Selective plasticity of callosal neurons in the adult contralesional cortex following murine traumatic brain injury

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Traumatic brain injury (TBI) results in deficits that are often followed by recovery. The contralesional cortex can contribute to this process but how distinct contralesional neurons and circuits respond to injury remains to be determined. To unravel adaptations in the contralesional cortex, we used chronic in vivo two-photon imaging. We observed a general decrease in spine density with concomitant changes in spine dynamics over time. With retrograde co-labeling techniques, we showed that callosal neurons are uniquely affected by and responsive to TBI. To elucidate circuit connectivity, we used monosynaptic rabies tracing, clearing techniques and histology. We demonstrate that contralesional callosal neurons adapt their input circuitry by strengthening ipsilateral connections from pre-connected areas. Finally, functional in vivo two-photon imaging demonstrates that the restoration of pre-synaptic circuitry parallels the restoration of callosal activity patterns. Taken together our study thus delineates how callosal neurons structurally and functionally adapt following a contralateral murine TBI.
M oderate traumatic brain injury (TBI) is often followed by regional neuronal cell loss and disruption of neuronal circuits that result in behavioral and cognitive deficits. Both in humans and in animal models, the functional deficits can be followed by spontaneous recovery, suggesting that plasticity of the neuronal circuits disrupted by the injury can compensate for the lost function. One region that has been implicated in the recovery process following traumatic or ischemic cortical lesions, is the homotopic contralesional cortex. Both clinical and experimental studies point to plastic reorganization and changes in neuronal activity levels in the contralesional cortex. Interestingly, several reports indicate different anatomical sites of plasticity including the formation of new axonal projections from the cortex to neighboring areas or to subcortical areas. While these observations suggest that the contralesional cortex and its efferent fibers can in principle contribute to recovery or to the emergence of compensatory behavioral strategies, it is worth noting that in some settings plasticity and increased activity in the contralesional cortex could also be detrimental and increase injury-mediated deficits. These findings indicate that a more refined understanding of the cortical response pattern and in particular of the specific neuronal populations and circuits that adapt to a contralateral lesion is needed.

Recently, it was reported that anatomically connected areas undergo microstructural changes in response to cortical ischemic injuries and that contralesional cortical areas take over the lost function following amputation via adaptive remodeling of callosal inputs. It is thus tempting to speculate that neuronal networks connecting the two hemispheres such as transcallosal connections could play a critical role in the response to cortical injury. This is important as, while some higher order information processing, such as attention and language, is lateralized to a region in one hemisphere, correlation of activity between homotopic regions of the two hemispheres is important for sensorimotor processing and hence recovery. Thus in this study, we used structural and functional in vivo imaging, selective labeling of neuronal subpopulations as well as circuit mono-synaptic tracing techniques to monitor the circuit rearrangements that take place in the contralesional cortex following TBI. Here, we reveal the selective structural and functional adaptation of callosal neurons and their input circuits to a contralateral brain injury.

Results
In vivo two-photon imaging reveals an early general loss of spines and changes in spine dynamics of contralesional cortical neurons following TBI. We induced TBI unilaterally in the sensorimotor cortex of mice using a controlled cortical impactor (TBI-0310 Impactor, precision Systems and Instrumentation, LLC; Fig. 1a) which produced hemispherical lesions that spanned the ipsilateral primary somatosensory cortex (mean lesion volume \(7.063 \pm 0.603 \text{ mm}^3, n = 4\); Supplementary Fig. 1a) and produced righting reflex and apnea recovery consistent with a moderate injury (Supplementary Fig. 1b). We first investigated the sensorimotor changes triggered by this brain injury over time using the irregular ladder rung, which allows us to appreciate skilled walking while minimizing the ability of the mice to compensate for impairments through learning. In this test, we observed that acutely following TBI, there is a marked increase in the number of mistakes made by the animals which then recovers over the following weeks (up to day 21 after TBI; Fig. 1b; *** \(p = 0.0001\) 3d vs Baseline; * \(p = 0.0481\) 7d vs Baseline; ** \(p = 0.0044\) at 14d, 21d vs 3d; Friedman test followed by uncorrected Dunn’s test). This established that, in our model, remodeling processes in the first few weeks after injury are likely critical for functional recovery. Such remodeling processes are likely to take place throughout the CNS and include corticospinal and intraspinal rewiring. Here we wanted to focus on changes that occur in the contralesional cortex so we first examined whether there were apparent changes to the cortical microanatomy. Our results indicated, however, that cortical thickness, neuronal density and soma size contralateral to the lesion were not different between control and injured mice at any time points investigated (Supplementary Fig. 2). As contralesional cortical neurons do neither die nor suffer from atrophy following TBI, we concentrated on analyzing changes to the morphology of individual neurons and in particular their dendritic spines. To do so we used GFP-M mice, which sparsely express EGFP in a subset of pyramidal neurons mainly in layer V of the cortex, and performed in vivo two photon imaging of the contralesional sensorimotor cortex before (baselines 1 and 2) and after injury (Fig. 1c). We were able to image and follow 81 dendritic stretches from 4 animals (Fig. 1d) and a total of 10,191 spines throughout the experiment. With two-photon imaging we could show that TBI induced a strong and significant reduction of the spine density that can be detected as early as 3 days following TBI and persisted throughout the study period (Fig. 1c; *** \(p = 0.0001\) B1 vs 3dpi and ** \(p < 0.0001\) B1 vs all other time points; one-way repeated measure (RM) Anova followed by Dunnett’s test). To assess whether the reduction in spine density was due to a pronounced loss of spines or a lack of newly formed spines, we then followed the fate of the spines and determined the proportion of stable spines, lost spines or newly formed spines (Fig. 1f and Supplementary Fig. 3). We found that while the rate of stable spines remained around 80% (Supplementary Fig. 3) during the entire study period, the rate of formation and elimination of spines fluctuated over time following injury (Fig. 1f). In particular, the elimination rate of spines was significantly increased compared to the formation rate at 3d post-injury (*) \(p = 0.0146\) Two-way RM Anova and Tukey test) while the formation rate was significantly increased compared to the elimination rate between days 12 and 18 (\(p = 0.0287\) 12–15d and \(p = 0.0146\) 15–18d Two-way RM Anova and Tukey test). This argues that adaptive structural plasticity can restore spine density and prompted us to perform additional experiments to determine the neuronal populations involved in these adaptations and the specific time course of these changes following TBI.

