Progress in Neurobiology 203 (2021) 102073

Contents lists available at ScienceDirect

Progress in Neurobiology

journal homepage: www.elsevier.com/locate/pneurobio

Original Research Article

Combining robotics with enhanced serotonin-driven cortical plasticity improves post-stroke motor recovery

S. Conti [a], C. Spalletti [b], M. Pasquini [a], N. Giordano [b], N. Barsotti [c], M. Mainardi [b], S. Lai [a], A. Giorgi [c], M. Pasqualetti [c,d], S. Micera [a,e,*], M. Caleo [b,f,**]

[a] Translational Neural Engineering Area, The BioRobotics Institute, Scuola Superiore Sant'Anna, Pisa, Italy
[b] Institute of Neuroscience, National Research Council (CNR), Pisa, Italy
[c] Unit of Cell and Developmental Biology, Department of Biology, University of Pisa, Italy
[d] Center for Neuroscience and Cognitive Systems, Istituto Italiano di Tecnologia, Rovereto, Italy
[e] Bertarelli Foundation Chair in Translational NeuroEngineering Laboratory, École Polytechnique Fédérale de Lausanne (EPFL), Centre for Neuroprosthetics and Institute of Bioengineering, Lausanne, Switzerland
[f] Department of Biomedical Sciences, University of Padova, Italy

ARTICLE INFO

Keywords:
Stroke
Robotic rehabilitation
Cortical plasticity
Motor rehabilitation
Serotonergic system
Buspirone

ABSTRACT

Despite recent progresses in robotic rehabilitation technologies, their efficacy for post-stroke motor recovery is still limited. Such limitations might stem from the insufficient enhancement of plasticity mechanisms, crucial for functional recovery. Here, we designed a clinically relevant strategy that combines robotic rehabilitation with chemogenetic stimulation of serotonin release to boost plasticity. These two approaches acted synergistically to enhance post-stroke motor performance. Indeed, mice treated with our combined therapy showed substantial functional gains that persisted beyond the treatment period and generalized to non-trained tasks. Motor recovery was associated with a reduction in electrophysiological and neuroanatomical markers of GABAergic neurotransmission, suggesting disinhibition in perilesional areas. To unveil the translational potentialities of our approach, we specifically targeted the serotonin 1A receptor by delivering Buspirone, a clinically approved drug, in stroke mice undergoing robotic rehabilitation. Administration of Buspirone restored motor impairments similarly to what observed with chemogenetic stimulation, showing the immediate translational potential of this combined approach to significantly improve motor recovery after stroke.

1. Introduction

Stroke is one of the major causes of long-lasting motor disabilities worldwide. Indeed, a substantial fraction of stroke patients does not recover the ability to perform daily activities (Hummel and Cohen, 2006). Several studies highlighted that motor recovery is associated with neural plasticity (Biernaskie, 2004; Langhorne et al., 2009; Allman et al., 2016). In this regard, training intensity is crucial for rehabilitation, because it has been shown to boost activity-dependent plasticity of the spared circuitry and hence improve motor recovery (Raineteau and Schwab, 2001; Kleim and Jones, 2008; Sun and Zehr, 2019). Since the use of robotic systems allows to train with high levels of intensity, repeatability, precision, and consistency, it is considered a promising approach in post-stroke neurorehabilitation (Lo et al., 2010; Klamroth-Marganska et al., 2014; Reinkensmeyer et al., 2016; Raffin and Hummel, 2018; Micera et al., 2020). Recently, clinical trials and meta-analysis exploiting robot-based treatments on stroke patients revealed small but significant upper limb improvements (Hesse et al., 2005; Lo et al., 2010; Veerbeek et al., 2017; Mehrholz et al., 2018; Rodgers et al., 2019), which however were not generalized to non-trained motor functions (Kwakkel et al., 2008; Panarese et al., 2012; Veerbeek et al., 2017; Rodgers et al., 2019). Therefore, coupling robotic rehabilitation with therapies that increase neural plasticity may facilitate cortical reorganization and increase functional restoration of motor abilities (Sonde and Lökk, 2007; Zeiler and Krakauer, 2013; Straudi et al., 2016; Tran et al., 2016; Alia et al., 2017). In line with this hypothesis, we recently showed (Spalletti et al., 2017) that combining robotic rehabilitation with transient inactivation of the contralateral

* Corresponding author at: Translational Neural Engineering Area, The BioRobotics Institute, Scuola Superiore Sant'Anna, Pisa, Italy.
** Corresponding author at: Institute of Neuroscience, National Research Council (CNR) Pisa, Italy.
E-mail addresses: silvestro.micera@epfl.ch (S. Micera), matteo.caleo@in.cnr.it (M. Caleo).

https://doi.org/10.1016/j.pneurobio.2021.102073
Received 6 February 2021; Received in revised form 22 April 2021; Accepted 6 May 2021
Available online 11 May 2021

hemisphere in a mouse model of cortical stroke improves recovery outcomes compared to robotic rehabilitation alone. The contralesional hemisphere has been suggested to be a potential target for neuromodulatory interventions after stroke (Di Pino et al., 2014; Spalletti et al., 2017). Experimental strategies for stimulating post-stroke plasticity often rely on invasive, intracerebral administration of drugs and blocking agents (Gherardini et al., 2015). Therefore, these approaches cannot be easily translated to current clinical protocols and while promising, they still present major implementation roadblocks.

In this study we aimed at designing a clinically-relevant therapeutic paradigm that could offer a realistic path for clinical implementation of combined rehabilitation therapies adding to robotics a plasticizing effect. However, in order to specify a targeted, safe, and effective intervention, a detailed identification of specific signal pathways that could be selectively modulated by clinically approved drugs in human patients is needed.

Specifically, we hypothesized that the serotonergic system might have a role in boosting plasticity and motor recovery. Serotonergic agonists have been associated to movement facilitation (Courtine et al., 2009) and recovery in animal models of stroke and spinal cord injury (Pariente et al., 2001; Chollet et al., 2011). Accumulating evidence suggests that Selective Serotonin Reuptake Inhibitors (SSRIs) can improve functional recovery by promoting post-stroke plasticity and modulating cortical excitability (Dam et al., 1996; Pariente et al., 2001; Zittel et al., 2008; Chollet et al., 2011; Siepmann et al., 2015). Indeed, clinical trials have showed the beneficial effects of combination of fluoxetine, a SSRIs, with physiotherapy on neurological deficit of stroke patients (Chollet et al., 2011). In contrast, when stroke patients were treated with fluoxetine but did not receive any kind of motor rehabilitation, no significant improvement in functional status (measured with the modified Rankin scale) were detected (FOCUS, AFFINITY and EFFECTS studies - (Dennis et al., 2019; Hankey et al., 2020; Lundström et al., 2020)). In light of these results, we hypothesized that serotonin may represent a promising pharmacological target to augment the beneficial effects of motor rehabilitation. The inclusion of physiotherapy for post-stroke treatment appears to be crucial, as it has been shown that the enhancement of plasticity without the guide of an appropriate motor rehabilitation regime is not effective at promoting recovery (Wahl et al., 2014). Particularly, the impact of combining serotonergic stimulation with robotic rehabilitation has not been tested yet.

