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Training of the impaired forelimb after traumatic brain injury enhances hippocampal neurogenesis in the *Emx1* null mice lacking a corpus callosum

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Abstract

Unilateral brain injury is known to disrupt the balance between the two cortices, as evidenced by an abnormally high interhemispheric inhibitory drive from motor cortex $M1_{intact}$ to $M1_{lesioned}$ transmitted transcallosally. Our previous work has shown that the deletion of homeobox gene *Emx1* not only led to the agenesis of the corpus callosum (cc), but also to reduced hippocampal neurogenesis. The current study sought to determine whether lacking the cc affected the recovery of forelimb function and hippocampal plasticity following training of the affected limb in mice with unilateral traumatic brain injuries (TBI). One week after TBI, produced by a controlled cortical impact to impair the preferred limb, *Emx1* wild type (WT) and knock out (KO) mice were subjected to the single-pellet reaching task with the affected limb for 4 weeks. Both TBI and *Emx1* deletion had overall adverse effects on the successful rate of reaching. However, TBI significantly affected reaching performance only in the WT mice and not in the KO mice. Both TBI and *Emx1* gene deletion also negatively affected hippocampal neurogenesis, demonstrated by a reduction in doublecortin (DCX)-expressing immature neurons, while limb training enhanced

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DCX expression. However, limb training increased DCX cells in KO mice only in the TBI-treated group, whereas it induced neurogenesis in both WT mice groups regardless of the treatment. Our finding also suggests that limb training enhances neuroplasticity after brain injury at functionally remote regions including the hippocampus, which may have implications for promoting overall recovery of function after TBI.

Keywords

controlled cortical impact; DCX; *Emx1*; neuroplasticity; skill reaching

Introduction

The two cerebral hemispheres are functionally balanced; however, unilateral dysfunction resulting from injury often disrupts this balance (Oliveri et al., 1999, Kobayashi et al., 2004). In humans, cortical excitability in the unaffected motor cortex can increase after a unilateral brain injury like stroke or after a transient suppression of cortical excitability such as hemispherectomy or transection of the corpus callosum (Shimizu et al., 2000), the principal fiber tract that connects the left and right hemispheres. The exact role of contralesional cortical activity in mediating functional recovery is not completely understood, although in healthy human subjects, suppression of excitability of one motor cortex via repetitive transcranial magnetic stimulation can enhance motor performance of the ipsilateral hand (Kobayashi et al., 2003, Kobayashi et al., 2004). Hyperexcitability in the contralesional hemisphere after stroke is thought to occur as a consequence of disinhibition due to loss of interhemispheric connections from the affected cortex, an action largely mediated by transcallosal fibers (Liepert et al., 2000, Liepert, 2003). Indeed in some stroke patients, an abnormally high interhemispheric inhibitory drive from M1 (intact hemisphere) to M1 (lesioned hemisphere) is observed during the process of generation of a voluntary movement by the paretic hand (Murase et al., 2004). In rats, acute cortical lesions lead to an increase in excitability of homotopic areas of the contralateral hemisphere and facilitation of motor skill learning with the unaffected forelimb (Bury and Jones, 2002), further supporting the concept of interhemispheric rivalry. In suppressing the contralesional activity, we found that combining physical therapy and limb restraint with botulinum toxin significantly improved function in rats with TBI (Lam et al., 2013).

The mammalian homeobox *Emx1* gene family is involved in the development of the rostral brain. We previously found that germ line deletion of the *Emx1* gene reduced the size of the dentate gyrus (DG) and decreased the number of proliferating cells and immature neurons found within, but it did not affect baseline neurogenesis in the subventricular zone during adulthood (Hong et al., 2007). Despite the acallosal phenotype, the adult naïve *Emx1* mutant mice displayed normal basic motor coordination and spatial memory function compared to wild type mice. However, *Emx1* gene deletion impaired performance in a skilled reaching task and attenuated training-induced hippocampal neurogenesis. Because brain injury recapitulates gene expression patterns that are observed during development or regeneration (Emery et al., 2003), the current study sought to determine whether unilateral TBI modifies functional impairment in the *Emx1* mutant mice under conditions of acallosal phenotype and

reduced neurogenesis. We found that TBI appeared to impair limb reaching function less in mice lacking an intact interhemispheric connection compared to wild type mice. Enhanced neuroplasticity in response to limb training occurred in the acallosal mice only in the TBI group, in contrast to the wild type mice, which displayed this effect with or without brain injury.

