine serum albumin (BSA) conjugate) that cannot access the nuclear receptors that regulate transcription (Fig. 5c). Because circadian glucocorticoid oscillations cause cycles of activation and deactivation of the type II corticosteroid receptor (glucocorticoid receptor; GR)^{1,32}, we reasoned that spine formation during the circadian peak may depend on GR activity. Indeed, we found that cotreatment with corticosterone and a GR antagonist (mifepristone) blocked the enhancement of spine formation (Fig. 5c). To evaluate the role of this GR-dependent, non-transcriptional pathway during learning, we administered mifepristone (20 mg kg⁻¹ i.p.) immediately after training during the circadian peak. Mifepristone interfered with learning-induced spine formation (Fig. 5d).

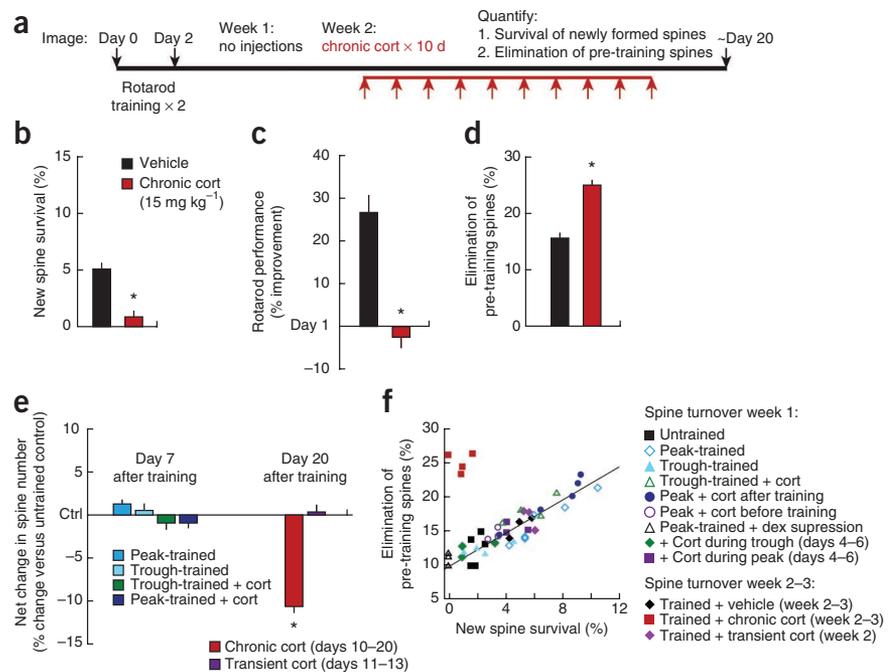
In contrast, glucocorticoid effects on spine elimination were relatively delayed and accumulated slowly over the course of 24 h (Fig. 5b), pointing to a transcriptional mechanism of action. We also found that the enhancement of spine elimination by glucocorticoids was not affected by cotreatment with mifepristone (Supplementary Fig. 8), suggesting that glucocorticoid effects on spine pruning may be mediated through the type I corticosteroid receptor (mineralocorticoid receptor; MR). To test this, we measured spine elimination rates 24 h after direct cortical application of aldosterone, a selective MR agonist, or spironolactone, a selective MR antagonist. Whereas spironolactone significantly reduced 24-h spine elimination rates, aldosterone increased spine pruning, and this effect was blocked by cotreatment with an inhibitor of transcription (actinomycin D) or translation (anisomycin; Fig. 5e). To determine whether this MR-dependent transcriptional mechanism also affects learning-related spine pruning, we used spironolactone (20 mg kg⁻¹ i.p. daily) to block MR-activity during the trough on days 4, 5 and 6 after training. We found that spironolactone caused a significant reduction in learning-related spine pruning (Fig. 5f). These findings suggest that glucocorticoids enhance learning-related spine elimination through an MR-dependent, transcriptional mechanism that is distinct from the non-transcriptional mechanism that promotes spine formation.