The decrease in spine density is specific to callosal neurons. To determine whether changes in dendritic spine density can be found in all contralesional neurons or are specific to a given population of neurons, we used GFP-M mice and used retrograde tracing with fluorogold. We injected fluorogold into the ipsilesional area prior to TBI to retrogradely label and analyze spine density of callosal neurons located homotopically in the contralesional hemisphere (Fig. 2a). Non-callosal neurons (not retrogradely-labeled with fluorogold but whose location was overlapping with the retrogradely-labeled callosal neurons in the contralesional hemisphere) were investigated as controls. We focused on layer II/III and layer V neurons and analyzed spine density in the proximal dendrite, distal dendrite (only for layer V neurons) and apical tufts. We observed that following brain injury dendritic spine density in the apical tufts of callosal neurons is decreased at 7 and 14d (layer II/III 7d \(p = 0.0124\), 14d \(p = 0.0303\); layer V 14d \(p = 0.0329\) One-Way Anova and Dunnett’s test) and subsequently recovers to control values by 42d (Fig. 2b–d; left panel). In contrast, dendritic spine density of the apical tufts of non-callosal layer II/III and layer V neurons remained unchanged over the study period (Fig. 2e, f, left panels). We then investigated changes in spine density of proximal
dendrites of callosal and non-callosal neurons (Fig. 2b–d, right panels and Fig. 2e,f right panels). We found a decrease in spine density at 14d in layer II/III callosal neurons (p = 0.0288) and a decrease in spine density at 7d (p = 0.0286) and 14d (p = 0.0288) in layer V callosal neurons while no such changes were observed in layer II/III and layer V non-callosal neurons (One-Way Anova and Dunnett’s test for all comparisons). Finally we examined the spine density of distal dendritic segments of layer V callosal neurons (Fig. 2d, middle panel) and could observe a significant albeit transient decrease in spine density at 7d post-injury (p = 0.0341 One-Way Anova and Dunnett’s test). In contrast, similarly to the apical tufts, no changes in spine density could be seen on the distal compartment of the dendritic trunk of layer V non-callosal neurons (Fig. 2f, middle panel). To confirm that the
observed changes are indeed selective for neurons that project to the lesioned hemisphere. We also examined corticospinal tract (CST) neurons, which were retrogradely labeled from the spinal cord. Here, no differences in spine density could be seen in CST neurons before and 7d after injury (proximal: 2.11 ± 0.22 vs 1.95 ± 0.12; distal: CST 2.1 ± 0.14 vs 2.19 ± 0.12; apical: CST 1.00 ± 0.07 vs 0.96 ± 0.07/μm) or between non-callosal and corticospinal neurons before and 7d after injury (proximal control: CST 2.11 ± 0.22 vs NC 2.28 ± 0.23; distal control: CST 2.1 ± 0.14 vs NC 1.75 ± 0.15; apical control: CST 1.00 ± 0.07 vs NC 0.94 ± 0.09; proximal 7d: CST 1.95 ± 0.12 vs NC 1.86 ± 0.10; distal 7d: CST 2.19 ± 0.12 vs NC 2.02 ± 0.11; apical 7d: CST 0.96 ± 0.07 vs NC 1.06 ± 0.08/μm). Finally, we investigated how the balance of excitatory and inhibitory inputs onto callosal...
Fig. 2 The decrease in spine density is specific to contralesional neurons directly connected to the lesion site (callosal neurons). a Scheme of the experimental paradigm to visualize the confo-cal ex vivo imaging (red: retrogradely-labelled neurons; green: GFP positive neurons). b Confocal images of a layer V cortical neuron (left) and magnification of the dendritic parts boxed in the left images (right) and representative example illustrating the spine quantification using Neuronstudio. Scale bars: 150 μm (left panel), 5 μm (top panels and bottom right panel) and 10 μm (bottom middle panels). c Scheme of layer II/III callosal cortical neuron and dendritic spine density (mean ± SEM) of layer II/III callosal population in the apical tuft (left) and proximal dendrite (right) in control (white column) and injured mice (red column) at several time points following injury. n = 6–9 independent dendritic stretches from 4 to 5 mice for Apical and n = 6–9 independent dendritic stretches from 4 to 5 mice for Proximal. Apical: p = 0.0124 and p = 0.0303 Ctrl vs 7d and Ctrl vs 14d respectively one-way Anova and Dunnett’s test. Proximal: p = 0.0288 Ctrl vs 14d one-way Anova and Dunnett’s test. d Scheme of layer V callosal cortical neuron and dendritic spine density (mean ± SEM) of layer V callosal population in the apical tuft (left), distal dendrite (middle) and proximal dendrite (right) in control (white column) and injured mice (green column) at several time points following injury. n = 5–6 independent dendritic stretches from 4 to 5 mice for Apical and n = 5–7 independent dendritic stretches from 4 to 5 mice for Proximal. e Scheme of layer V non callosal cortical neuron and dendritic spine density (mean ± SEM) of layer V non callosal population in the apical tuft (left) and proximal dendrite (right) in control (white column) and injured mice (green column) at several time points following injury. n = 10–17 independent dendritic stretches from 4 to 5 mice for Apical, n = 11–17 independent dendritic stretches from 4 to 5 mice for Proximal, n = 9–12 independent dendritic stretches from 4 to 5 mice for Control. f Quantification (mean ± SEM) of the density of excitatory and inhibitory spine density following TBI (p = 0.0001 Mann–Whitney test, Fig. 2g, h).

The decrease in spine density is accompanied by alterations of spine morphology. To further characterize the changes triggered by TBI to contralesional dendritic spines we then characterized the spine morphology as an indicator of the degree of spine maturation. We divided spines into three types: mushroom, thin and stubby spines (Fig. 3a) with mushroom spines generally considered as the most stable and mature structures. We investigated spine morphology in the apical tuft of control and injured contralesional callosal layer II/III and layer V neurons in GFP-M mice. We found that the proportion of the more stable mushroom spines decreased acutely following the injury (layer II/III: p = 0.0040 at 7d, p = 0.0102 at 14d; layer V: p = 0.0271 at 7d and p = 0.0015 at 14d; One-Way Anova and Dunnett’s test) and partially recovered by 42d in the apical tuft of contralesional callosal neurons. In parallel, the proportion of stubby spines transiently increased at 14d in the apical tufts of layer V neurons (p = 0.0026; One-Way Anova and Dunnett’s test) before returning to baseline levels at 42d (Fig. 3b,c) arguing for a shortening of the neck. The proportion of thin spines did not change overtime following TBI (Fig. 3b, c). We then investigated spine morphology in the distal and proximal dendrites of callosal neurons in GFP-M mice. Overall, we did not find any changes in the proportion of mushroom, thin and stubby spines following injury in the distal or proximal dendrites of layer II/III and layer V contralesional callosal neurons (Fig. 3b, c). In contrast to callosal neurons, non-callosal neurons do not demonstrate any changes of spine morphology following TBI even on apical tufts (Fig. 3d, e). To complement our spine analysis of contralesional callosal neurons, we also investigated the response of the callosal axons to injury. To do so, we quantified axons branches of callosal neurons as an indicator of the axonal response to TBI. Both at acute (7d) and chronic (42d) time points, we could see no differences to control animals (0.34 ± 0.05au in control animals vs 0.28 ± 0.04au 7d post-injury vs 0.35 ± 0.05au 42d post-injury). This suggests that the response of contralesional callosal neurons primarily involves dendritic remodeling with an initial loss and later recovery of pre-synaptic input to those neurons.

Newly formed spines on callosal neurons after TBI are more stable that those that form on non callosal neurons. As the spine density of callosal neurons recovers at later timepoints, we wanted to investigate the contribution of spines that form newly after injury to this process. To do so we used two-photon in vivo imaging to track the fate of the newly formed spines of callosal neurons as well as of corticospinal projection neurons (CST neurons), which are located in a similar anatomical location but do not project to the injured cortex. To selectively visualize these neuronal populations, we injected a retrograde adeno-associated virus expressing EGFP (retroAAV-EGFP) either in the ipsilesional cortex (to label callosal neurons, Fig. 4a–c) or in the spinal cord (to label CST neurons, Fig. 4d–f). Using this approach we could follow 23 apical dendrite stretches (8724 spines followed at each time points) for callosal neurons and 13 apical dendrite stretches (4376 spines followed at each time points) for CST neurons. We then analyzed the persistence of pre-existing (spines present before the injury) and newly formed spines and confirmed that spines that already exist (both on callosal and CST neurons) before the injury are significantly more stable than newly formed spines (p < 0.0001; Mann–Whitney test, Fig. 4g). When we specifically compared the stability of the spines that form after injury on callosal neurons to non callosal neurons (CST neurons) we found that the persistence index was significantly higher for newly formed spines on callosal neurons (p < 0.0001, Mann–Whitney test, Fig. 4g). This indicates that an increased survival of newly formed spines on callosal neurons can contribute to the recovery of spine density after injury and...
underlines the functional significance of the associated rewiring of the callosal input circuitry.

Callosal neurons adapt their input circuitry by re-establishing ipsilateral connections from pre-connected areas following TBI. The observation that callosal neurons can remodel their input connections prompted us to study how the input circuitry of callosal neurons evolves after TBI. To answer this question, we first implemented retrograde mono-synaptic tracing using rabies virus\(^{40,41}\) (Fig. 5a) combined with tissue clearing\(^{42}\) (Fig. 5b).