Materials and methods

Animals, housing and general considerations

This study was conducted in accordance with the animal care guidelines issued by the National Institutes of Health and by the San Francisco Veterans Affairs Medical Center Animal Care and Use Committee. Adult male mice 2.5 months of age, weighing 24 to 30 g, derived from cryopreserved embryos at Jackson Laboratory were bred and maintained in house in the institutional standard cages (4 mice per cage) on a 12-hr light/12-hr dark cycle with *ad libitum* access to water and food before and during experimental procedures. All procedures including surgery, behavioral assessment and histological quantification were conducted by examiners blinded to experimental conditions.

Induction of traumatic brain injury

Emx1 wild type (WT) and *Emx1* knock out (KO) mice were randomly assigned to either a TBI group or a sham group. Animals were anesthetized with isoflurane/O_2/N_2O (1.5/30/68.5%) during surgery and their core temperature was maintained within 37± 0.5°C with a heating blanket and rectal thermistor servo-loop during both the surgical and the postoperative recovery period.

Controlled cortical impact (CCI) was conducted as described (Neumann et al., 2009, Wang et al., 2011). Secured in a stereotaxic frame (Kopf instrument, Tujunga, CA) followed by a midline skin incision, a 3 mm diameter circular craniotomy was performed with a dental drill, lateral (right side) to the mid-sagittal suture centering at [AP: −2.0 mm; ML: 2.0 mm] relative to Bregma. The mouse was then subjected to a CCI using an impactor of 2.0 mm in diameter, operated by a linear motor and microprocessor controller (Linmot, Zurich, Switzerland). The impactor tip was first centered over the craniotomy and was slowly lowered till the tip just contacted the dura (confirmed by an operating microscope). The impact injury was generated using the following parameters: 1.5 m/sec strike velocity, 1.00 mm depth of penetration, and a 155 msec contact time. The scalp was then closed with sutures and each animal was given 1.0 ml of isotonic saline subcutaneously to prevent dehydration. Sham animals received craniotomy but no impact.

Single pellet reaching task

The skill reaching training was performed as described (Whishaw et al., 1991, Bury and Jones, 2002) (Hong et al., 2007). Mice were reduced to 90% of their starting body weight and maintained at this level throughout the experiment. To determine limb preference, mice were placed on a restricted diet one day prior to training, followed by a brief shaping period for 3 days during which the frequency of using left versus right limb was recorded. Shaping and training required the mouse to reach a single banana-flavored food pellet placed into a

well in front of the clear Plexiglas chamber (13.3 cm long x 21.4 cm high x 9.4 cm wide) where the animal resided. To prevent possible interaction between handedness and the performance of reaching, training was conducted on the preferred limb. To reinforce training of the preferred limb, an aluminum wall was placed ipsilateral to the reaching limb approximately 2 cm from the reaching window, preventing reaches with the non-preferred forelimb. For each reaching trial, mice were permitted up to five reach attempts until the pellet was grabbed successfully, dropped, knocked from its well or not touched at all. Performance was measured as the percentage of successful reaches divided by the total number of reaching trials (successful reaches + missed reaches + dropped pellets). A successful reach was one in which the mouse grabbed the pellet from the well and either brought it to its mouth and ate it, or put it to the floor for later consumption after properly grabbing. Missed reaches include reaches that did not contact the pellet at all or in which the mice knocked the pellet to the outside of the chamber. Drops were considered to be reaches in which the mouse grabbed the pellet but could not properly hold on to it so that it fell down. Training periods consisted of two daily sessions of 30-min or 40 single-pellet trials, whichever came first, for 28 days beginning at one week after CCI or sham surgery. Mice that did not reach for at least 30 pellets per session after 2 weeks of training were excluded from the experiment. Mice were euthanized at the end of the 28-day training.

BrdU Labeling

To determine the effect of motor learning on the survival of newborn hippocampal cells, mice received a single daily intraperitoneal injection of thymidine analog 5-bromo-2′-deoxyuridine-5′-monophosphate (BrdU) (Sigma, St. Louis, MO) at 50 mg/kg for 14 consecutive days, beginning on the first day of skill reaching training.