Corticosterone promotes spine formation via LIMK1-cofilin

Previous studies have extensively characterized the effects of glucocorticoids on gene expression linked to synaptic plasticity and loss^{19–21}. However, relatively few studies have investigated non-transcriptional mechanisms of action. To explore potential non-transcriptional pathways underlying glucocorticoid effects on spine formation, we obtained cortical biopsies 20–25 min after direct cortical application of corticosterone and examined changes in protein expression. We observed rapid increases in expression of the phosphorylated forms of LIM kinase-1 (LIMK1) and its substrate cofilin (Fig. 6a–c) that correlated with expression levels of phospho-GR, a marker of GR activity (Supplementary Fig. 9). Comparable effects occurred after coadministration of corticosterone and actinomycin D

Figure 4 Chronic glucocorticoid exposure causes spine loss and memory impairment. (a) Experimental procedure. (b) Prolonged corticosterone (cort) exposure disrupted the survival of learning-related spines that were present for at least 1 week ($F_{1,5} = 54.2$, $P < 0.001$). New spine survival represents the number of spines formed during the 2-d training period and persisting on day 20, expressed as a percentage of the total number of spines at baseline. (c) Prolonged cort exposure was associated with corresponding deficits in retention of the motor skill ($F_{1,16} = 42.2$, $P < 0.001$). (d) Elimination rates for spines present before training also increased ($F_{1,5} = 66.6$, $P < 0.001$). Elimination rates describe the number of spines that were present before training (day 0) and eliminated on day 20, expressed as a percentage of the total number of spines at baseline. (e) Chronic cort ($F_{1,5} = 125.9$, $P < 0.001$) but not transient cort or learning ($F_{4,19} = 2.07$, $P = 0.13$), caused significant spine loss. Net change in spine number represents the combined effects of elimination and formation on total spine number, relative to the small (4–5%) rate of net spine loss observed in untrained controls (ctrl). (f) The survival of new spines formed during training was strongly correlated with the elimination of pre-existing spines ($r = 0.91$, $P < 0.001$), but this balance was disrupted after chronic cort exposure (red). * $P < 0.05$ (corrected) versus vehicle-treated control. Error bars, s.e.m.

See **Supplementary Table 4** for statistics and details.



(Fig. 6b,c), indicating a non-transcriptional process. Phosphorylated cofilin stabilizes actin polymers and has been linked to spine growth, whereas the dephosphorylated form has opposing effects³³. Thus, the effect of glucocorticoids on spine formation is likely mediated through the LIMK1-cofilin pathway.

To determine whether glucocorticoids mediate this effect through mechanisms that require neuronal GR signaling, we studied protein phosphorylation in a cortical culture system, transfecting neurons with a GFP-interfering RNA construct specific for GR. Whereas corticosterone increased dendritic expression of phospho-GR, phospho-cofilin and phospho-LIMK1 in cells transfected with a scrambled control construct, there was no effect after transfection with the interfering RNA (Fig. 6d,e). This suggests that glucocorticoids modulate spine growth on cortical pyramidal cells through the LIMK1 and cofilin pathway and direct effects on neuronal GRs.

To determine whether glucocorticoid effects on spine formation require LIMK1, we tested the effects of direct cortical application of