We first verified that the mono-synaptic tracing was specific and does not show any leakage by injecting either the rabies virus in absence of the complementing G and EnvA proteins as well as the cre recombinase or either injecting the AAV expressing the envA and G proteins (AAV1-synP-DIO-sTpEpB-eGFP) without rabies virus. Trans-synaptic retrograde tracing and tissue clearing allowed us to identify the general location of presynaptic inputs of callosal neurons before and following injury (Fig. 5c). This approach allowed us to determine that the major presynaptic input of contralesional callosal neurons is located in the
contrallesional somatosensory cortex and showed that presynaptic cortical areas that are strongly connected were largely similar between control and TBI mice, with only subtle changes (Supplementary movie and Fig. 5c). As only presynaptic cells could be visualized and starter cells could not be detected using clearing techniques, we complemented this analysis using conventional immunohistological approaches. We sectioned, stained and analyzed the entire mouse brain and obtained connectivity ratios by analyzing the counted number of presynaptic cells to the normalizing the counted number of presynaptic cells to the number of starter cells (Fig. 5d, e). Starter neurons (mCherry + / GFP +) were located exclusively within the injected area in the somatosensory cortex (Fig. 5e) and were surrounded by many mCherry + only, monosynaptic input neurons (Fig. 5e). We analyzed more than 20 brain regions and identified the areas that provide input to callosal neurons in controls, in both ipsilesional and contrallesional cortices (Fig. 5f), with the somatosensory cortex exhibiting the highest connectivity ratio. Other cortical inter-areal connectivity included afferents from the motor cortex, from the auditory area, the retrosplenial area or the posterior parietal association area, among others (Fig. 5f). Subcortical inter-areal connectivity included mostly the thalamus and at much lower ratios the hypothalamus and caudate putamen (Fig. 5f). Importantly, all of these regions have been found to project to the somatosensory cortex before and we detected no aberrant input.

Fig. 4 Differential stability of newly generated spines formed on callosal and non callosal neurons following traumatic brain injury. a Scheme of the experimental design with retroAAV injected in the lesion area and timeline of the two-photon in vivo imaging. b Confocal images of retrogradely labeled callosal neurons in the cortex (Green: retroAAV-EGFP; red: NT435). Insets are four times magnified (top raw overlay; middle raw: retroAAV-EGFP; bottom raw: NT435). Scale bar equals 50 µm. c Representative timelapse series of an apical dendrite from a callosal neuron retrogradely-labeled with retroAAV-EGFP. Each image shows the same dendrite stretch at a specific experimental time point before and up to 42d post-injury (23 independent dentritic stretches and 8724 spines were followed). Cyan arrowheads indicate disappearing pre-existing spines. Gray arrowheads indicate disappearing newly-formed spines. Green dot indicate stable pre-existing spines. Magenta dots indicate stable newly-formed spines. B1: Baseline 1; B2: Baseline 2; Scale bar: 10 µm. d Scheme of the experimental design with the retroAAV injected in the spinal cord and timeline of the two-photon in vivo imaging. e Confocal images of retrogradely labeled CST neurons in the cortex (Green: retroAAV-EGFP; red: NT435). Insets are two times magnified (top raw overlay; middle raw: retroAAV-EGFP; bottom raw: NT435). Scale bar equals 50 µm. f Representative timelapse series of an apical dendrite from a CST neuron retrogradely-labeled with retroAAV-EGFP. Each image shows the same dendrite stretch at a specific experimental time point before and up to 42d post-injury (13 independent dentritic stretches and 4376 spines were followed). Cyan arrowheads indicate disappearing pre-existing spines. Gray arrowheads indicate disappearing newly-formed spines. Green dot indicate stable pre-existing spines. Magenta dots indicate stable newly-formed spines. B1: Baseline 1; B2: Baseline 2; Scale bar: 10 µm. g Quantifications comparing the persistence of all pre-existing spines (n = 377 spines analyzed) to all newly formed spines (n = 187 spines analyzed) (top panel) and the persistence of newly formed spines on callosal (n = 134 spines from nine dentritic stretches from four animals) and CST neurons (n = 55 spines analyzed from eight dentritic stretches from four animals) (bottom panel). All data (top and bottom) are analyzed using two-tailed Mann-Whitney test (p < 0.0001 in both cases) and presented as box plots (top panel: Pre-existing spines: minima:0, maxima:1; median = 0.8889; 25% percentile = 0.6667; 75% percentile = 1; newly formed spines: minima:0, maxima:1; median = 0.2111; 25% percentile = 0; 75% percentile = 0.7708; bottom panel: CST: minima:0, maxima:1, median = 0; 25% percentile = 0; 75% percentile = 0.3333; Callosal: minima:0, maxima:1, median = 0.3333; 25% percentile = 0; 75% percentile = 1) Source data are provided as a Source Data file.
Acutely (7d) following the lesion we observed a general reduction in connectivity within the contralesional somatosensory area, motor area (p = 0.0419), retrosplenial area (p = 0.0405), the auditory cortex (p = 0.0021) and temporal association area (p = 0.0503), in particular (all two-tailed t-test, Fig. 5f). Additional contralesional areas, albeit whose connectivity ratio was lower initially showed no significant change (anterior cingulate area, visual and perirhinal cortices). All areas located in the ipsilesional cortex fully dropped in connectivity, as expected, as this is the site of the brain injury. At chronic time points, 42d post TBI, these ipsilesional areas remained low in connectivity. Remarkably, during the chronic phase following TBI, we observed a pronounced recovery of the connectivity in the contralesional hemisphere with a normalization of input from the
motor, auditory, retrosplenial and temporal association areas (all two-tailed \( t \)-test, Fig. 5f) when injured animals at 42d were compared to their matching controls. Subcortical areas, which had originally low connectivity ratios with the examined contralateral somatosensory cortex, demonstrated relatively minor changes both acutely and chronically following TBI (Fig. 5f). Our in situ analysis therefore underscores that callosal neurons restore their pre-synaptic connectivity largely by strengthening ipsilateral connections from pre-connected areas and is therefore largely homeostatic in nature, thereby restoring hitherto lost connections.

**TBI causes a widespread, transient reduction in neuronal activity in the contralateral cortex.** As we have shown that TBI causes dendritic remodeling of callosal neurons that include the loss of excitatory inputs early after lesion, we then asked whether these changes might also translate into alterations of neuronal function. To address this question, we longitudinally assessed neuronal activity in vivo using two-photon calcium imaging using the genetically encoded calcium indicator GCaMP6m in callosal and non-callosal neurons in the contralateral cortex (Fig. 6a, b). Callosal neurons were retrogradely labelled by the injection of retroAAV-tomato into the lesion area prior to brain trauma (Fig. 6a). At the level of the proportion of active cells a clear decrease was detected 10 days after the injury for both neuronal populations (Fig. 6c, baseline (B) vs 10d: callosal neurons \( p = 0.029; \) non-callosal neurons \( p = 0.015, \) two-tailed \( t \)-test). The fraction of active cells normalized after 42d both in callosal as well as in non-callosal neurons (Fig. 6c, 10d vs 42d: callosal neurons \( p = 0.027, \) non-callosal neurons \( p = 0.047, \) two-tailed \( t \)-test) and were not significantly different from the baseline recording anymore. When we followed the same neurons over time we found that TBI also caused a pronounced reduction of neuronal activity in both callosal and non-callosal neurons 10 days after injury (Fig. 6d, baseline vs 10d: callosal neurons \( p = 0.013, \) non-callosal neurons \( p < 10^{-10}, \) KS test). After 42 days we observed a recovery in activity levels primarily in non-callosal neurons (10d vs 42d: callosal neurons \( p = 0.23, \) non-callosal neurons \( p = 0.012, \) KS test). The activity levels, however, were still reduced compared to the baseline recordings in non-callosal (\( p < 10^{-4}, \) KS test). More specifically, the activity of cells active at baseline was strongly decreased 42 days after injury for both non-callosal and callosal neurons (Fig. 6c, callosal neurons \( p < 10^{-5}, \) non-callosal neurons \( p < 10^{-12}, \) two-tailed \( t \)-test). The initial activity levels were also determining the likelihood of remaining active until at least 42 days after the injury. Neurons with a baseline activity level of \( \geq 1 \) transient per minute (‘high’) were significantly more likely to remain active as opposed to neurons with a lower activity level (<1 transient/min, ‘low’) (Fig. 6f, callosal neurons \( p < 0.05, \) non-callosal neurons \( p < 0.05, \) nonparametric bootstrapping).