Tissue preparation, immunohistochemistry staining and cell counting

Animals were anesthetized with ketamine (80 mg/kg; Parke-Davis, Morris Plains, NJ) and xylazine (20 mg/kg; Butler, Columbus, OH) and perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed, fixed overnight in 4% PFA-PB and placed in 20% sucrose for 48 hrs. Coronal sections were cut at 40 μm on a microtome and collected serially. Some sections were stained with lipophilic fluorescence dye Fluoromyelin Red (1:300 dilution; Molecular Probes, Eugene, OR) to verify the presence of absence of corpus callosum. Others were immunostained using the following reagents: goat anti-doublecortin (DCX) (1 μg/ml; Santa Cruz Biotechnologies, Santa Cruz, CA); mouse anti-BrdU (0.25 μg/ml; Roche, Indianapolis, IN); mouse anti-NeuN (1:1000 dilution; Chemicon, Temecula, CA); biotinylated donkey anti-goat, anti-rat and anti-mouse (1000x dilution; Jackson ImmunoResearch Labs, West Grove, PA); ABC Solution (Vector laboratories, Burlingame, CA); diaminobenzine-tetrachloride (DAB Fast; Sigma, St Louis, MO). Unbiased stereological estimation of cell number (Stereo Investigator, MicroBrightField) was determined by quantifying every 6^{th} coronal section, spanning the septal hippocampus. To achieve a coefficient of error (CE) of less than 0.10, counting frames of 15 x 15 x 20 μm were used in a 40 x 40 μm matrix that were randomly superimposed onto the region of interest by the program as described previously (Hong et al., 2007, Wang et al., 2011).

Assessment of TBI lesion volume

TBI lesion cavity size was determined as previously described with modifications (Lam et al., 2013). Briefly, NeuN-stained coronal sections (1 in 6 series) were photographed, and the perimeters of the lesioned hemisphere and the contralateral hemisphere were outlined and areas calculated using NIH-Image J. The area of each lesioned hemisphere was then subtracted from the contralateral hemisphere, summed and multiplied by the section thickness to yield a lesion volume for each brain.

Statistical analyses

Data were expressed as mean ± standard error of mean (SEM). All statistical tests were carried out with Statview software (SAS Institute Inc., Cary, NC, USA). Statistical significance was evaluated using two-way or three-way analyses of variance (ANOVA), or two-way repeated measure ANOVA (RANOVA). Post hoc tests were conducted when appropriate. Differences between groups were considered significant when $p<0.05$.

Results

TBI or acallosal genotype impairs skill-reaching performance

The *Emx1* gene deletion resulted in an acallosal phenotype (Fig 1A), although it did not have an overall effect on the extent of brain injury produced by CCI (Fig 1B), suggesting that *Emx1* does not influence the vulnerability of the brain to TBI. One week after unilateral TBI that impaired the preferred limb, *Emx1* WT and KO mice were subjected to the single-pellet reaching task for a period of 4 weeks using the affected limb (Fig 2). Over the training period, all groups improved on the task as evidenced by a progressive increase in the successful rate of reaching and grasping the pellet (Fig 3; Two-way RANOVA: Day effect: $F_{13, 507}=18.7$, $p<0.0001$). TBI significantly reduced the overall successful rate of reaching (Two-way RANOVA TBI effect: $F_{1, 39}=7.5$, $p<0.01$). The effect of brain injury on reaching was only significant in the WT mice (post hoc: $p<0.05$), compared to the trending non-significant effect in the KO mice (post hoc: $p=0.18$). *Emx1* KO mice performed more poorly in general over the training period compared to their wild type counterparts (Two-way RANOVA Genotype effect: $F_{1, 39}=6.2$, $p<0.05$). Specifically, uninjured WT mice performed better than the uninjured KO mice (post hoc: $p<0.05$). However, *Emx1* KO mice with TBI did not perform significantly worse than WT mice with TBI (post hoc: $p=0.16$), particularly during the first two weeks (Fig 3). Taken together, this suggests that the effect of TBI on impairing skill reaching function is less apparent in the acallosal *Emx1* KO mice compared to WT mice.