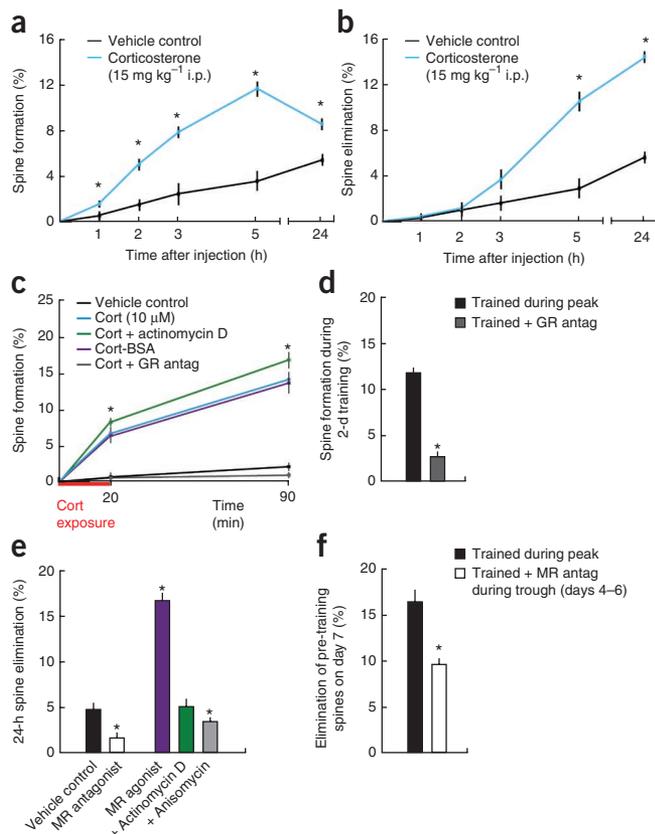


Figure 5 Glucocorticoids promote spine formation and pruning through distinct signaling pathways. (a) Corticosterone (cort) increased spine formation rapidly ($F_{1,40} = 124.0$, $P < 0.001$). (b) Cort caused a delayed increase in spine elimination ($F_{1,40} = 102.3$, $P < 0.001$). (c) Glucocorticoids promote spine formation rapidly through a GR-dependent, non-transcriptional mechanism. Direct cortical application of cort caused rapid increases in spine formation ($t = 14.6$, $P < 0.001$), with comparable effects evident after cortical application of cort plus actinomycin D ($50 \mu\text{g ml}^{-1}$; $t = 18.8$, $P < 0.001$) or a membrane-impermeant cort-BSA conjugate ($t = 4.83$, $P < 0.001$). Co-administration of the GR antagonist (antag) mifepristone ($100 \mu\text{M}$) blocked this effect. (d) Mifepristone (20 mg kg^{-1} i.p.) administered immediately after training reduced learning-dependent spine formation ($t = 8.46$, $P < 0.001$). (e) Glucocorticoids cause a delayed increase in spine elimination through a MR-dependent, transcriptional mechanism. A selective MR antagonist (spironolactone, $10 \mu\text{M}$) reduced 24-h spine elimination ($t = 6.19$, $P < 0.001$), whereas a selective MR agonist (aldosterone, $10 \mu\text{M}$) had the opposite effect ($t = 18.3$, $P < 0.001$). This effect was blocked by cotreatment with either actinomycin D or anisomycin, consistent with a transcriptional mechanism of action. (f) Spironolactone administered during the circadian trough on days 4, 5 and 6 after training interfered with learning-induced spine elimination ($t = 4.84$, $P = 0.002$). * $P < 0.05$ versus corresponding control. Error bars, s.e.m. See **Supplementary Table 5** for statistics and details.

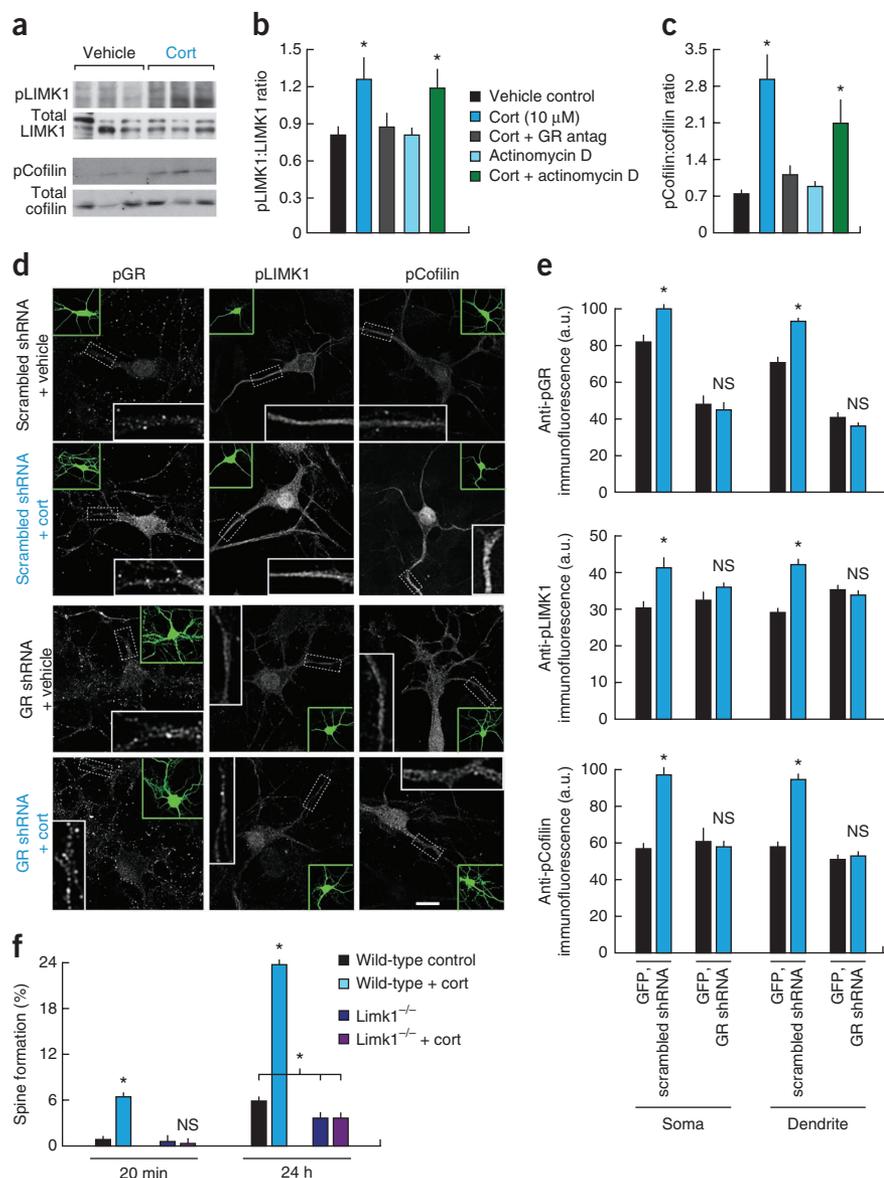
Figure 6 Glucocorticoids promote spine formation rapidly through non-transcriptional regulation of LIMK1-cofilin activity.