We further investigated the fate of neurons initially active at baseline. This investigation revealed that a larger fraction of callosal compared to non-callosal neurons went silent at 10d after TBI (\( p < 0.05, \) nonparametric bootstrapping), while the fraction of persistently active neurons or neurons that regained activity after 42d, or those turning silent only at d42 did not differ between the two populations (Fig. 6g). When investigating the history of cells active at day 42, we found that a large majority of these cells only became active after TBI (‘newly active’) both in callosal as well as in non-callosal neurons (Fig. 6h). Only \( \sim 20\% \) of cells active at day 42 were persistently active cells, while 24 and 29\% in callosal and non-callosal neurons regained activity after turning silent at day 10 (\( p = ns; \) Fig. 6h).

**Discussion**

The adaptation of contralateral cortex following brain injury is an important paradigm that has been studied to understand how the adult brain responds to damage. However, if and how such adaptive responses contribute to functional recovery is not entirely resolved\textsuperscript{26,28} and the specific neuronal populations and circuits that underlie these responses have not been identified. In this study, we used a combination of techniques ranging from retrograde viral labeling, in vivo timelapse two-photon imaging of spine turnover, trans-synaptic circuit tracing, tissue clearing and in vivo calcium imaging to reveal the specific adaptation of contralateral cortical callosal neurons directly connected to the injury site following TBI (Table 1). We find, using two-photon longitudinal imaging over 18 days that there is a strong early decrease in spine density over at least 2 weeks with signs of recovery thereafter. The plasticity of contralateral areas following brain injury has been established previously thanks to observations in patients\textsuperscript{44–46} and in animal models\textsuperscript{47–51}. In particular changes in neurotransmitters\textsuperscript{52}, dendritic growth and synapse formation have been described\textsuperscript{53,54}. Reports also point out changes in neuronal activity in the contralateral cortex that are correlated with functional impairments in the acute and subacute phases following injury\textsuperscript{10,18,55}. In our study, we find, among others, that callosal neurons, which homotopically connect both cortical hemispheres, show a unique vulnerability and adaptation following TBI with a homeostatic reintegration into existing circuits. Recent clinical observations reported a specific vulnerability of remote brain regions directly connected to ischemic brain lesion\textsuperscript{16,58}. Likewise, recent experimental work indicates a specific and profound plasticity of callosal input following peripheral injury that underlies cortical takeover\textsuperscript{17}. In line with this view, our study now reveals the selective adaptation of callosal neurons and their input circuits after a contralateral brain injury. Interestingly, clinical observations following visual cortical damage that leads to blindness also point toward a similar compensatory mechanism in which areas in the visual cortex of
the contralesional hemisphere are recruited to compensate for altered visual function. One aspect of the adaptation of callosal neurons is that the spines that are lost early after the TBI lesion persist longer than the spines that are formed over the same time period in non-callosal neurons. This underlines the functional significance of callosal input circuitry plasticity. Such a re-establishment of stable spines has previously been described in response to sensory deprivation in mice and might be an attempt to re-establish a response to spared inputs or to recruit new inputs. Notably the spine morphology of callosal neurons evolves in line with the changes of spine density with more mature and stable spine morphologies emerging later on. This is interesting as it suggests a biphasic plastic and adaptive response of callosal spines after injury. It therefore demonstrates that connected neurons in the contralesional cortex are uniquely involved in the formation of stable spine plasticity. Such a re-organization of the callosal input circuitry constituting the cortical renaissance.
addition, that the changes triggered in the contralesional hemisphere can depend on the lesion extent as previous studies have shown that the balance between adaptive and mal-adaptive responses are affected by the size of the initial brain injury. It is therefore quite possible that the structural and functional alterations of callosal neurons that we observe here as well as their contribution to recovery can be affected by the type and size of the brain injury and could well be different in case of smaller or bigger lesion sizes26,27,61 or for lesions induced in the developing or aged CNS58. As the TBI destroys axonal projections of the contralesional callosal neurons, we reasoned that plasticity at the level of those axons could also occur as a response to the injury and therefore investigated this possibility. It is known that during development callosal neurons extend profuse axonal projections till about post-natal day 8, and these connections are then refined62,63 in an activity-dependent process till p21 to obtain the adult pattern of cross-hemispheric homotopy64. Here we have found no additional branching of callosal axons neither at acute nor at chronic time points following TBI indicating that the plastic processes could be restricted to the dendritic remodelings in the contralesional hemisphere.

As callosal neurons are uniquely affected by and responsive to TBI, we wondered whether and how their input circuitry would re-arrange at acute and chronic time points following injury. To answer this question we made use of the rabies virus, which monosynaptically labels pre-synaptic neurons that project to callosal neurons. We took a two-step approach: we first identified the brain regions connected to contralesional callosal neurons using tissue clearing and large scale imaging. Next we then determined the connectivity ratios before and after brain injury of the connected areas identified in tissue clearing analysis. Not surprisingly, we find that the main input to the somatosensory cortex is provided by cortical areas and in particular the somatosensory cortex, the motor cortex and the auditory cortex. This is in agreement with previous studies33,65. We further observed remarkably little pre-synaptic input at 42d to ectopic cortical or subcortical regions, as most brain regions connected before the injury were also found to provide input at 42d. This is important as restored connectivity to somatomotor areas such as the primary or secondary somatosensory cortex and primary motor cortex might underlie favorable rather than mal-adaptive adaptations17. Finally, we sought out to understand the state of activity of those callosal neurons and performed functional imaging experiments using genetically encoded calcium sensors. Functional imaging revealed that the fraction of active callosal neurons initially decreased but recovered over time. This initial decrease in neuronal activity in callosal neurons is in line with the synaptic structural changes seen in those neurons early following the injury. Indeed, we demonstrate that concomitantly to the decrease in activity, callosal neurons also demonstrate a specific loss of excitatory input onto their dendrite. This structural loss of excitatory input is consistent with the functional decrease in neuronal activity also observed in other paradigms66,67. In addition, while the callosal neurons active at baseline mostly fail to maintain and regain activity, a large part of the activity seen at 42d is due to newly active cells. This probably reflects activity-dependent functional refinements as circuit plasticity is correlated with increased spine dynamics43,68, which we observe during this period. In line with this, monosynaptic tracing revealed that input in discrete regions increases from 7 to 42 dpi also, supporting the notion of ongoing synapse refinement. Our functional imaging data also show that callosal neurons did not behave fundamentally differently from non-callosal neurons. Why callosal and non-callosal neurons behave similarly can probably be explained by intracortical communication, as non-callosal neurons receive input from callosal neurons and vice versa.

Taken together, our study reveals important structural and functional principles of remodeling following TBI: it identifies a specific neuronal population (callosal neurons) that are particularly responsive to contralateral lesions and are thus prime targets for therapeutic interventions. Considering the importance of cortical remodeling processes for the functional recovery not only after traumatic but also after ischemic nervous system injuries, this has important implications for many common neurological conditions.

Methods
All experiments were approved by the Regierung von Oberbayern under the protocol number AZ.55.2.1.54-2532.135-15.

Animals. GFP-M mice (24 mixed gender randomized equally per group) and 78 female C57Bl6 mice, 8–12 weeks-old, were used for this study. All animals were housed under controlled standard housing conditions (dark/light cycle of 12 h, temperature 22 ± 2 degrees and humidity of 55 ± 10%) with food and water ad libitum.