Limb training enhances hippocampal neurogenesis in acallosal mice

To determine how limb training affected hippocampal plasticity, we quantified the number of DCX immunoreactive cells in the dentate gyrus of the hippocampus immediately after four weeks of the reaching training in both TBI and sham-operated mice. Consistent with our previous findings, TBI resulted in a reduction in hippocampal neurogenesis (Wang et al., 2011), as demonstrated by a reduction in DCX-expressing immature neurons in the dentate gyrus (Three-way ANOVA: TBI effect: $F_{1, 40}=14.2$, $p<0.001$) (Fig 4A). Limb training

increased hippocampal neurogenesis (Three-way ANOVA: Reaching effect: $F_{1, 40}$=17.5, p<0.0005), while *Emx1* gene deletion had a very strong negative impact on neurogenesis (Three-way ANOVA: genotype effect: $F_{1, 40}$=105.3, p<0.0001). Interestingly, limb training significantly increased hippocampal DCX cells in uninjured *Emx1* WT *mice (p<0.05),* whereas it enhanced hippocampal neurogenesis in the *Emx1* KO mice with TBI (post hoc: p<0.005). The cell proliferation study corroborated that there were fewer surviving new born cells remaining in the hippocampus in *Emx1* KO mice compared to WT mice at the time of detection (Fig 4B; three-way ANOVA: genotype effect: $F_{1, 40}$=16.2, p<0.0005). Our data imply that the limb training had a stronger effect in overcoming TBI-attenuated neurogenesis in the acallosal *Emx1* KO mice as compared to WT mice. It also suggests that TBI synergizes with behavioral training in inducing neuroplasticity in the *Emx1* KO mice, which normally do not respond to training in the absence of brain injury.

Discussion

Indisputable evidence suggests that the *Emx1* transcription factor is involved in mammalian brain development. Although *Emx1* deletion led to structural abnormalities including an acallosal phenotype and smaller dentate gyri, it did not affect basic motor coordination or memory function in naive adult mice (Hong et al., 2007). Interhemispheric inhibition, mediated via transcallosal fibers, is implicated in functional recovery following unilateral brain injury. However, it is unclear whether the acallosal phenotype of the *Emx1* KO mice would exert an effect on forelimb skill reaching function after unilateral TBI. To the best of our knowledge, this is the first study demonstrating a lack of disparity in motor skill reaching function between WT and *Emx1* mice groups that had undergone TBI treatment in contrast to their non-treated counterparts, which showed a significant level of impairment in the acallosal group compared to the wild type. Our results also indicate that skill-reaching training induces hippocampal neurogenesis in the acallosal mice only in those with TBI, unlike in WT mice, which are affected regardless of the brain injury state. This suggests that TBI activates additional neurogenesis signals that normally don’t exist in the naïve acallosal mice, which are typically afflicted by an innate impaired neurogenesis in contrast to their wild type counterparts.

Loss of interhemispheric inhibition, mediated by transcallosal fibers, from the affected cortex can lead to disinhibition of the contralesional hemisphere and its functional consequences (Liepert et al., 2000, Liepert, 2003). A recent study suggests that mirror movements caused by dysfunction of the motor cortex is a likely result of the reduction in commissural inhibition from the affected side, which concomitantly enhanced the activity of the cortico-motoneuronal pathway of the intact side and led to the mirror movements (Tsuboi et al., 2010). Consistent with the literature, we found that TBI had a lesser effect on reaching in the acallosal mice compared to WT mice, suggesting that it was likely attributed to lacking interhemispheric inhibition from the healthy hemisphere to TBI-affected hemisphere in the former mice, particularly during the first two weeks after TBI. This notion is supported by a series of MRI studies in rats with intact corpus callosum showing that there was a reduced functional activation of the ipsilateral side early on after unilateral stroke, and the reinstatement of the ipsilateral sensorimotor cortex around two weeks later was correlated with functional recovery (Dijkhuizen et al., 2003). Nonetheless, the recovery of