Based on these considerations, here we combined robotic rehabilitation therapies with chemogenetic stimulation to boost serotonin release in transgenic mice expressing a modified muscarinic receptor (hM3Dq) in serotonergic neurons (Giorgi et al., 2017). Controls were mice expressing the inverted open reading frame of this receptor. We showed that mice experiencing a selective increase in serotonin release during the rehabilitation protocol exhibited superior motor performances on a robotic platform and on two sensitive motor tests (Gridwalk and Schallert Cylinder Test) of forelimb performance. To further clarify the receptor pathway that is associated to the plasticizing effects of serotonin, we focused on the serotonin 1A receptor (Vetencourt et al., 2008). Buspirone, a clinically approved 1A agonist, was administered to wild type stroke mice during robotic-rehabilitation training. These mice showed a recovery of motor performances comparable to that achieved via chemogenetic stimulation. These results indicate a pathway towards clinical application of combined therapies for stroke rehabilitation in human patients.

2. Materials and methods

2.1. Experimental design

All experiments respected ARRIVE guidelines and the EU Council Directive2010/63/EU, and were approved by the Italian Ministry of Health. hM3Dq double-floxed inverse open reading frame (DIO-hM3Dq) (Giorgi et al., 2017) mice were used as control. DIO-hM3Dq were compared with hM3Dq/Pet1-Cre (SER-hM3Dq) (Giorgi et al., 2017) mice expressing Designer Receptor Exclusively Activated by Designer Drugs (DREADD) in serotonergic neurons. For the Buspirone experiments, wild type C57BL/6J mice were used. Animals were randomly allocated to the experimental groups (robotic training, serotonergic stimulation, robot+serotonergic stimulation, etc.). A small cohort (n = 11) of mice were excluded in advance because they failed to reach a sufficient performance (40% of correct reachings) in the pellet grasping task before stroke and were not subjected to photothrombosis. All mice that received the ischemic lesion were included in the study. Before the ischemic lesion, we tested mice for baseline measurements on motor tests (Gridwalk test, Schallert Cylinder test and pellet grasping task). Mice were then tested 2 days after stroke induction to assess the early effect of the lesion before treatment. Five days after stroke, mice started the robotic training for 4 days per week and received either CNO or Buspirone (saline) 90 and 30 minutes before the robotic session, respectively. All the behavioral tests were conducted in the same day of the week, when no therapeutic treatment (robot, CNO, or Buspirone) was applied.

2.2. Surgical procedures

Photothrombotic lesion was induced as previously described (Lai et al., 2015). After stroke induction, a metal post was placed on the occipital bone for head fixation (Spalletti et al., 2014, 2017; Lai et al., 2015).

2.3. Motor tests

To assess general motor performance in mice, we selected the Gridwalk and Schallert cylinder test since they display very little recovery to baseline performance in mice with CFA photothrombosis up to 30 days post-surgery (Clarkson et al., 2010; Lai et al., 2015; Spalletti et al., 2017).Animals were tested on Gridwalk Test and Schallert Cylinder test for 5 minutes once per week as previously described (Lai et al., 2015). The video recordings were analyzed off-line, frame-by-frame by means of a custom designed Graphical User Interface implemented in Matlab. Percentage of foot faults in Grdiwalk Test was computed according to Lai et al. (2015). Performance on the Schallert Cylinder Test was evaluated analyzing the reliance on the ipsilesional forelimb (right forelimb). The number of contacts (on the wall and on the floor) performed by each paw of the animal was counted and the use of the right forelimb is expressed as a % of total use. Skilled Reaching Test and Kinematic Analysis of the whole reaching movements were performed as previously described (Lai et al., 2015). Animals were trained for three weeks to perform a skilled reaching task with their left paw until they reached a plateau in the performance. The baseline value was obtained using the average of the last 3 sessions prior to the stroke. After the lesion, animals were tested once a week. Off ;-line reconstruction of paw trajectories was performed as previously described in Lai et al. (Lai et al., 2015). To ensure consistency, only trajectories from successful trials were considered.

2.4. Robotic Rehabilitation

Mice were trained in daily sessions on a robotic platform (M-Platform). The animals started the daily rehabilitative treatment at day 5 post lesion and continued for 4 days a week until day 37 (5 weeks). A detailed description is provided in Spalletti at al. (2014). Briefly, the M-Platform is a mechatronic device that allows intensive and highly repeatable retraction movements. Each retraction movement is composed by two phases: a passive one, when the actuator extends the injured forelimb of the mouse, and an active one, when the animal has to pull back the handle. In **Supplementary Fig. 1I**, we show representative examples of the position of the movable handle across several trial during the active phase. Animals performed at least 10 cycles of

extension/retraction during each sessions of the first week, and at least 15 cycles during the subsequent 4 weeks. The force threshold to overcome to move the handle was 0.2 N. Position and speed signals were subsequently extracted from video recordings and synchronized with the force signals recorded by a load-cell. Example of speed, position, force and acceleration during a single retraction task are shown in **Supplementary Fig. 1L**. Different parameters about motor performance were automatically computed. Computation and statistical analysis were performed in Matlab.

2.5. PCA analysis

To identify the most important physiological variables for recovery assessment in the retraction task and in the single pellet reaching task, a multistep statistical process based on principal component analysis (PCA) was implemented (Courtine et al., 2009; Takeoka et al., 2014). After selecting all relevant parameters from kinetic and kinematic measures according to the investigated task, a z-score standardization was applied to set zero mean and standard deviation 1 for all parameters. This operation set all data on the same scale, avoiding bias related to the disparate ranges of different parameters. PCA was applied on data from all single trials for all animals: the mathematical transformation allowed the identification of a new orthogonal system where the variance is maximized on each axis. The correlation between parameters and PCs was quantified by factor loadings. For each animal, the Euclidian distance between different conditions along each individual PC was computed.

2.6. Pharmacological treatments

CNO (Clozapine-N-Oxide; Sigma Aldrich, 0.5 mg/kg, 0.125 μg/ml saline) was injected intraperitoneally one hour and a half before the training from day 5 to day 37. Buspirone hydrochloride (Tocris, 8 mg/kg, saline) was delivered daily 30 minutes before the training.