Controlled cortical impact (CCI). Mice were anaesthetized via an intraperitoneal (i.p.) injection of MMF (Medetomidin 0.5 mg/kg, Orion Pharma; Midazolam 5.0 mg/kg, Ratiopharm; Fentanyl 0.05 mg/kg, B.Braun). When pedal reflex disappeared, animals were fixed on the stereotactic frame (Precision Systems & Instrumentation, LLC). The skin was incised and a square craniotomy (4 mm × 4 mm), positioned between Lambda, Bregma, the sagittal suture and the side of the skull was made. The injury to the brain was induced using a TBI-0310 impactor (Precision Systems & Instrumentation, LLC), in the area of the somatosensory cortex (flat-edged rod of 3 mm diameter, velocity of 6 m/s, a dwell time of 150 ms and a depth of 0.3–0.5 mm). The removed bone piece was re-positioned to its previous location and glued to the skull with Vetbond (3 M™ Vetbond™, 3 M United States). The skin was sutured. The mean apnea duration and righting reflex

### Table. 1 Summary table of the changes and adaptations of callosal neurons following contralateral traumatic brain injury.

| Manipulation                              | Acute time points | Later time points | Figure panel |
|-------------------------------------------|-------------------|-------------------|--------------|
| Sensorimotor function                     | Decreased         | Normalized        | 1b           |
| Spine density in the contralesional cortex | Decreased         | Normalized        | 1e           |
| Spine density of callosal neurons          | Decreased         | Normalized        | 2c & d       |
| Spine density of non callosal neurons      | Not changed       | Not changed       | 2e & f       |
| Excitatory input onto callosal neurons     | Decreased         | Normalized        | 2g & h       |
| Inhibitory input onto callosal neurons     | No changed        | Normalized        | 2g & h       |
| Spine morphology of callosal neurons       | Altered           | Normalized        | 3b & c       |
| Spine morphology of non callosal neurons   | Not changed       | Not changed       | 3d & e       |
| Persistence of new spines on callosal neurons | Decreased         | Higher in callosal neurons compared to control neurons | 4g |
| Connectivity ratios of callosal neurons    | Decreased         | Normalized        | 5            |
| Activity patterns callosal neurons         | Decreased         | Normalized        | 6c           |
| Activity patterns non callosal neurons     | Decreased         | Normalized        | 6c           |
duration (time for a mouse to flip onto its feet from a supine position after the injury) were recorded in order to evaluate the severity of the injury.

Evaluation of sensorimotor function. Ladder rung test: For assessment of fine paw placement, the ladder rung test was used69. In this test, the animals had to cross 1 m horizontal grid ladder and mistakes were counted by an investigator blinded to time points based on video recordings frame-by-frame of three consecutive crossings. We evaluated the animal’s ability for fine coordinated paw placements using irregular spacing of the rungs (irregular walk task). Only consecutive steps of the hindlimbs were analyzed. Therefore, the last step before or after any interruptions were not scored. Placements were considered as a mistake when mice either totally missed a rung or if they slipped from a rung (deep or slight slip). Placements were considered as correct when the mice correctly placed all the foot or only a part of the foot on the rungs. Then the number of slips over a standard distance was calculated quantitatively.

Evaluation of neuronal density, soma size, spine density, spine morphology and synaptic input onto callosal dendrites in fixed tissue

Tracer injections. For experiments on fixed tissue imaged with the confocal microscope, we retrogradely labeled callosal projection neurons by stereotactically injecting 0.3 μl of FluoroGold; (1% in 0.1 M Cacodylate buffer, Fluorochrome LLC) in the ipsilateral somatosensory cortex (coordinates from Bregma: rostrocaudal −1.5 mm, lateral 1.7 mm, depth 0.3 mm and timeline is depicted in Fig. 2). In short, a glass capillary micropipette tip was slowly inserted into the brain through a small hole drilled in the skull. In order to avoid backflow, the pipette remained in the brain for a minimum of three minutes after injection completion70. To retrogradely label corticospinal (CST) projection neurons, laminectomy was performed at thoracic level 8 of the spinal cord and 0.5 μl of FluoroGold were stereotactically injected with a glass capillary into the right side of the spinal cord, 2 mm lateral from spinal midline at a depth of 0.3 mm. The micropipette remained in place for at least 3 min after completing the injection to minimize backflow. Subsequently, the muscles and skin were sutured and saline was administered subcutaneously. For the experiment aiming at observing the changes in synaptic input onto hilar dendrites, we injected the somatosensory cortex with 0.5 μl of a retrograde AAV expressing tdTomato (Retro-AAV-tdTomato, Addgene #59462) at the following coordinates from Bregma (rostrocaudal −1.5/lateral 1.7/deepth 0.3 mm).

Tissue preparation for spine imaging. Mice were sacrificed at 7, 14, and 42 days post-injury or at 12 days after injection (control non-injured group) and perfused with 4% paraformaldehyde (PFA) in 0.1 M PBS. Brains were kept in 4% PFA for 24 h, dissected, immersed in 3% low-melting point agarose and cut with a Leica VT 1000 s vibratome. Coronal sections (100 μm) were cut and washed three times with 1× PBS for 10 min before free-floating staining with NeuroTrace 435 (Thermo Fischer Scientific; 1:500 in 0.1% Triton PBS) at 4 °C, overnight. The next day sections were washed three times in 1 × PBS for 10 min and mounted on gelatin subbed slides, using VectaShield (Vector Laboratories).

Tissue preparation for synaptic input onto callosal dendrite quantification. For thin paraffin sections, mouse brains were paraffin embedded and cut into 2 μm thin sections. After deparaffinization, antigen retrieval was performed using a Pancy Citrate buffer at pH 6.0. To inactivate endogenous peroxidases, slides were incubated with Dako REAL peroxidase-blocking solution (Dako, K0672) and subsequently blocked with 10% FCS in PBS.

Immunohistochemistry. To quantify neuronal density and soma size, we stained free-floating (Iacob et al., 2015). To do so, the sections were washed for 10 min in 1× PBS three times followed by 1 h incubation in blocking buffer (1× PBS with 0.5% Triton and 10% goat serum) at room temperature. Brain sections were then incubated overnight at 4 °C in the primary antibody solution: 1× PBS containing 0.1% Triton, 1% goat serum and mouse anti-NeuN Ab (1:1500; Thermo Fischer Scientific; 1:500 in 0.1% Triton PBS) at 4 °C, overnight. The next day the tissue was washed three times for 10 min in 1× PBS 0.1% Triton before the application of the secondary antibody solution goat anti-mouse AlexaFluor® 594 Ab (1:500; Invitrogen) in 1× PBS − 0.1% Triton, 1% goat serum. After overnight incubation at 4 °C the tissue was again washed three times for 10 min in 1× PBS, mounted on gelatin coated glass slides in VectaShield (Vector Labs). To quantify synaptic input onto callosal dendrites in vivo, paraffin sections (2 μm thin) we first stained retrogradely-labeled callosal neurons with rabbit anti-RFP antibody (1:400; abcam, ab124754) diluted in Dako REAL antibody diluent (Dako, S2022) at 4 °C o/n. To visualize the specific signal, anti-rabbit HRP (Dako, K4003) together with Opal-570 (1:600; Akyoa, F4188001KT) was used as secondary antibody and amplification system. After washing, slices were incubated with goat-anti-mouse IgG Fab-fragments (1:100; Jackson ImmunoResearch, 115-007-003) and goat serum to avoid subsequent unspecific binding. To perform the synaptic staining the sections were incubated with mouse IgG2b anti-Gephyrin antibody (1:200; Santa Cruz, sc-25311) and mouse IgG1 anti-GAD65/67 antibody (1:100; Santa Cruz, sc-365318) or chicken anti-Homer1 antibody (1:500; Synaptic System, 1600006) and mouse IgG1 anti- BNPI antibody (1:100; Santa Cruz, sc-377425), respectively. Conjugated secondary antibodies corresponding to the species and isotype of the primary antibody pairs were used to visualize specific staining. Nuclei were stained with DAPI (Invitrogen, D1306). Slides were mounted with Fluoromount aqueous mounting medium (Sigma-Aldrich, F4880).