(a) Expression of phospho-LIMK1 (pLIMK1; upper panels) and phospho-cofilin (pCofilin; lower panels) in cortical lysates 20 min after corticosterone (cort). (b) Cort rapidly increased the expression of phospho-LIMK1 *in vivo* ($F_{4,68} = 2.74$, $P = 0.036$). This effect was blocked by mifepristone but not by actinomycin D. (c) Similar effects of cort on pCofilin ($F_{4,68} = 6.95$, $P < 0.001$). (d) Cortical pyramidal cells in culture were transfected with a construct encoding GFP plus an interfering RNA (shRNA) specific for GR (lower panels) or a scrambled control construct (upper panels). Phospho-GR (pGR; left), pLIMK1 (middle) and pCofilin (right) immunofluorescence (white) and GFP expression in the same frame (green insets), which identifies pyramidal cells, are depicted after treatment with cort (1 μ M) or vehicle for 20 min. White insets show 3 \times magnifications of boxed representative dendritic segments. Scale bar, 10 μ m. (e) Cort increased dendritic and somatic pGR, pLIMK, and pCofilin immunofluorescence after transfection with a scrambled RNA construct ($P < 0.001$) but not the GR-specific shRNA construct ($P > 0.21$). A.u., arbitrary units. (f) Direct cortical application of cort (10 μ M) increased spine formation within 20 min in wild-type (WT) controls, but not in *Limk1* knockout mice (main effect of cort: $F_{1,11} = 58.7$, $P < 0.001$; interaction between genotype and cort: $F_{1,11} = 71.1$, $P < 0.001$). Spine formation at 24 h in vehicle-treated *Limk1* knockouts was also reduced ($t = 5.34$, $P < 0.001$ versus wild-type). * $P < 0.05$ versus corresponding control; NS, not significant. Error bars, s.e.m. See **Supplementary Table 6** for statistics and details.

corticosterone (10 μ M) in LIMK1 knockout (*Limk1*^{-/-}) mice, which show abnormal spine morphology and learning impairments³⁴. *Limk1*^{-/-} mice are known as a model of Williams syndrome, a neurodevelopmental disorder characterized by learning disability and abnormal *LIMK1* expression^{34,35}. We found that spine formation was significantly reduced in *Limk1*^{-/-} mice compared to wild-type controls, and corticosterone had no effect on spine formation in *Limk1*^{-/-} (Fig. 6f). Together with the data showing the efficacy of a membrane-impermeant glucocorticoid (corticosterone-BSA) and the lack of an actinomycin D effect, these results show that the rapid effect of glucocorticoids on spine formation occurs through a non-transcriptional pathway that requires LIMK1 activity.

DISCUSSION

Glucocorticoid secretion varies phasically with stressful environmental triggers and tonically with the circadian rhythm^{1,2}. It is well established that excessive glucocorticoid exposure causes dendritic atrophy and spine loss, with complex effects on learning and memory^{3-7,14-18}. Here we have identified new functions for circadian glucocorticoid oscillations in forming and consolidating stable structural correlates of learning experiences. Specifically, our findings indicate that circadian glucocorticoid peaks are important for forming new spines after learning, whereas troughs are required for stabilizing a subset of new



spines during a critical period after their formation. On the one hand, learning-induced spine remodeling and memory are enhanced when learning occurs during the circadian peak or coincides with elevated glucocorticoid secretion and when subsequent glucocorticoid troughs remain intact. On the other hand, disruption of the glucocorticoid trough reduces the survival of learning-associated new spines and impairs memory retention, and prolonged, excessive exposure leads to a substantial loss of spines formed early in life. These findings suggest that loss of the glucocorticoid oscillation may contribute to learning and other cognitive deficits in neuropsychiatric disease and chronic stress states^{1,2,36}. Furthermore, alterations of the glucocorticoid milieu may contribute to pathophysiology in many immunological and neurological diseases, for which long-term maintenance on synthetic glucocorticoids and related receptor modulators are often mainstays of treatment.