finger movement does involve the bilateral primary motor cortex, as evidenced by focal inactivation of the individual cortical regions with microinjected muscimol, a γ-aminobutyric acid type A agonist (Nishimura et al., 2007). The concept of transcallosal inhibition has offered great insight not only in understanding the mechanism underlying functional impairment, but also in developing therapies to promote functional recovery after brain injury. For example, anesthetizing the healthy hand of chronic stroke patients, thereby possibly reducing the abnormal level of inhibition to the affected hemisphere, improves the motor performance of the paretic hand (Floel et al., 2004). Another example is how constraint-induced movement therapy (CIMT), the forced use of the affected limb by immobilization of the healthy limb, which has been shown to enhance functional recovery in patients with motor deficits after stroke (Liepert et al., 1998, Kunkel et al., 1999, Taub and Morris, 2001, DeBow et al., 2003, Taub et al., 2003, Wittenberg et al., 2003, Grotta et al., 2004, Park et al., 2004). The benefits of CIMT are believed to be associated with the decrease of excitability of the healthy motor cortex and an increase in excitability of the affected motor cortex (Traversa et al., 1997). Reduction of excitability in the healthy motor cortex is the consequence of the immobilization, and may lead to decreased transcallosal inhibition and the subsequent increase of the cortical excitability in the affected motor cortex (Liepert et al., 1998). To apply this approach to subjects with TBI, we have recently achieved a similar restraining effect on the forelimb ipsilateral to the side of the TBI via botulinum toxin and obtained significantly improved function in rats subjected to unilateral controlled cortical impact when combined with physical therapy (Lam et al., 2013).

Germ line deletion of the *Emx1* gene decreased the number of proliferating cells and immature neurons in the dentate gyrus subgranular zone during adulthood (Hong et al., 2007). The impaired neurogenesis was also observed in the *Emx1* KO embryonic neural stem cells (NSC) cultured in vitro (Kobeissy et al.), leading to reduced neurosphere-forming frequency and secondary renewal capacity when compared to WT NSCs. The disparity in neurosphere-forming frequency between WT and KO *Emx1* was even greater in the presence of FGF. Our previous comparative proteomics study suggests that *Emx1* deletion might have resulted in alterations in signaling pathways affecting various aspects of neurogenesis including neuroprogenitor cell proliferation and migration. One of the alterations lies in a defect in cofilin phosphorylation induced by VEGF or other growth factors, which may have contributed to the reduced proliferation and migration of NSCs in the embryonic brains of *Emx1* null mice. The third possible affected pathway may be that of the fibroblast growth factor (FGF) signaling, since FGF is involved in progenitor proliferation (Dono, 2003, Reuss et al., 2003, Reuss and von Bohlen und Halbach, 2003), as well as in the development of the corpus callosum in both mice and humans (Dode et al., 2003, Dode and Hardelin, 2004). Additionally, a number of studies also suggest that FGFs are involved in learning and memory (Sasaki et al., 1994, Oomura et al., 1997).

It is reported that CIMT has unexpectedly increased hippocampal gray matter volume bilaterally in stroke patients (Gauthier et al., 2008). CIMT or simple forelimb skill training has also enhanced neurogenesis and functional recovery in rats after experimental stroke (Wurm et al., 2007, Zhao et al., 2009). Consistent with these observations, we found that reaching training increased hippocampal DCX cells in both WT mice groups underwent either TBI or sham surgery, but it did so only in the KO mice with brain injury. This suggests

that TBI may have induced a permissive environment for behavior-induced brain plasticity to occur. TBI-induced permissive environment includes, but not limited to, growth factors such as BDNF, NGF, bFGF, HGH, VEGF, neurotrophin-3, and neurotrophin 4/5, all of which are capable of inducing neurogenesis (Chen et al., 2002, Chodobski et al., 2003). Also in this case, lacking the cc seems to benefit limb training after TBI, which induced more hippocampal neurogenesis. Our results also suggest that limb training enhances neuroplasticity after unilateral brain injury at functionally remote regions, such as the hippocampus, which may have implications for promoting overall recovery of function that is needed for brain injury. In light of the salutary effect of CIMT on motor recovery after stroke, modified CIMT was shown to improve upper limb use and function in patients with TBI as evaluated by the Wolf Motor Function Test (WMFT), Motor Activity Log (MAL) and The Action Research Arm Test (Page and Levine, 2003), or by the WMFT, Fugl-Meyer Motor Performance Assessment and MAL (Shaw et al., 2005). CIMT has also significant efficacy in children in improving the recovery of upper limb function following TBI (Cimolin et al., 2012) or in those with hemiplegic cerebral palsy (Hoare et al., 2007a, Hoare et al., 2007b).