Image acquisition and image processing. Callosal neurons were identified as GFP and Fluorogold positive, while GFP-positive but Fluorogold-negative neurons located in the same area than callosal neurons were considered non-calllosal. CST-neurons were identified as double positive neurons for Fluorogold and GFP.

Cortical thickness, cell density and cell size. To measure the cortical thickness, cell density and soma size in the contralesional cortex, confocal stacks of 10 μm were taken (four sections per animals) with a step size of 1 μm using the FV1000 Olympus microscope (10× objective NA 0.4, zoom of 1.0 and resolution of 1024 × 1024). Fluorogold labeling was imaged using the 405 nm laser (with emission wavelength set at 610 nm), GFP with 488 nm laser and NeuN with 543 nm laser, in sequential scans.

Spine density and morphology. To measure spine density and morphology we relied on the intense and sparse labeling of GFP-M mice (essentially layer V neurons and fewer layer II/III neurons). In order to quantify the spine density/morphology of individual dendrites, we acquired z-stacks using the FV1000 Olympus microscope (60× oil objective NA 1.35, zoom of 3.5 and resolution of 800 × 800). We imaged three areas for each individual layer V neuronal dendrites and two areas for layer II/III neuronal dendrites (average length of 60 μm): proximal dendrite (at a distance of ~370–450 μm from soma, only for layer V neurons) and apical tuft (at a distance up to 75 μm below the SI cortical surface). The images underwent deconvolution using the Huygens Essential software (16.05 Scientific Volume Imaging, NL) and Z projections of the deconvolved stacks were obtained using Neuron Studio.

Data analysis. All analysis were performed by an investigator blinded to the injury status and injury time points.

Cortical thickness. To calculate cortical thickness the distance between the bottom of Layer VI and top of Layer I, using the straight line tool in Fiji was made at three different coordinates in the medio-lateral direction (1 mm, 1.5 mm and 2 mm from midline). An average of those three measurements was then made.

Cell density and cell size. Images were processed and analyzed using the Definiens Developer XD™ software from Definiens, Version 2.7.0 which allowed a precise cell count. The cell counts were then divided by the area to obtain the cell density results.

Spine density and morphology analysis. Each deconvoluted stack containing one proximal, distal or apical tuft dendritic segment was opened in NeuronStudio (version 0.9.92) (Fig. 2b) and the dendritic trunk was semi-automatically traced resulting in a series of green vertices superimposed on volume-rendered data. Thereafter, dendritic spines were detected and classified. This automatic quantification was manually controlled to remove falsely detected spines, add falsely undetected or reclassifi misclassified spines. The resulting numbers of spines were divided by the length of the dendritic segment as calculated by the program.

Analysis of synaptic input onto callosal dendrites. Immunofluorescence staining was performed to visualize excitatory or inhibitory synaptic pairs together with RFP positive neuronal structures. Images were acquired on a Leica M125P TCS SP8 confocal microscope (63× oil objective, zoom of 3.5 and resolution of 1024 × 1024, z-stack of 0.3 μm). Quantification was carried as follows: the perimeter of the dendrites was outlined and measured. Then the number of excitatory or inhibitory synaptic pairs (in which both the pre-synaptic and post-synaptic markers co-localize) that co-localize with the outlined dendritic segments were counted. The density of synaptic pairs along the dendritic segments was then calculated.

Lesion volume. To characterize the lesion volume, we cut 50 μm coronal brain sections starting from the first visibly “damaged” section to the last. Lesion volume was calculated according to Yu et al71, by outlying in 15–17 consecutive sections the area of the lesion 42 days following the TBI.

In vivo 2 photon imaging of spine density in the contralesional cortex

Chronic cranial window implantation. Before in vivo two-photon imaging, all animals were implanted with a chronic cranial window (CW), following a slightly modified protocol by Holtmaat et al. (2009)72. Mice were anaesthetized and fixed into a stereotactic frame. The skin above half of the skull was permanently removed with scissors. The remaining skin, surrounding the exposed skull was glued to the sides of the skull with Vetbond (3 M Vetbond™ tissue adhesive, 3 M United States). The CW (a circle of 4 mm of diameter) was positioned onto the somatosensory cortex between both coronal sutures and lambda and bregma, respectively. Then the bone was thinned until it was possible to remove the bone flap from the skull with forceps leaving the sagittal suture intact. A 4 mm diameter glass coverslip was
then positioned on top of the exposed and then glued and fixed onto to remaining skull, by applying another cyanoacrylate tissue adhesive (Histacycol®, B.Braun) on the edges of the glass. Dental cement was then placed on the remaining skull, as well as at the edges of the glass coverlip, thereby securing the coverlip further. After the surgery, a resting period of at least 2–3 weeks before starting the imaging procedure was required to minimize inflammation.

Two-photon microscopy. All animals were imaged using a two-photon microscope (Olympus FV1000 MPE), equipped with a Mai Tai DeepSee femto-second pulsed Ti Sapphire laser (SpectralPhysics). In more detail, the apical dendritic tufts of GFP-labelled neurons of the somatosensory cortex were imaged. Before every imaging session animals were anaesthetized with an i.p. injection of MMF. Mice were placed under the two-photon microscope objective (Olympus XPLan N 25X W MP). To visualize GFP-fluorescence, a green/red Olympus filter cube (FV10-MRVR/GR; BA595-540, BA575) was used. The mice were imaged at a wavelength of 940 nm. A low-resolution overview stack was taken over regions of interest (resolution: 640 × 640 pixels; Zoom: 1; step 3 or 5 μm). This overview stack was later used to find back the same ROIs and dendrites each subsequent imaging session. In addition, the unique vascular arrangement, visible in superficial layers of the brain through the fluorescence lamp of the microscope, helped locate the same dendrites each following imaging period. For a detailed stack of individual dendrites and their spines, which were later used for spine analysis, a higher resolution image was acquired (resolution: 2048 × 2048 pixels; Zoom: 2; step 1 μm). In general, at least two detailed images with a varying number of analyzable dendrites (ranging from 3 to 20) were imaged per animal. Particular care was taken to keep a similar fluorescence intensity and laser power for each ROI, but also in between animals. Image acquisition was performed for baseline measurements and every 3 days after the induction of TBI. In baseline conditions before injury, baseline 1 (B1) and baseline 2 (B2), with 3 days in between each other. We imaged and followed 81 dendritic stretches from 4 animals (Fig. 1d) and a total of 10,191 spines throughout the experiment. The average length of the dendritic stretches quantified was 63.31 ± 1.54 μm.

Analysis of in vivo spine dynamics and counting criteria. Image analysis was performed only on the high resolution stacks with Fiji (ImageJ) software. Images were median filtered (“Despeckle”), and suitable dendrites for counting were determined. The length of each dendrite was traced and measured with Fiji at baseline. Dendritic spines were counted using the Fiji Cell Counter Plugin at each time point, by going through stacks manually and looking at caudal tuft. In short, only structures clearly marked by the GFP fluorescence and protrusion length of 0.4 μm were defined and counted as spines72. Structures, however, that fulfilled these criteria but coincided with another crossing dendrite and could not be distinctly attributed to the chosen dendrite were not included in the analysis. The total spine number, number of eliminated and formed spines, as well as the stable number of spines were determined. A spine was defined as eliminated, if it was not visible anymore in the next time point or as formed, if a new structure appeared, at the same location or where no spine had been counted before. Spines that were visible in successive time points at the same location were considered as stable. In addition, an elimination and formation rate was calculated, by dividing the eliminated or formed spines at one time point by the total number of spines in the previous time point and multiplied by 100 (thus, rates were expressed as percentages). Spine density in turn was calculated relative to the length of the dendrite (number of spines/μm).