Our findings also underscore the importance of the timing of training relative to the endogenous glucocorticoid rhythm for optimal synaptic remodeling and maintenance. This effect may account in part for the observation that motor skill learning in humans varies with the time of day^{37,38}. Studies in both rodents and humans have

identified circadian variation in other forms of cognition, including working memory, associative learning, declarative memory and fear conditioning^{39–41}. Future work will be required to determine whether and how circadian glucocorticoid oscillations regulate spine remodeling in these contexts.

Our results define a new, non-transcriptional pathway by which glucocorticoids promote spine formation after learning. Specifically, they indicate that GR-dependent, non-transcriptional regulation of LIMK1 and cofilin is critical in generating new spines. These signaling mechanisms may generate new spines through direct effects on the dendritic cytoskeleton, by modulating neuronal network activity, or by some combination of the two. Previous studies indicate that spine formation can occur within minutes in brain slice cultures⁴². Thus, the non-transcriptional mechanisms described here could facilitate the process of new spine formation after learning. In addition, deficits in linking this pathway to experience-dependent plasticity may contribute to spine abnormalities and learning disability in Williams syndrome, a genetic neurodevelopmental disorder caused by the deletion of LIMK1 and other genes on the long arm of chromosome 7 (refs. 34,35). The LIMK1 and cofilin signaling pathway may likewise contribute to cortical spine abnormalities in stress-related neuropsychiatric diseases. Future imaging studies in awake animals will be required to characterize the time course of spine formation minutes to hours after training, to better understand the action of this non-transcriptional pathway in learning-dependent spine formation under normal and disease conditions.

Our results also show that glucocorticoids promote spine elimination through MR-dependent transcriptional mechanisms, which regulate the expression of dozens of genes linked to synaptic plasticity²⁰. On the one hand, spine pruning may serve to compensate for the addition of new synapses after training. On the other hand, it may also contribute actively to learning by fine-tuning connections in neuronal circuits, as has been demonstrated in the developing nervous system and in other forms of behavioral learning^{12,43,44}. Future studies are needed to elucidate the function of spine elimination in learning and the involvement of other mechanisms in this process. For example, histone deacetylases are sensitive to stress^{45,46} and are established regulators of synapse number⁴⁷. They may influence synapse loss in part through interactions with mitogen enhancer factor 2, leading to transcription of *Arc*, *Syngap1* and other genes implicated in synaptic plasticity^{48,49}. Furthermore, glucocorticoid secretion exhibits ultradian oscillations that are superimposed on the circadian rhythm, leading to high-frequency fluctuations in transcription that may interact with circadian rhythms to regulate both spine formation and elimination^{21,50}. Amid these complexities, our findings highlight a central, glucocorticoid-regulated pathway that is important for remodeling excitatory synapses in the cortex after learning. Future use of genetic approaches to activate and inactivate MR and GR pathways rapidly in specific cell types will aid in further dissecting the underlying mechanisms.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Supplementary information is available in the [online version of the paper](#).

ACKNOWLEDGMENTS

We thank B.S. McEwen and Gan laboratory members for critical comments on this manuscript. This work was supported by grants from the US National Institutes of Health (R01 NS047325 and a subcontract of R01MH085324) to W.-B.G. C.L. is supported by grants from the US National Institute of Mental

Health (K99 MH097822) and the DeWitt Wallace Reader's Digest Foundation at Weill Cornell Medical College. F.J. and M.V.C. are supported by grants from the US National Institutes of Health (MH086651 and NS21072).