2.7. Immunohistochemical analysis

Anesthetized mice were transcardially perfused with 4% paraformaldehyde (PFA). Brains were cut using a sliding microtome (Leica, Germany) and cortical coronal sections (50-μm thickness) were used for immunostaining. To quantify the lesion volume we used randomly chosen mice from a cohort that was sacrificed at 37 days post-stroke and were not included in the follow-up. We applied a stereological method: one out of every six sections was stained with Hoechst (33258, Sigma Aldrich, USA) and the ischemic area was contoured and measured using a fluorescence microscope (Zeiss, Germany) with a 10x objective. The lesion volume for each mouse was calculated by summing up all damage areas and multiplying this by section thickness and spacing factor (6). A total infarction volume in mm3 is given as the mean±standard error of all analyzed animals. To quantify the number of Parvalbumin and Somatostatin positive neurons, we acquired images with a fluorescence microscope (Zeiss, Germany) using a 10x objective. To ensure consistency, immunopositive cells were counted in all animals in a 200 μm-wide cortical column spanning all layers at the lateral edge of the ischemic tissue, following the methods reported in Alia et al. (2016), using StereoInvestigator software. Similarly, V-GAT, V-GluT1 and PV fluorescence were assessed in regions of interest located at the lateral edge of the ischemic lesion and acquired with a 63x objective and a 2.5 digital zoom, in deep regions (layer V) of the perilesional tissue. Layer V hosts indeed output cells that most directly triggers body movements, and any alterations in their synaptic inputs has a direct impact on movement control. Three sections per animal were examined. For V-GAT and V-Glut1, off-line analysis was performed with a custom designed Graphical User Interface (Matlab). For the fluorescence of PV+ puncta, we imaged serial optical sections and generated maximum intensity projections from 5 consecutive sections; each image was analyzed using Puncta Analyzer plugin (ImageJ; Caleo et al., 2018). To perform immunohistochemical analyses, brain coronal sections were exposed to antibodies against Parvalbumin (1:300, Synaptic Systems, Germany), Somatostatin (1:400, Millipore, Germany), V-GAT (1:800, Synaptic Systems, Germany), and V-GluT1 (1:1000, Synaptic Systems, Germany). *In situ* hybridization (ISH) was performed following standard protocols, as described in Pratelli et al. (Pratelli et al., 2017).

2.8. Patch-clamp electrophysiology

For mIPSC recordings, we used an adaptation of the method described in Testa et al. (2019) to prepare acute slices comprising the perilesional primary motor cortex. Mice were cervically dislocated and the brain was quickly dissected out in ice-cold, oxygenated cutting solution containing (in mM): sucrose 262, HEPES 20, glucose 10, $MgCl_2$ 10, Na-ascorbate 5, Na-pyruvate 3, KCl 2, $CaCl_2$ 0.5, pH 7.4. A Leica VT1200S vibratome was used to cut 300 μm-thick coronal sections, which were transferred to a recovery chamber filled with oxygenated artificial cerebrospinal fluid (aCSF), containing (in mM): NaCl 119, HEPES 10, glucose 10, NaHCO3 6.2, KCl 2.5, CaCl2 2, MgCl2 1.2 NaH2PO4 1, Na-ascorbate 1, pH adjusted to 7.4, held at 32 °C for 30 min. After an additional 60 min at room temperature, sections were placed in a recording chamber continuously perfused with oxygenated aCSF at 32 °C. Tetrodotoxin (1 μM), NBQX (10 μM) and D-AP5 (50 μM) were added to the bath to block evoked synaptic activity, AMPA receptor-mediated currents, and NMDA receptor-mediated currents, respectively, in order to isolate miniature Inhibitory Post Synaptic Currents (mIPSCs). Patch-clamp recordings were performed using borosilicate glass micropipettes having a 4-6 MΩ resistance when filled with an internal solution containing (in mM): CsCl 135, HEPES 10, Na-phosphocreatine 5, Mg-ATP 2.5, EGTA 1.1, Na-GTP 0.25, $CaCl_2$ 0.1, pH 7.3 with CsOH). Recordings were performed in whole-cell configuration while holding the neuron at -70 mV.

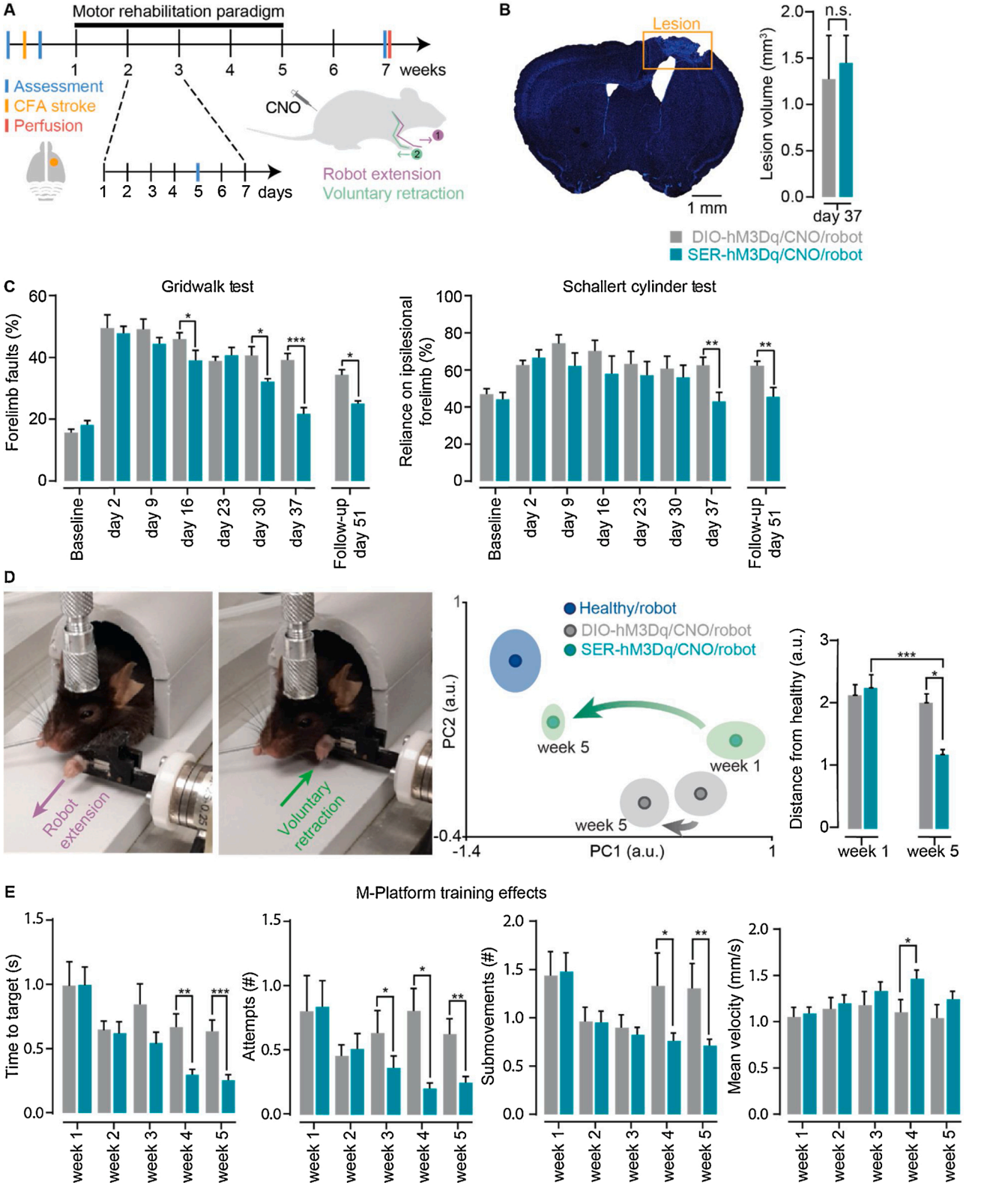
2.9. Statistical analysis

All data are expressed as mean±standard error of the mean (s.e.m.). Statistical tests were performed using Matlab (Mathwork, USA) and SigmaPlot 11.0 (Systat Software Inc, USA). For the longitudinal assessment of motor performance (Gridwalk test, Schallert Cylinder Test, M-Platform) we used two way repeated measures ANOVA (followed by Holm-Sidak test), or Kruskal-Wallis for datasets not normally distributed. For correlation analysis, Spearman correlation was applied. For patch-clamp data, cumulative distributions were tested using Kolmogorov-Smirnov two-sample test. All statistical analyses were performed on raw data. The level of significance was set at *p 0.05, **$p < 0.01$, ***$p < 0.001$.