In vivo 2 photon imaging of newly formed spine persistence index. Imaging of the specific cell population of callosal and non callosal CST neurons (Fig. 3) was carried out, as described above. For detecting callosal neurons, we first injected the somatosensory cortex with 0.5 μl of a retrograde AAV expressing GFP (Retro-AAV-GFP #37825 Addgene; Fig. 3a) and then implanted a CW 4 days later. To visualize AAV-GFP fluorescence, a green/red Olympus filter cube (FV10-MRVR/GR; BA595-540, BA575) was used. The mice were imaged at a wavelength of 940 nm. A low-resolution overview stack was taken over regions of interest (resolution: 640 × 640 pixels; Zoom: 1; step 3 or 5 μm). This overview stack was later used to find back the same ROIs and dendrites each subsequent imaging session. For a detailed stack of individual dendrites and their spines, which were later used for spine analysis, a higher resolution image was acquired (resolution: 2048 × 2048 pixels; Zoom: 2; step 1 μm). In general, at least two detailed images with a varying number of analyzable dendrites (ranging from 3 to 20) were imaged per animal. Particular care was taken to keep a similar fluorescence intensity and laser power for each ROI, but also in between animals. Image acquisition was performed for baseline measurements and every 3 days after the induction of TBI. In baseline conditions before injury, baseline 1 (B1) and baseline 2 (B2), with 3 days in between each other. We imaged and followed 81 dendritic stretches from 4 animals (Fig. 1d) and a total of 10,191 spines throughout the experiment. The average length of the dendritic stretches quantified was 63.31 ± 1.54 μm.

Neuronal connectivity

Viral tracing. For labeling and analysis of neuronal cell connectivity using clearing methods, we applied a mono-synaptic rabies virus tracing technique. For this purpose, callosal axons on the ipsilesional side of the injury were first selectively infected with 0.5 μl of AAV expressing the cre recombinase (“AAV-cre” [AAV1-IRES-Cre-WPRE.EGFP]), titer: 5.0 × 10^13 vg/ml supplied by the Penn Vector Core; dilution 1:1 in PBS; coordinates from Bregma: rostrocaudal −1.5 mm, lateral 1.7 mm, depth 0.3 mm). For the acute time point groups (4 days after AAV injection), 35 days after AAV-cre injection. In a final step, pre-synaptic partners of callosal neurons were visualized by injecting 1 μl of a rabies virus (SAD-ΔG-mcherry (EnvA); TBI was induced on the same day as the rabies virus injection (2 weeks after the AAV-cre and AAV-TVA-G-eGFP injections), while animals in the chronic time point group were injured 2 weeks after AAV-cre injection, 1 week before the AAV-TVA-G-eGFP and 35 days before the rabies virus injection. Control uninjured animals were generated similarly, with one early and one late injection group.

Tissue clearing. Brains underwent staining and clearing using vDISCO protocol as described in Cai et al.26. Briefly, after perfusing the animals with 1× PBS and 4% PFA, brains were collected and post-fixed for one night in 4% PFA and the next day were washed with 1× PBS.

Then whole brains were placed in 5 mL tubes (Eppendorf, 0030 119.401) and incubated in a permeabilization solution containing 1.5 vol/vol% goat serum (Gibco, 16210027), 0.5 vol/vol% Triton X-100 (AppliChem, A9475100), 0.5 mM of methyl-β-cyclodextrin (Sigma, 352615), 0.2% wt/vol% trans-1-acyl-4-hydroxy-1-proline (Sigma-Aldrich, 41562E) and 0.05 wt/vol% sodium azide (Sigma, 71290) in 1× PBS for 1 day at 37 °C in dark with gentle shaking. Then samples were incubated in 4.5 mL of a solution containing 1.5 vol/vol% goat serum, 0.5 vol/vol% Triton X-100, and 0.05 wt/vol% sodium azide in 1× PBS + 4× of anti-RFP nanobody conjugated with Atto647N (Chromotec, rhb647N-100, lot 71290) in 1× PBS for 1 day at 37 °C in dark with gentle shaking. Then samples were incubated in 4.5 mL of a solution containing 1.5 vol/vol% goat serum, 0.5 vol/vol% Triton X-100, and 0.05 wt/vol% sodium azide in 1× PBS for 4 times every hour and finally with 1× PBS times every hour. Both washing procedures were carried at room temperature in the dark and with gentle shaking. Samples were next cleared by incubating them in the following sequential solutions for 2–3 h each step: 50 vol% THF, 70 vol% THF, 80 vol% THF in distilled water, 100 vol% THF overnight and again 1 h in 100 vol% THF, followed by 2 h in dichloromethane and finally in BABB until samples turned completely transparent after 5–6 h. All the clearing steps were performed with gentle shaking, at room temperature and by protecting the samples from light.

Light-sheet microscopy imaging and whole brain analysis. Cleared brains were imaged as tilting scans (10% overlap) with the light-sheet microscope Ultra microscope-II (LaVision BioTec) using a x2 objective Olympus MPLLAP02X/0.5 NA (WD = 6 mm) coupled to an Olympus MVX10 zoom body that was kept at zoom magnitude x2.5 using the following parameters: 240 × 240 × 240 μm volume of tile scans was performed by using Fiji’s (ImageJ, v.1.52 h, https://fiji.sc/) stitching plugin. Stitched images were saved in tiff format. Fiji’s TrakEM2 plugin and Imglb2 library were used to correct acquisition shifting. Scans were pre-processed in Fiji software for background equalization via pseudo-flat-field correction function from Bio-Voxcel toolbox, for background removal (to remove particles bigger than cells) via median option from the same toolbox, for noise reduction via two-dimensional medial filter and for signal amplification via the unsharpen mask. Cells were visualized as heatmaps after Clearmap software analysis as described by Renier et al.31. TBI and control heatmaps colored with two different colors were overlaid over the brain atlas provided by Clearmap and visualized in Fiji and displayed as image sequence in Fig. 5b and in the Supplementary movie.

Tissue processing and immunohistochemistry. For circuit connectivity analysis, mice were transcardially perfused either 7 or 42 days after TBI, as described above. Control mice were sacrificed on the same day as TBI injuries. After the 24 h period of post-fixation in 4% PFA, brains were microdissected and kept in ependorf receptacles with 30% sucrose until sinking to the bottom. They were sectioned coronally with a cryostat (Leica CM1850) at a thickness of 60 μm. Next, a GFP- amplification-protocol combined with a NeuroTrace (NT) staining was performed on the selected sections. To that end, solutions were prepared as follows: 1× PBS & 10 min. In the final washing step, 1 × PBS was replaced by blocking buffer (1 × PBS with 0.5% Triton and 10% goat serum) and sections were incubated in it for 1 h at room temperature. After removing the blocking buffer, primary antibody solution (1× PBS containing 0.1% Triton, 1% goat serum and 1:500 chicken anti-GFP Ab (Abcam)) was added. Overnight incubation at 4 °C ensued. The following...
day, sections were washed three times for 10 min with 1× PBS/0.1% Triton and later incubated with the secondary antibody solution (1× PBS – 0.1% Triton, 1% goat serum and 1:500, Goat Anti-Chicken Alexa Fluor® 488 Ab (Abcam); 1:500 NT345 (Invitrogen)) overnight at 4°C. After a last washing step, three times à 10 min with 1xPBS, sections were mounted on gelatine submerged slides with VectaShield (Vector Laboratories) and finally coverslipped as described above.

**Imaging of cleared tissue.** Images of brain sections of 2 mm thickness were acquired on a light-sheet microscope (UM II LaVision BioTe-UltraMicroscope II), with a z-step of 3 μm, using a 4× objective (0.28 NA, Olympus XFLUOR04), with a lens provided with a 10 mm working distance dipping cap. Images were processed with Imaris (Bitplane1) in order to generate 3D-projections, while HeatMap Histograms were created using Fiji.