AUTHOR CONTRIBUTIONS

C.L. and W.-B.G. conceived the project and designed all experiments. C.L. collected, quantified and analyzed the *in vivo* spine remodeling data. J.M.C. and F.J. contributed to the design of the cortical culture experiments, and J.M.C. carried them out and analyzed the results. C.L. obtained cortical biopsies and plasma samples. F.J. and M.V.C. contributed to the biochemical analysis of the samples. Z.J. developed and shared the LIMK1 knockout mouse. C.L. and W.-B.G. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Experimental animals. Mice expressing YFP in pyramidal cells predominantly in cortical layer V⁵¹ were purchased from Jackson Laboratory (YFP-H line) and group housed (3–5 mice per cage) with a 12-h light:dark cycle (lights on 7 a.m., lights off 7 p.m.). Some experiments also used LIMK1 knockout mice³⁴. Both male and female mice at 1 month of age were used in most experiments unless noted otherwise. They were afforded *ad libitum* access to food and water for all experiments. All experimental protocols were conducted in accordance with guidelines established by the Institutional Animal Care and Use Committee at the New York University School of Medicine.

Surgery and *in vivo* transcranial two-photon imaging. This procedure has been described in detail elsewhere⁵². In brief, surgical anesthesia was achieved using an intraperitoneal injection of ketamine and xylazine. A midline incision of the scalp exposed the periosteum, which was manually removed with a microsurgical blade. The area to be imaged was identified in terms of stereotaxic coordinates: 1.3 mm anterior to the bregma, 1.2 mm lateral from the midline for the forelimb area of motor cortex. These coordinates were confirmed to lie within the forelimb area of motor cortex in previous reports^{10,53}. The head was then immobilized, and a high-speed micro-drill and microsurgical blade were used to thin a circular area of skull over the area of interest to a thickness of approximately 20 μm . This was performed under a dissection microscope. Image stacks of dendritic segments projecting to superficial cortical layers were obtained using a two-photon laser scanning microscope tuned to 920 nm (10–30 mW measured at the sample) and an Olympus 60 \times (NA 1.1) objective immersed in artificial cerebrospinal fluid. High-magnification imaging (66.7 $\mu\text{m} \times 66.7 \mu\text{m}$; 512 \times 512 pixels; 0.75 μm step; ~6- μs pixel dwell time) was performed using a 3 \times digital zoom to generate images suitable for quantification of dendritic spines. Repeated time-lapse imaging over minutes to hours was performed in a single imaging session. For repeated imaging over intervals of days to weeks, the procedure above was repeated, and the region to be imaged was identified by referring to vascular landmarks in light photomicrographs of the thinned area of skull. Previous studies have shown that, in motor cortex, YFP is expressed almost exclusively in layer V pyramidal cells in this transgenic line (YFP-H)⁵¹.

Manipulations of glucocorticoid activity. All of the reagents described below were obtained from Sigma Aldrich. Corticosterone and various synthetic glucocorticoid receptor modulators were injected intraperitoneally in a DMSO vehicle at doses described in the text and in **Supplementary Tables 1–6** below. Vehicle-treated control animals were injected with DMSO alone. The timing of the injections varied by experiment and is described in the main text. Corticosterone was injected at a dose of 2.5 mg kg⁻¹ to achieve a plasma level comparable to that observed after an acute stressor or after handling during the circadian peak. The validity of this manipulation was confirmed by ELISA (**Fig. 1b**; see below). Corticosterone does not penetrate the blood-brain barrier at this concentration (stress level), and doses in this range have been found to influence memory⁵⁴.

Corticosterone at physiological concentrations binds two receptors: the type I (MR) and type II (GR) corticosteroid receptors. To dissociate these effects, spironolactone (20 mg kg⁻¹ i.p.) and mifepristone (20 mg kg⁻¹ i.p., formerly known as RU-486) were used to selectively antagonize MR and GR, respectively, with doses selected on the basis of previously published reports. We also tested the effects of suppressing endogenous glucocorticoid secretion using injections of low-dose dexamethasone (0.1 mg kg⁻¹). Dexamethasone is a synthetic glucocorticoid that does not penetrate the blood-brain barrier at this dose but suppresses corticosterone secretion through negative feedback effects on the anterior pituitary gland⁵⁵.

Experiments depicted in **Figures 5c,e** and **6a–c,f** were designed to test the mechanisms by which glucocorticoids stimulate cortical spine formation after direct cortical application of corticosterone through a small craniotomy. Here, imaging was performed through a thinned but intact area of skull as described above. A small craniotomy, ~500 μm in diameter, was performed on an adjacent area of skull, 2–4 mm from the imaged area. The imaged area was left intact. Corticosterone was administered by bathing the craniotomy in approximately 200 μl of a 10 μM solution of corticosterone in ACSF for 20 min. After 20 min, the corticosterone solution was removed and the craniotomy was rinsed three times in ACSF. The same procedure was used to apply the following solutions