3. Results

3.1. Combined therapy is required to boost motor recovery

The experimental design is summarized in Fig. 1A. Following the induction of photothrombotic stroke in the motor cortex (caudal forelimb area, CFA, Fig. 1B), we trained adult mice to perform a retraction task on a robotic platform: the M-Platform (Spalletti et al., 2014) (Supplementary Fig. 1A, Supplementary Video 1). The M-Platform is a simple, one-degree-of-freedom rehabilitation device that mimics one of the first robots for rehabilitation in humans, i.e. the Arm-guide (Reinkensmeyer et al., 2000). We previously characterized this platform and demonstrated that the retraction task is skill dependent (i.e., animals progressively learn to perform better and faster) and that mice are impaired in completing the task after a cortical ischemic lesion (Supplementary Fig. 1B, C, Spalletti et al., 2014). Our rehabilitation paradigm consisted of intensive training (4 days a week for 5 weeks) with this robotic device that allows highly-repeatable retraction movements



(caption on next page)

Fig. 1. Combined therapy is required to boost motor recovery. (A) Experimental time schedule. (B) Hoechst-stained coronal brain section showing the lesioned motor cortex, 5 weeks after stroke. Bar graph shows no significant difference in lesion size between the 2 groups examined (DIO-hM3Dq/CNO/Robot group, n=4; SER-hM3Dq/CNO/Robot group, n=7). (C) Motor assessment on generalized motor tests (Gridwalk, on the left and Schallert Cylinder test, on the right), measured longitudinally (once per week) on DIO-hM3Dq/CNO/Robot and SER-hM3Dq/CNO/Robot mice. Percentage of contralesional forelimb faults in the GridWalk test pre-lesion (Baseline), once per week until 37 days post stroke and during the Follow up (7 weeks post stroke). Only the SER-hM3Dq/CNO/Robot group showed significant improvement of forelimb function compared to pre-lesion values (DIO-hM3Dq/CNO/Robot group, n=7; SER-hM3Dq/CNO/Robot group, n=7). Statistical analysis was carried out with two way repeated measures ANOVA. Bar plot on the right shows the percentage of reliance on the ipsilesional forelimb on the Schallert Cylinder test, measured pre-lesion (Baseline), once per week until 37 days post stroke, and during the Follow up (7 weeks post stroke) (DIO-hM3Dq/CNO/Robot group, n=12; SER-hM3Dq/CNO/Robot group, n=12). Statistical analysis was carried out with Kruskal-Wallis. (D) (Left) Retraction task on the M-Platform. (Middle) Principal component analysis (PCA) applied on 19 quantitative parameters for DIO-hM3Dq/CNO/Robot mice, SER-hM3Dq/CNO/Robot mice and a group of mice with no lesion (Healthy/Robot). Parameters are shown in the space defined by PC1 and PC2 for the first and fifth week of rehabilitation. (Right) Bar plots reporting the mean distance between week 1 and week 5 of the 19 parameters of SER-hM3Dq/CNO/Robot and DIO-hM3Dq/CNO/Robot mice compared to the healthy group (DIO-hM3Dq/CNO/Robot group, n=16; SER-hM3Dq/CNO/Robot group, n=23). (E) Bar graphs of average values (DIO-hM3Dq/CNO/Robot group, n=16; SER-hM3Dq/CNO/Robot group, n=23) for four of the quantitative parameters (Time to target, Attempts, i.e. force peaks not overcoming the static friction force, Submovements, i.e. the suprathreshold speed peaks, and Mean velocity) evaluated on the M-Platform. Statistical analysis was carried out with two way repeated measures ANOVA. Data are presented as mean ± SEM. Asterisks indicate significances: *p<0.05, **p<0.01, ***p<0.001.

of the impaired paw (Fig. 1A, D).

To selectively enhance serotonergic signaling, we employed a transgenic mice (Giorgi et al., 2017) expressing a modified muscarinic receptor (hM3Dq) selectively in serotonin (SER) neurons (Supplementary Fig. 1D). Previous studies have shown that delivery of an hM3Dq agonist (clozapine-*N*-oxide, CNO) leads to sustained firing of raphe neurons and robust activation of motor cortical areas in these mice (Giorgi et al., 2017). As a control, we employed mice carrying hM3Dq in an inverted, inactive orientation (hM3Dq-double-floxed inverse open reading frame, DIO-hM3Dq; Supplementary Fig. 1D).

We first examined the effect of our combined robotic therapy against each intervention administered independently. Neither robotic therapy nor stimulation of serotonergic neurons alone had a significant impact on post-stroke motor recovery (Gridwalk test and Schallert Cylinder test, Supplementary Fig. 1E). In agreement with other studies (Clarkson et al., 2010; Spalletti et al., 2017), we found that mice undergoing any of the therapies independently remained impaired up to 37 days post-lesion.

We then analyzed functional recovery in animals that underwent a combined activation of serotonergic neurons and robotic training (SER-hM3Dq/CNO/robot). Specifically, we administered CNO (0.5 mg/kg) before each session with the M-Platform in both SER-hM3Dq and DIO-hM3Dq mice. Mice were tested once per week during the course of the rehabilitation paradigm on the Gridwalk and Schallert Cylinder test to assess motor recovery in generalized motor tasks. Although lesion volumes were superimposable across the two groups at the anatomical level (Fig. 1B), longitudinal analysis of motor performance showed significantly higher functional gains in SER-hM3Dq than DIO-hM3Dq mice (Fig. 1C). Indeed SER-hM3Dq mice showed almost full levels of motor recovery. Interestingly, these motor improvements emerged gradually over time, suggesting a synergistic interaction between robotic training and serotonergic activation (Fig. 1C). To verify that our results were not biased by neuroprotective effects or inter-individual variability in lesion volumes, we correlated the forelimb faults in the Gridwalk test at 37 days with stroke volume and found no correlation between these 2 parameters (Supplementary Fig. 1F). Next, we investigated whether the beneficial effect of the combined treatment was retained over time. Mice underwent an additional follow up performance assessment two weeks after the end of the therapy (follow-up). In both the Gridwalk test and Schallert Cylinder tests, SER-hM3Dq mice that underwent our combined therapy retained their functional improvements (Fig. 1C) showing that motor gains persisted beyond the window of treatment.