**Imaging.** Starter cells were evaluated as follows: A total of six sections per brain, beginning from the section with the first detectable starter cells to the section with the last detectable starter cells, were scanned using a Leica SP8 confocal microscope (magnification: ×20; Zoom 1; step 1 μm; resolution: 512 ×512 pixels). Presynaptic cells were evaluated as follows: Here, 20 sections per brain were selected according to the Allen Mouse Brain Atlas from a 1.15 to 2.85 mm relative to Bregma2. All sections were imaged consecutively with a Leica DM B microscope (Leica Microsystems), using the MBF Stereo Investigator 2017 software and a 2×5 objective when cells were sparse. In cases where cell labelling was too dense we used a 10X objective. All 2.5 and ×10 magnification images were stitched with the Fiji Particle Stitching Plugin. The 20X image stacks were further processed using ImageJ (Fiji).

**Quantifications.** Starter cells were manually quantified using the Fiji Cell Counter Plugin in all imaged sections and interpolated to the total number of sections evaluated in the whole brain. For quantification, each imaged section was subdivided into different brain areas, as defined by the Allen Mouse Brain Atlas. In total, 120 different brain regions, ranging from cortical to subcortical regions as well as white matter tract structures. Within those, pre-synaptic cells (m-Cherry positive) were manually counted with the Fiji Cell Counter Plugin. For areas in which cell aggregation was too dense and manual counting was not accurate enough we employed Fiji’s Particle Analyzer Plugin for automatic quantification, after image processing for background subtraction. We then made a ratio between the total number of pre-synaptic neurons in that specific area to the total number of starter cells in the whole brain. This ratio was termed connectivity ratio, as previously described28. We then compared the connectivity ratios of acute and chronic injured animals with their respective controls (acute and controls generated at the respective virus injections timelines as for injured animals).

**Calcium imaging experiments**

**Viral injections and window implantation.** For in vivo calcium imaging, callous neurons were selectively traced and labelled using viral vectors. To do so, we injected mice with a retrograde adenovirus vector (retro AAV-Cherry Tomato, Addgene, 59462-AAV; Titer ≥1011 vg/mL; dilution 1:3 with 1× PBS) as described above. AAV1.hSyn-GCaMP6mWPRE.SV40 (Addgene 100838, Titer ≥1012 vg/mL) was injected in the contralateral site with the following coordinates (rostro-caudal to Bregma: −1.8; lateral to Bregma 2.0; 0.5 mm depth) and a CW (diameter 4 mm) was implanted above described as above.

**Two-photon imaging in anesthetized mice.** In vivo two-photon imaging was performed on a resonant scanning two-photon microscope (Hyperscope, Scientifica, equipped with an 8 kHz resonant scanner) and a 16× water-immersion objective (Nikon), yielding frame rates of 30 Hz at a resolution of 512 × 512 pixels. We recorded 18,000 frames (10 min) within cortical layer II/III (cortical depths of (Nikon), yielding frame rates of 30 Hz at a resolution of 512 × 512 pixels. We recorded 18,000 frames (10 min) within cortical layer II/III (cortical depths of (Nikon), yielding frame rates of 30 Hz at a resolution of 512 × 512 pixels. We recorded 18,000 frames (10 min) within cortical layer II/III (cortical depths of 28 nm) covering a field of view (FOV) of 230 × 230 μm. Light source was a Ti Sapphire laser with a DeepSee pre-chip spectum (Spectra Physics MaiTai eHP), GCaMP6 and tdTomato were excited at 930 nm, with a laser power not exceeding 40 mW (typically 10–40 mW). During imaging, mice were anesthetized with isoflurane (0.5%–1% pure) at a stable respiratory rate in the range of 110–140 breaths per minute. Body temperature was maintained at 37 degrees as a physiological monitoring system (Harvard Apparatus). In total 663 non-callous and 139 callous neurons were analyzed within 13 experiments in 6 mice.

**Image processing and data analysis.** All image analyses were performed using custom-written routines in Matlab (R2018b)39. In brief, full frame images were corrected for potential x and y brain displacement occurred during the in vivo recording period, and regions of interest (ROIs) were manually selected based on the maximum and mean projection of all frames. Those ROIs werereadjusted, if necessary, over the different recorded frames following our time course. Fluorescence signals of all pixels within a selected ROI were averaged, the intensity traces were low pass filtered at 10 Hz. Contamination from neuropil signals was accounted for, as described using the following equation: FROI_comp = FROI – 0.7 × Fneuropil + 0.7 × median (Fneuropil).

\[ F_{\text{ROI}_{\text{comp}}} \text{ stands for neuropil-compensated fluorescence of the ROI, } F_{\text{ROI}} \text{ and } F_{\text{neuropil}} \text{ represent the initial fluorescence signal of the ROI and the signal from the neuropil, respectively. A neuron was defined as ‘active’ if it displayed at least one prominent calcium transient over 20 frames (corresponding to ~0.7 s).} \]

**Statistical analysis.** All results are given as mean ± standard error of the mean (SEM), unless otherwise stated. Statistical analysis, as well as graphs illustrating data, was carried out with GraphPad Prism 7.01 for Windows (GraphPad Software). Ladder rung behavior data were analyzed with a Friedman test followed by post-hoc test. In vivo two-photon imaging data were analyzed using one-way and two-ways RM ANOVA followed by post-hoc test. In experiments without data of a “repeated-measures-type”, we performed an ordinary one-way ANOVA and post-hoc test. In addition, two-tailed t-test were carried out for connectivity analysis and in vivo functional imaging experiments (unless otherwise stated). To compare cumulative distributions we used the Kolmogorov-Smirnov (KS) test. When data were unparametric we used Mann–Whitney test to compare two groups. Statistical significance levels are indicated as follows: *p < 0.05; **p < 0.01; ***p < 0.001.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Source data are provided with this paper and have also been deposited in the Zenodo database, under https://doi.org/10.5281/zenodo.4730201 including supplementary data. Raw data are available from the corresponding author upon reasonable request.

Received: 26 February 2021; Accepted: 11 April 2022; Published online: 15 May 2022

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Acknowledgements
We thank B. Fiedler and L. Schödel for excellent technical assistance as well as D.Matzek and B. Stahr for animal husbandry. F.M.B. is supported by the Deutsche Forschungsgemeinschaft (DFG, SFB 870 Project ID 118803580). F.M.B. is supported by the Wings for Life Foundation, the International Foundation for Research in Paraplegia (IRP) and the DFG, TRR274 (Project ID 40885537). F.M.B. and S.L. are supported by the Munich Center for Systems Neurology (DFG, SyNergy; EXC 2145/ID 390857198). S.L. is supported by the Emmy Noether program (DFG). V.V.S. is supported by the Humboldt foundation and J.F. is supported by the Wings for Life Foundation. The authors declare no competing financial interests. We thank the Core Facility Bioimaging at the Biomedical Center (BMC) for excellent imaging support.

Author contributions
F.M.B. designed the experiments. L.E., A.C., M.C., W.Y.V.K. contributed all surgical procedures. L.E., A.C., M.C., W.Y.V.K., S.W., V.V.S., J.F., M.M. collected and analyzed data. A.G., P.B., and K.K.C. contributed rabies viruses. R.C., A.G., A.E. contributed clearing experiments. M.C., W.I.V.K., and S.L. contributed calcium imaging and analysis. I.W., M.K., and D.M. analyzed cell density in the cortex and performed paraffin-embedded immunohistochemistry. L.E., A.C., S.L., and F.M.B. wrote the paper. All authors approved the final version of the paper.

Funding
Open Access funding enabled and organized by Projekt DEAL.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-29992-0.

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Peer review information Nature Communications thanks Ramesh Raghupathi and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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