in other experiments in the same figure panels: actinomycin D, 50 $\mu\text{g ml}^{-1}$; mifepristone, 100 μM ; corticosterone-BSA, 10 μM ; spironolactone, 100 μM ; aldosterone, 10 μM ; anisomycin, 50 $\mu\text{g ml}^{-1}$. Because small molecules diffuse rapidly in the cortex, we estimated that the effective cortical concentration was at least 10 times lower than in the solution applied to the craniotomy⁵⁶, such that the effective cortical concentration of these drugs would be comparable to those used in prior studies⁵⁷. In all cases, the mice appeared healthy, ambulating and feeding normally on recovery from the anesthetic and free of any overt signs of distress or discomfort. There were no apparent differences between vehicle-treated controls and drug-treated animals in general health or appearance.

Habituation procedure. Prior to the training procedure described below, mice were habituated to handling and to the Rotarod apparatus^{10,58} (EZRod System, Accuscan Instruments) for 3 d to minimize the effects of training on the diurnal corticosterone rhythm. The habituation procedure involved 15 trials on a rod that rotated slowly at a constant speed of 15 r.p.m. Each trial ended after falling from the rod or after 30 s and was followed by a 30-s rest, such that each day involved about 15 min of habituation. To validate the habituation procedure, we obtained blood samples 30–45 min after the circadian trough (~8 a.m.) or the circadian peak (~8 p.m.), from mice that were untrained, trained but not habituated, or trained after 3 d of habituation (**Supplementary Fig. 1**; see below for plasma corticosterone quantification).

Rotarod training procedure. Training sessions occurred in the same Rotarod apparatus but on an accelerating rod that increased steadily in speed from 0 to 100 r.p.m. over the course of 2 min (refs. 10,58). Each trial ended after the mouse fell from the rod or after 120 s. Each session included 15 trials. Mice were trained for two sessions during the circadian corticosterone peak or trough (approximately 1 h after lights off or on, respectively), as described in the text. The latency to falling off the rod and maximum speed achieved were recorded for each trial, with the latter serving as a measure of performance. Between-session learning was quantified in terms of the percentage change in mean performance from day 1 to day 2 (**Supplementary Fig. 3**). Retention of the learned motor skill was measured in a third session occurring on day 7 (or later in some cases, as noted in the text). To control for effects of time of day on recall, subjects in each group were initially tested in the morning or in the evening on day 7. There were no time-of-day effects on performance on day 7 ($F_{1,60} = 0.17$, $P = 0.69$) and no interactions between time of day and training condition ($F_{1,60} = 1.25$, $P = 0.27$; **Supplementary Fig. 4**).

Plasma corticosterone assay. To confirm the validity of our manipulations of the circadian glucocorticoid rhythm, plasma corticosterone was quantified using a commercially available ELISA kit (AssayMax Corticosterone ELISA Kit, AssayPro). Plasma corticosterone was measured from trunk blood collected from separate cohorts of mice that were trained (but not imaged) approximately 1 h after lights off (circadian peak, ~8 p.m.) or approximately 1 h after lights on (circadian trough, ~8 a.m.), with i.p. injections of corticosterone or vehicle immediately after training. Blood samples were collected ~45 min after training to provide time for peritoneal absorption of the hormone⁴. Circadian variations and effects of exogenous corticosterone are depicted in **Figure 1b**. Effects of training and habituation on these circadian oscillations are depicted in **Supplementary Figure 1**.

Characterization of corticosterone effects on phosphorylation of GR, LIMK1 and cofilin. Corticosterone (10 μM) with or without mifepristone (100 μM) or vehicle (DMSO) was administered directly to the cortex *in vivo* through a 2- to 3-mm craniotomy as described above. Twenty to 25 min later, cortical tissue underlying the craniotomy was biopsied by careful dissection using a microsurgical blade and forceps, placed immediately on dry ice and stored at -80°C . Specimens were processed as described elsewhere⁵⁹. Phosphorylated and total GR, LIMK1 and cofilin were quantified in cortical lysates using the following antibodies: phospho-GR, Ser211-P, ref. 57 (1:1,000); total GR, M18, Santa Cruz (1:400); phospho-cofilin, Ser3-P, ab12866, Abcam (1:1,000); total cofilin, ab54532, Abcam (1:1,000); phospho-LIMK1/2, T508/505-P, Bioworld (1:500); total LIMK, ab55414, Abcam (1:500); HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies, Jackson ImmunoResearch (1:10,000).