We then exploited the capabilities of the M-Platform to execute a detailed movement analysis. We computed a principal component analysis (PCA) on several movement parameters that provide a quantitative assessment of a forelimb retraction movement (Fig. 1D). This analysis revealed that motor recovery in SER-hM3Dq mice was accompanied by the restoration of several parameters such as the time required to accomplish the task and the decrease in number of failed movements ("attempts"). In the analysis we included these failed movements since the corticospinal circuit is activated during sub-threshold force peaks as well; indeed, clear cortical motor evoked potentials are detected also during attempts (Pasquini et al., 2018). All animals rapidly learned the retraction task, as shown by the reduction in the movement time over training session (Fig. 1E). However, SER-hM3Dq mice exhibited a more pronounced and quicker learning curve (reduced movement time, decrease in number of attempts and submovements, enhanced velocity; Fig. 1E). In contrast, DIO-hM3Dq mice did not show any significant change compared to the first week of training (Fig. 1E). Overall, these results highlight that robotic rehabilitation and enhanced serotonin release in combination, but not individually, are beneficial for motor recovery after stroke (Spalletti et al., 2017).

3.2. *Evidence for "true" recovery*

To further characterize whether improvements triggered by our therapies were generated by the development of compensatory strategies, we trained mice in a "Skilled Reaching Task" (Lai et al., 2015) (Fig. 2A). The analysis of the paw trajectories during a reaching and grasping movement is a useful tool to gain information about the movement strategy and patterns (Zeiler and Krakauer, 2013; Lai et al., 2015). Animals were pre-trained for 3 weeks pre-stroke and then tested weekly after the lesion. We found that the percentage of correct reaching movements (endpoints) was reduced 2 days after the cortical infarct and remained persistently impaired in both SER-hM3Dq and DIO-hM3Dq mice. Next, we computed a PCA on several movement parameters to study the kinematic trajectory (Fig. 2B) of successful reaching movements. This analysis shows that the kinematic of SER-hM3Dq mice is closer to pre-lesion movement patterns compared to the control group (Fig. 2C). These results suggest a regain of motor abilities that goes beyond simple compensatory strategies and is near to what we can define a "true recovery" (Zeiler and Krakauer, 2013; Spalletti et al., 2017).

3.3. *Combined treatment reduces neuroanatomical and electrophysiological markers of GABAergic inhibition*

To identify the molecular mechanisms underlying the improved motor recovery observed in SER-hM3Dq mice subjected to combined therapy, we quantified the expression of plasticity markers in the perilesional cortex at the end of our rehabilitation paradigm (37 days post-stroke). As the GABAergic system is one of the principal modulators of post-stroke neuronal plasticity (Clarkson et al., 2010; Alia et al., 2016), and is potently impacted by serotonergic stimulation (Vetencourt et al., 2008), we carried out immunohistochemical analysis for Somatostatin (SOM) and Parvalbumin (PV), which are markers of two key populations of GABAergic interneurons (Deidda et al., 2015). We found a significant decrease in SOM + cells density in SER-hM3Dq mice with respect to

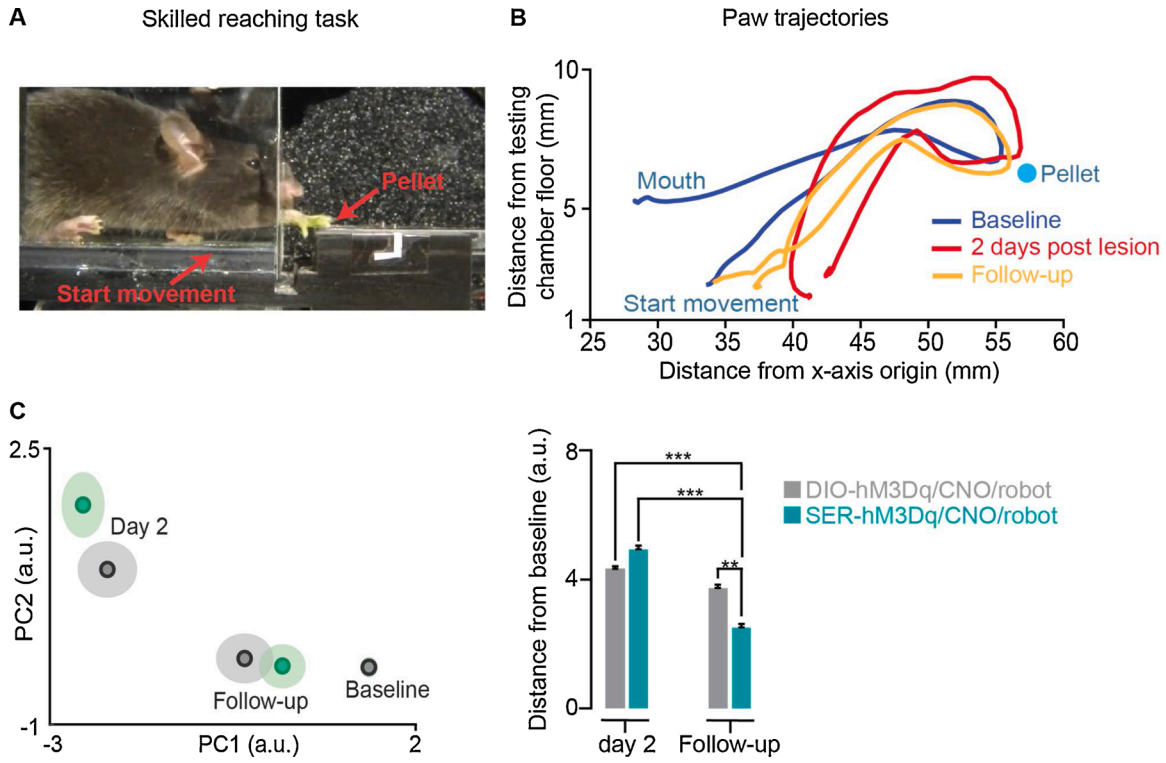


Fig. 2. Combined therapy restores reaching kinematics to prelesion level. (A) Behavioral kinematics. (B) Hand position of the transgenic mice (SER-hM3Dq/CNO/Robot group) performing the Single Pellet Retrieval task before the lesion (Baseline), 2 and 51 (Follow Up) days after the lesion. (C) Principal component analysis (PCA) applied on 26 parameters for DIO-hM3Dq/CNO/Robot and SER-hM3Dq/CNO/Robot mice. Parameters are shown in the space defined by PC1 and PC2 for three different time-points: baseline (before lesion), 2 days and 51 days (Follow up) after the lesion. On the right, bar graph reporting the mean distance between day 2 and Follow up of the 26 parameters of SER-hM3Dq/CNO/Robot and DIO-hM3Dq/CNO/Robot mice compared to the healthy group. Values are plotted as the mean ± SEM. Statistical analysis was carried out with Kruskal-Wallis. Asterisks indicate significances: *p<0.05, **p<0.01, ***p<0.001. (DIO-hM3Dq/CNO/Robot group, n=4; SER-hM3Dq/CNO/Robot group, n=4).

control mice (Fig. 3A). Though PV + cells density appeared unaltered (Fig. 3B), when we analyzed PV expression in "puncta rings" surrounding the soma of perilesional pyramidal neurons, we found a decrease in the mean fluorescence in the SER-hM3Dqgroup (Fig. 3C). To investigate the excitation/inhibition ratio in perilesional cortex, we quantified the mean fluorescence of inhibitory and excitatory terminals impinging on the soma of pyramidal neurons. Vesicular GABA Transporter (V-GAT) significantly decreased in SER-hM3Dq mice compared to DIO-hM3Dq mice (Fig. 3D). We next quantified the mean fluorescence of Vesicular Glutamate Transporter 1 (V-GluT1) and found no changes between the two groups (Fig. 3E). Overall, these neuroanatomical data demonstrate significant reductions in GABAergic markers at the termination of therapy.