In conclusion, unilateral TBI elicited a lesser impairment in forelimb reaching function in mice without an interhemispheric connection compared to mice with an intact corpus callosum. Furthermore, TBI induces a permissive environment that synergizes with signals induced by behavioral training to enhance neuroplasticity at remote brain regions that may improve overall recovery of function.

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Highlights

- Performance of the skill reaching task is adversely affected by traumatic brain injury
- Unilateral traumatic brain injury induces less limb impairment in mice without an intact hemispheric connection compared to wild type mice
- TBI induces neuroplasticity in the remote brain regions, which may have a wider implication in functional recovery
- TBI induces neuroplasticity and neurogenesis signals that recapitulate the state during brain development

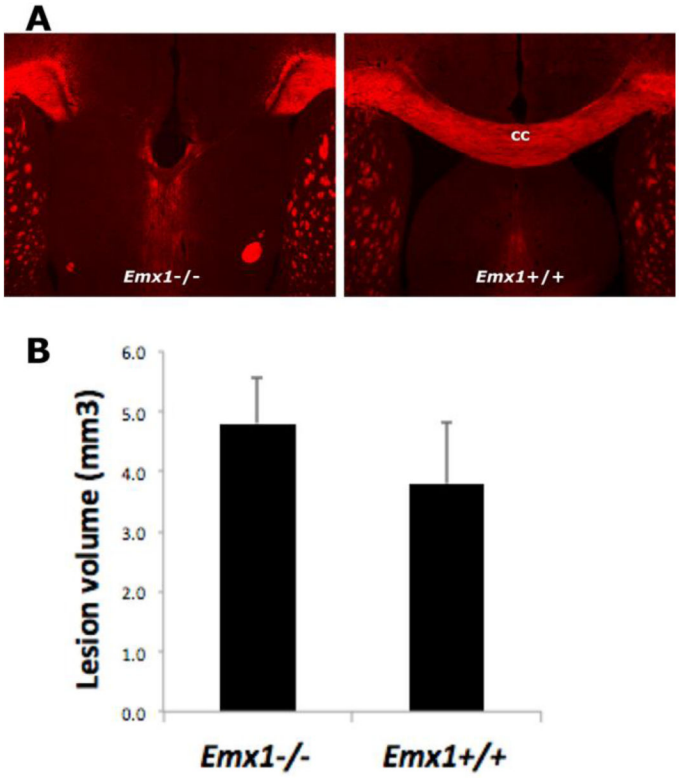

Figure 1. There is no significant difference in the extent of brain injury caused by CCI between the acallosal and wild type mice

A, Representative fluorescent images of coronal sections stained with lipophilic dye Fluoromyelin Red showing the agenesis of the corpus callosum in the *Emx1* / mice compared to their *Emx1*+/+ littermates. ***B***, CCI produced a similar brain lesion volume between the *Emx1* / mice and *Emx1*+/+ mice (p=0.52). N=5–6/group.

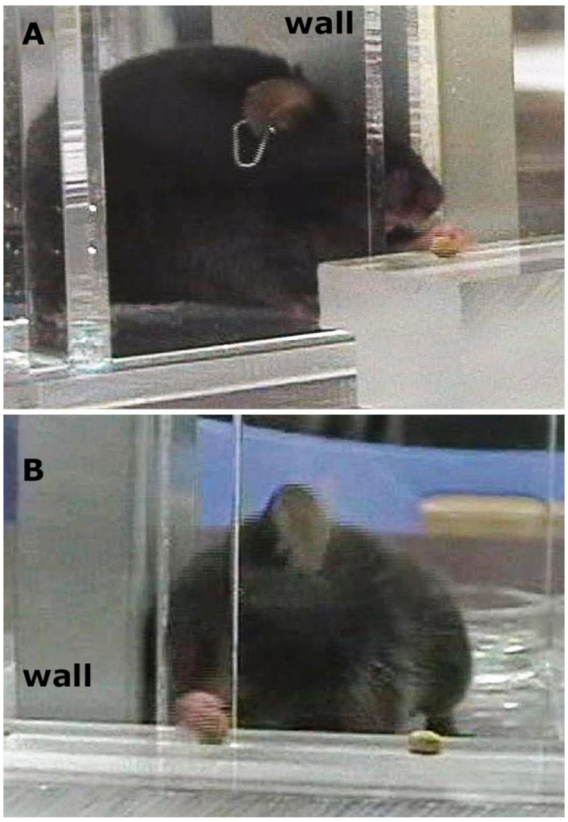

Figure 2. Single pellet reaching task

Photomicrographs showed two mice performing the single pellet reaching task using left (**A**) or right (**B**) forelimb. An aluminum plate (labeled as “wall”) was placed 2 cm in distance from window slits to reinforce the use of the affected limb for each mouse.