Cortical co-culture experiments. Cortical tissue was dissected from postnatal day 1 mouse embryos (P1) of either sex and dissociated in 0.05% trypsin-EDTA solution. After enzyme treatment, tissue blocks were triturated gently by using a fire-polished Pasteur pipette. After centrifuging (900 r.p.m. for 5 min) and discarding the supernatant to remove the debris of dead cells or connective tissues, we resuspended the cells and plated them at low density on coverslips coated with poly-D-lysine (1 mg ml⁻¹) covered by a monolayer of astrocytes. The culture medium contained 500 ml MEM (Invitrogen, Eugene, OR), 5% FBS (HyClone, Logan, UT), 10 ml B-27 supplement (Invitrogen), 100 mg NaHCO₃, 20 mM D-glucose, 0.5 mM L-glutamine and 25 U ml⁻¹ penicillin/streptomycin. Cells were maintained in a 5% CO₂/95% air incubator for up to 3 weeks. 5-Fluorouracil (5 μM) was added to cultures after 2–3 d of plating to inhibit the proliferation of astrocytes.

Transfections. Cortical cultures were transfected with a GR-specific GFP-shRNA or a scrambled control construct⁵⁷. Calcium phosphate transfection was performed as described elsewhere⁶⁰. Transfection efficiency was optimized through short incubation of neurons with calcium phosphate/DNA mixture (25 min) and a washing phase with 10% CO₂ pre-equilibrated transfection medium.

Immunostaining. Cells were rinsed with PBS twice and fixed for 15 min in a solution of 4% paraformaldehyde, pH 7.4. Coverslips were then rinsed three times in PBS and permeabilized with 0.1% Triton X-100 in PBS for 3 min. Permeabilization solution was removed and washed three times with PBS. Primary antibodies were added together with a blocking solution (10% goat serum, 2% BSA, 0.25% fish skin gelatin, pH 7.4) in PBS and set to incubate overnight (12–15 h) at 4 °C. The following day, coverslips were rinsed three times in PBS for 15 min. Subsequently, samples were incubated for 30 min at ~20 °C in anti-rabbit Alexa 647-conjugated secondary antibodies (Invitrogen). Phospho-GR was immunostained with rabbit polyclonal antibody (M. Garabedian, New York University School of Medicine, 1:1,000). Phospho-LIMK was immunostained with rabbit polyclonal antibody (1:200, Bioworld, St. Louis Park, MN). Phospho-cofilin was immunostained with rabbit polyclonal antibody (1:500, Abcam, Cambridge, MA). Coverslips were then rinsed three times in PBS for 15 min and mounted with 50% glycerol, 50% 0.1 M NaHCO₃, pH 7.4 on glass slides.

Quantification. Confocal images of cultured neurons were obtained at 60× with digital zoom (2.0–2.4×). Immunofluorescence was quantified for phospho-GR, phospho-LIMK1 and phospho-cofilin in regions of interest (ROIs) using the ImageJ software package. ROIs were drawn manually around cell somas after selecting the section from a confocal z-stack that best encompassed the plane of each cell. Regions of interest for dendrites were drawn manually along small segments (~10–20 μm) showing clear continuity with the cell body.

Data analysis. The procedure for quantifying spine dynamics has been described elsewhere⁵². In brief, image stacks were analyzed using the freely available ImageJ

software (<http://rsbweb.nih.gov/ij/>). For each dendritic segment analyzed, filopodia were identified as long, thin protrusions with ratio of length to neck diameter >3:1. Corticosterone effects on filopodia are depicted in **Supplementary Figure 2**. The remaining protrusions were classified as spines. Raters blinded to experimental condition compared pairs of images of the same dendritic segment and identified stable spines (present in image 1 and 2), eliminated spines (present in image 1 but not in image 2) and formed spines (present in image 2 but not in image 1). In some experiments, the survival of newly formed spines was characterized by examining an image obtained after the pair in question. Glucocorticoid effects on spine elimination rates, formation rates, survival rates and Rotarod performance (see above) were tested using one- or two-way ANOVA as specified in the text. For each analysis, we used Levene's statistic to test for homogeneity of group variances and either Student's *t*-tests or Welch's (unequal variance) *t*-tests, as indicated, to assess the significance of differences between each glucocorticoid-treated condition and a vehicle-treated control condition. All contrasts were specified a priori. For analyses involving more than two contrasts with a control, we used a Holm-Bonferroni correction for multiple comparisons. See **Supplementary Tables 1–6** for statistical details. All analyses were performed using the SPSS statistical software package.

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