To test the idea that the combined treatment triggers an early reduction of GABAergic signaling which allows rehabilitation-induced remodeling of spared circuits, we used patch-clamp recordings to analyze miniature Inhibitory Post Synaptic Currents (mIPSCs) of perilesional pyramidal neurons 21 days after the infarct. We chose this time point as it precedes the appearance of motor improvements in SER-hM3Dq mice as observed in our behavioral data (Fig. 1C). We found that the frequency of mIPSCs recorded in brain slices from SER-hM3Dq mice was significantly lower than in DIO-hM3Dq mice (Fig. 3F, G). These results indicate an early decrease of spontaneous inhibitory drive onto pyramidal neurons in perilesional areas. Interestingly, the amplitude of mIPSCs was significantly higher in SER-hM3Dq mice compared to DIO-hM3Dq mice (Fig. 3F, H). Altogether, the anatomical and electrophysiological data concur in indicating a downregulation of presynaptic inhibitory markers in the perilesional cortex of SER-hM3Dq mice compared to DIO-hM3Dq mice after the combined treatment.

3.4. Activation of serotonin 1A receptor during training promotes recovery in a translational experiment

Finally, we sought to investigate whether the beneficial effects of this approach could be reproduced using clinically relevant compounds affecting specific neuromodulatory pathways. The serotonergic system can be stimulated in humans with pharmacological treatments including SSRIs (Pariente et al., 2001). To test a more targeted approach, we focused on the 1A serotonin receptor (5-HT_{1A}) which is known to play a major role in the neuroplastic effects of serotonin (Vetencourt et al., 2008; Maya Vetencourt et al., 2011). We confirmed expression of the 5-HT_{1A} receptor in peri-infarct tissue at 5 days (i.e., during the "critical period" for post stroke plasticity (Zeiler and Krakauer, 2013); Fig. 4A), suggesting its possible involvement in motor recovery. To test this hypothesis, we engaged the serotonergic network pharmacologically by systemic administration of the clinically approved drug Buspirone, a 5-HT_{1A} receptor agonist. Wild type mice with CFA-targeted photothrombotic lesion received daily Buspirone or Saline with intra-peritoneal injections and underwent robotic training starting after 30 min (Lee et al., 2017). Administration of Buspirone in combination with robotic rehabilitation resulted in enhanced motor improvement after stroke compared to control groups, thus mimicking the effects that we obtained in SER-hM3Dq mice (Gridwalk and Schallert Cylinder Test, Fig. 4B and Supplementary Fig. 1G). Importantly, these functional gains were maintained during a follow-up of two weeks in which therapeutic treatments were suspended (Fig. 4B).