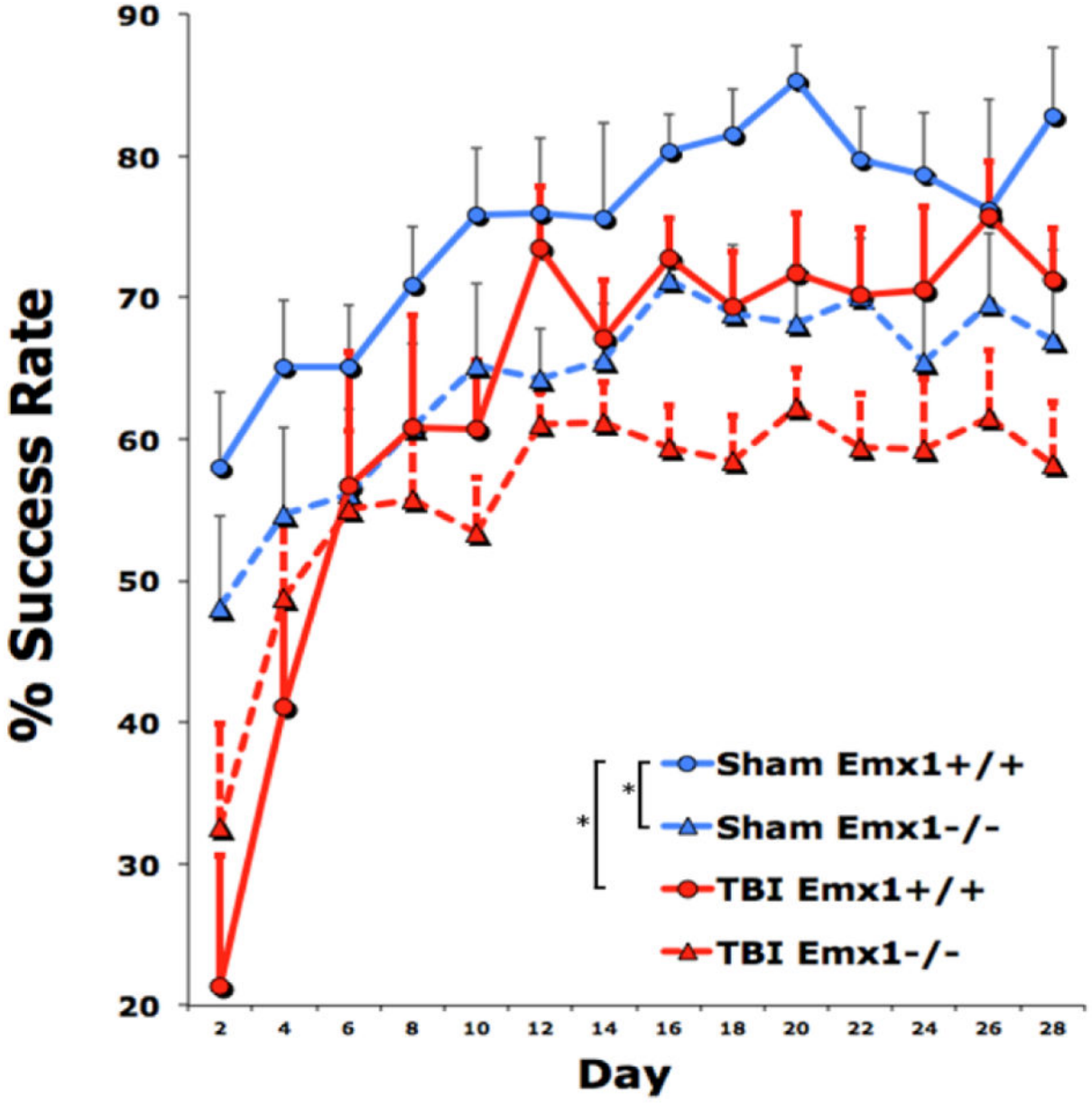

Figure 3. TBI or *Emx1* deletion reduces overall successful rate during skill reaching performance