4. Discussion

Significant efforts are currently devoted to identify effective

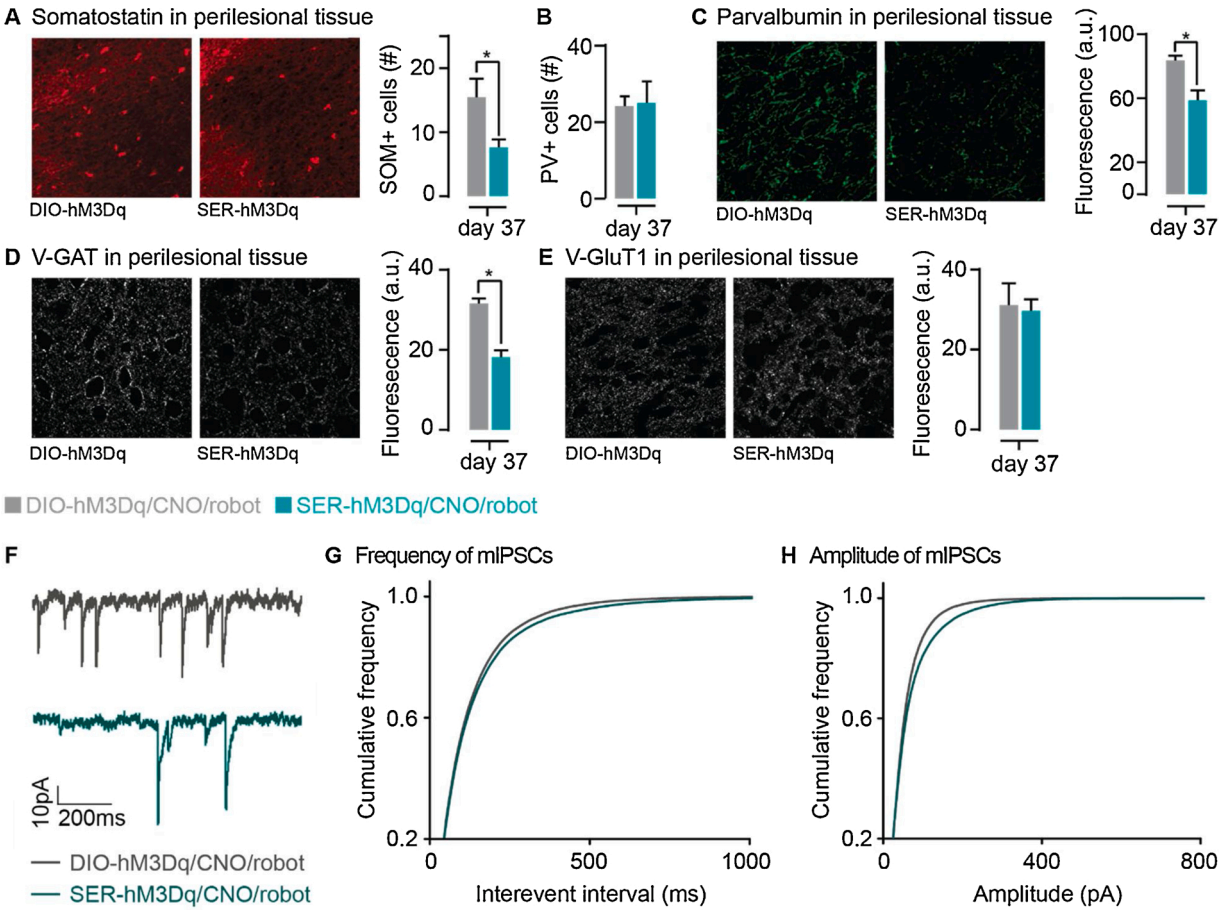


Fig. 3. Changes in plasticity markers in perilesional areas. (A) Representative SOM immunostainings taken in perilesional areas in DIO-hM3Dq/CNO/Robot and SER-hM3Dq/CNO/Robot at 37 days post stroke. Bar plot represents the number of SOM+ cells in 200μm wide column lateral to the lesion at 37 days post injury. SOM+ cells are significantly reduced in SER-hM3Dq animals. (B) Number of PV-positive cells in 200μm wide column lateral to the lesion at 37 days post injury. No significant difference in PV+ cells has been found between the 2 groups. (C) Representative PV immunostainings taken in perilesional areas in DIO-hM3Dq/CNO/Robot and SER-hM3Dq/CNO/Robot at 37 days post stroke. The bar graph shows the quantification of fluorescence in PV puncta rings; fluorescence is decreased in SER-hM3Dq mice. (D) Mean fluorescence intensity of V-GAT-positive and (E) V-Glut1-positive profiles in puncta rings surrounding the soma of pyramidal neurons, lateral to the stroke. While a significant reduction in fluorescence for V-GAT is observed 37 days after injury in Ser-HM3+/CNO/Robot group, V-Glut1 staining is similar between the two groups. Data are presented as means ± SEM. Statistical analysis was carried out with Kruskal-Wallis. Asterisks indicate significances: *$p<0.05$. (n=4 mice per group). (F) Representative traces from patch-clamp recordings of mIPSCs in perilesional pyramidal neurons with quantifications of (G) lower frequency (i.e., longer inter-event interval; DIO-hM3Dq n=45,648 events, 10 cells, 3 mice; SER-hM3Dq n=36,540 events, 12 cells, 3 mice; Kolmogorov-Smirnov test, $p<0.0001$) and (H) higher amplitude (DIO-hM3Dq n=45,673 events, 10 cells, 3 mice; SER-hM3Dq n=36,565 events, 12 cells, 3 mice; Kolmogorov-Smirnov test, $p<0.0001$) in SER-hM3Dq mice compared to DIO-hM3Dq mice.

rehabilitation strategies to improve motor recovery after stroke. Recent studies provided important evidences that optimal functional repair after brain injuries requires a combination of different therapies (Zeiler and Krakauer, 2013; Spalletti et al., 2017) – such as pharmacological/neuromodulatory approaches to increase cortical plasticity and physical therapy.

Unfortunately, the approaches proposed to increase plasticity are often difficult to translate to patient studies (Ward, 2017). Several pharmacological treatments, tailored for different neurotransmitters, have been explored as possible targets to augment neuroplasticity. In particular, SSRIs have been proven to modulate neural excitability and promote plastic changes that lead to an improved motor rehabilitation after stroke (Chollet et al., 2011).

However, the specific mechanisms underlying these beneficial effects of serotonin have not been completely elucidated. To this aim, in this study, we implemented a rehabilitation protocol based on the use of a robotic platform (Spalletti et al., 2014; Pasquini et al., 2018) and a targeted, chemogenetic mouse model able to selectively activate the serotonergic system. This approach allowed us to achieve two very important goals. First, we showed that a selective stimulation of the serotonergic system results in robust recovery of forelimb function after a focal cortical stroke, but only when combined with intensive (robot--based) rehabilitation training. This combination has a synergistic effect on motor recovery after stroke, resulting in an enhanced behavioral and functional outcome. Indeed, mice undergoing the combined treatment exhibited almost full restoration of forelimb function that persisted over time. In contrast, as shown in Suppl. Fig. 1E, mice undergoing single treatments showed no significant improvements in generalized motor tests over the 5 weeks of treatment. These results demonstrate that increased neuroplasticity is a supportive factor but it is not sufficient alone to promote motor recovery after stroke. Our neuromodulation strategy is able to boost the therapeutic effect of the robotic rehabilitation and, at the same time, robotic training channels plasticity of the spared circuits towards a successful functional output. Moreover, our kinematic analysis suggested the restoration of pre-lesion movement patterns rather than the development of compensatory strategies. The combined treatment is able to generalize the effect of the robotic training, leading to significant improvements in untrained motor tasks (Gridwalk and Schallert Cylinder tests). This is a key result that will trigger further studies aimed at validating the application of our

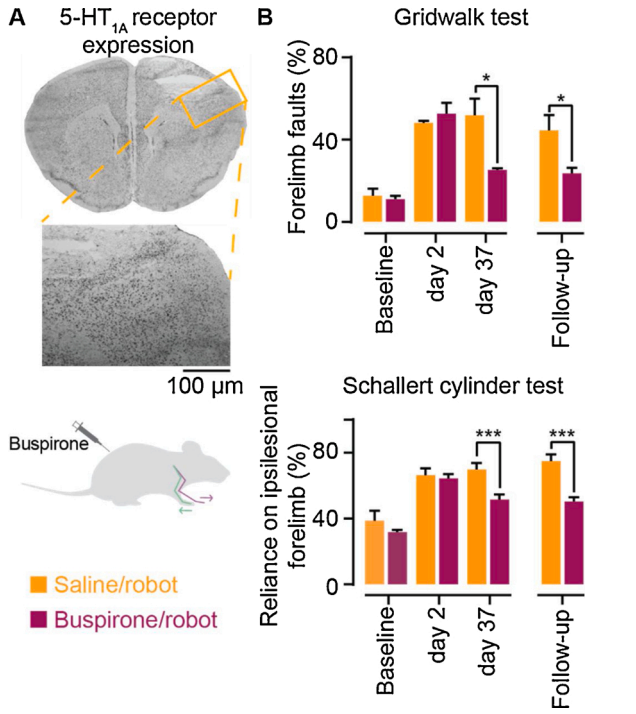


Fig. 4. (A) Representative coronal section showing expression of 5-HT1$_A$ receptor in perilesional areas at 5 days post stroke in wild type animals. (B) Stroke animals were trained daily in the M-Platform. Those that received Buspirone therapy (purple) performed significantly better in Gridwalk and in Schallert Cylinder test than stroke animals injected with saline (yellow). Data are presented as means ± SEM. Statistical analysis was carried out with Kruskal-Wallis for the Gridwalk test and with two way repeated measures ANOVA for the Schallert Cylinder test. Asterisks indicate significances: *p<0.05; saline group, n=3; Buspirone group, n=8.

therapeutic paradigm into clinical practice where it is crucial to assure motor recovery that translates to a broad spectrum of motor abilities.

Second, these experiments allowed us to dissect the pivotal role of serotonin in the enhancement of plasticity that would be unachievable with the delivery of unspecific pharmacological interventions. Here, following up on previous observations (Chollet et al., 2011), we provided strong evidence that the serotonergic system is a valid target for neuroplastic interventions. Interestingly, enhancing 5-HT signaling has been shown to facilitate the recovery of locomotion after a spinal cord injury in rodents (Courtine et al., 2009).One possible explanation for this result might be that one major function of the brain serotonergic system is to facilitate motor output, suggesting that 5-HT administration might be more efficient when paired with training (Jacobs and Fornal, 1997). We examined neuroplastic alterations in GABAergic neurons, a major target of serotonin action (Vetencourt et al., 2008) in peri-infarct regions, which are critical for functional restoration after stroke (Starkey et al., 2012; Harrison et al., 2013). We found that the behavioral recovery triggered by the combined therapy was associated with a significant downregulation of inhibitory GABAergic markers (i.e., SOM-+ interneurons, PV-+ boutons and VGAT-+ terminals), which act as "plasticity brakes" (Zeiler et al., 2013). Specifically, a decrease in PV labeling has been associated with improved behavioral outcome after stroke (Hakon et al., 2018). These neuroanatomical findings were paralleled by functional data, showing a dampened mIPSC frequency in perilesional pyramidal neurons. On the other hand, homeostatic compensation can explain the higher mIPSC amplitude in animals subjected to our combined therapy. The decrease in SOM-+ neurons, PV-+ boutons, VGAT-+ terminals and mIPSC frequency indicate that the enhanced recovery in animals with combined treatment is accompanied by modulation of GABAergic inhibitory neurotransmission. These results are in line with growing evidence that a decrease in GABAergic inhibition may trigger brain plasticity of the spared areas and facilitate motor recovery (Clarkson et al., 2010; Alia et al., 2016; Hakon et al., 2018; Johnstone et al., 2018).