Over the training period, all groups improved on the task as evidenced by a progressive increase in the successful rate in reaching and grasping the pellet (main effect of training). Both TBI and *Emx1* deletion had overall adverse effects on the performance of the reaching task (main effects of TBI and *Emx1* deletion, respectively). Specifically, WT TBI mice performed significantly worse than WT uninjured mice (*$p<0.05$), whereas WT uninjured mice performed better than KO uninjured ones (*$p<0.05$). Sample sizes are as following: *Emx1*+/+ Sham (N= 12); *Emx1*−/− Sham (N= 14); *Emx1*+/+ TBI (N= 9); *Emx1*−/− TBI (N= 8).

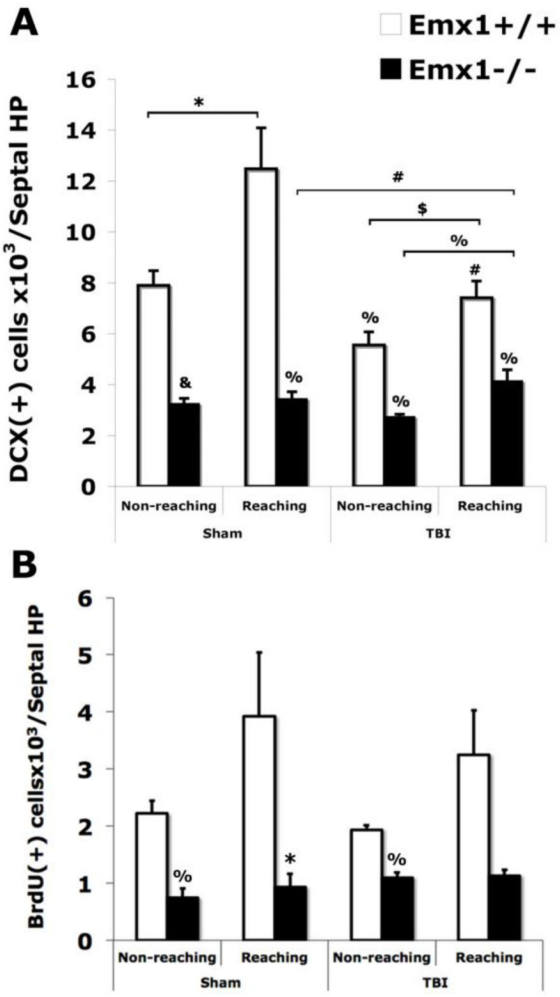

Figure 4. Skill reaching training induces hippocampal neurogenesis in uninjured *Emx1*+/+ mice or in *Emx1*−/− mice with TBI

There was a negative impact on hippocampal neurogenesis by TBI or *Emx1* gene deletion as demonstrated by a reduction in DCX-expressing immature neurons (**A**). Although reaching training had an overall enhancing effect on DCX (+) neurons, it specifically increased immature neurons in WT mice with sham surgery or in KO mice with TBI, suggesting that TBI provided a permissive environment in the KO mice for training-induced neuroplasticity. Significant differences in DCX (+) cells between genotypes are labeled above the columns of *Emx1*−/− as & ($p<0.005$), and % ($p<0.01$). Significant differences in DCX (+) cells between two *Emx1*+/+ non-reaching groups (Sham vs. TBI) and *Emx1*+/+ Reaching groups (Sham vs. TBI) are labeled above the columns of *Emx1*+/+ as % ($p<0.01$) and # ($p<0.05$), respectively. Other significant paired comparisons are as indicated. ***B***, There were also less surviving cells incorporating BrdU in the KO mice compared to WT mice as indicated above the columns of *Emx1*−/− (%$p< 0.005$; *$p<0.05$). Sample sizes are as following: *Emx1*+/+ Sham Non-reaching (N= 5); *Emx1*+/+ Sham Reaching (N= 5); *Emx1*+/+ TBI Non-reaching (N= 9); *Emx1*+/+ TBI Reaching (N= 9); *Emx1*−/− Sham Non-reaching (N= 6); *Emx1*−/−

Sham Reaching (N= 6); *Emx1*−/− TBI Non-reaching (N= 6); *Emx1*−/− TBI Reaching (N= 4).