While the reduced GABAergic signaling is a likely mechanism underlying recovery, modulation of inflammation and/or angiogenesis may also play a role. Inflammation has a dual role in stroke pathophysiology, since it may enhance infarct size but also contribute to improved neurological outcomes (Lambertsen et al., 2019). On the other hand, stimulation of angiogenesis is known to promote recovery after stroke by enhancing neuronal plasticity and reorganization of synaptic contacts. In line with this idea, our previous work has shown that a combined rehabilitation regime enhances vascular density in the peri-infarct zone (Allegra Mascaro et al., 2019).

Finally, this increased understanding of serotonin basic mechanisms allowed us to design a clinically relevant approach to target the 5-HT$_{1A}$ serotonin receptor, which is one key regulator of neuroplasticity (Lesch and Waider, 2012). One potential mechanism driving the plasticity-promoting effect of this receptor is the disinhibition of pyramidal cortical neurons mediated by reduced firing of GABAergic interneurons (Starkey et al., 2012). Specifically, our data demonstrate that activation of this receptor, through the delivery of Buspirone, a clinically approved 5-HT$_{1A}$ agonist (Harrison et al., 2013), in combination with intense robotic rehabilitation promotes functional recovery, thus opening perspectives for a targeted pharmacological intervention. Mice receiving Buspirone in combination with robotic rehabilitation performed better on the training task compared to control; moreover, their regained skills were transferred also to non-trained, generalized motor tasks. As already described for the experiments with transgenic mice, also in this case only the combination of both, Buspirone and robotic rehabilitation, was able to induce significant functional improvements 5 weeks after treatment, highlighting an important synergistic effect.

Challenges lie ahead. In fact, further translational studies are needed to adapt our approach to humans, in particular to investigate specific time windows, and drug dosing. However, the fact that this combined treatment enhance generalized motor recovery could represent a very important turning point for the development of more effective future stroke therapies with great potential for clinical application. Overall our results provide strong evidences about the therapeutic potentials of a new combined protocol and open promising avenues to significantly improve motor recovery in people with stroke.

Author contributions

SM, MC and SC conceived the study. SC conducted all the experiments and performed the data analysis. CS and NG conducted the experiments. AG and MP provided the transgenic mice. SL developed the M-Platform and performed the analysis of the results. MP performed the data analysis. NG and NB performed neuroanatomical experiments. MM performed the patch-clamp electrophysiology experiments. SC, SM and MC wrote the manuscript; all authors edited the manuscript. MP supervised the activities and the analysis related to the transgenic animals. SM supervised the activities related to the development of the M-Platform and to the analysis of the results. MC supervised the experimental activities and the analysis of the results.

Declaration of Competing Interest

The authors declare no competing interests.

Acknowledgements

The authors would like to thank Francesca Biondi for the care provided to the animals and Dr. Marco Capogrosso for the inspiring discussions. This work was supported by RONDA and PERSONA projects, funded by the Government of Tuscany – Bando FAS Salute 2014 and

Bando Salute 2018. The work was also supported by the Swiss National Science Foundation through the National Centre of Competence in Research (NCCR) Robotics and by the Bertarelli Foundation. MC has received funding from the H2020 EXCELLENT SCIENCE - European Research Council (ERC) under grant agreement ID n. 692943 (BrainBIT) and from Fondazione Cassa di Risparmio di Padova e Rovigo (Bando Ricerca Scientifica di Eccellenza 2018). Special thanks to the project "EBRI-CNR research agreement" for supporting research activity in CNR-Neuroscience Institute.

Appendix A. The Peer Review Overview and Supplementary data

The Peer Review Overview and Supplementary data associated with this article can be found in the online version, at doi: https://doi.org/10.1016/j.pneurobio.2021.102073.

References

Alia, C., Spalletti, C., Lai, S., Panarese, A., Micera, S., Caleo, M., 2016. Reducing GABA A-mediated inhibition improves forelimb motor function after focal cortical stroke in mice. Sci. Rep. 6, 1–15.

Alia, C., Spalletti, C., Lai, S., Panarese, A., Lamola, G., Bertolucci, F., et al., 2017. Neuroplastic changes following brain ischemia and their contribution to stroke recovery: novel approaches in neurorehabilitation. Front. Cell. Neurosci. 11, 76.

Allegra Mascaro, A.L., Conti, E., Lai, S., Di Giovanna, A.P., Spalletti, C., Alia, C., et al., 2019. Combined rehabilitation promotes the recovery of structural and functional features of healthy neuronal networks after stroke. Cell Rep. 28, 3474–3485 e6.

Allman, C., Amadi, U., Winkler, A.M., Wilkins, L., Filippini, N., Kischka, U., et al., 2016. Ipsilesional anodal tDCS enhances the functional benefits of rehabilitation in patients after stroke. Sci. Transl. Med.

Biernaskie, J., 2004. Efficacy of rehabilitative experience declines with time after focal ischemic brain injury. J. Neurosci. 24, 1245–1254.

Caleo, M., Spinelli, M., Colosimo, F., Matak, I., Rossetto, O., Lackovic, Z., et al., 2018. Transynaptic action of botulinum neurotoxin type a at central cholinergic boutons. J. Neurosci. 38, 10329–10337.

Chollet, F., Tardy, J., Albucher, J.F., Thalamas, C., Berard, E., Lamy, C., et al., 2011. Fluoxetine for motor recovery after acute ischaemic stroke (FLAME): a randomised placebo-controlled trial. Lancet Neurol. 10, 123–130.

Clarkson, A.N., Huang, B.S., Macisaac, S.E., Mody, I., Carmichael, T., South, D., et al., 2010. Reducing excessive GABAergic tonic inhibition promotes post- stroke functional recovery. Nature 468, 305–309.

Courtine, G., Gerasimenko, Y., Van Den Brand, R., Yew, A., Musienko, P., Zhong, H., et al., 2009. Transformation of nonfunctional spinal circuits into functional states after the loss of brain input. Nat. Neurosci. 12, 1333–1342.

Dam, M., Tonin, P., De Boni, A., Pizzolato, G., Casson, S., Ermani, M., et al., 1996. Effects of fluoxetine and maprotiline on functional recovery in poststroke hemiplegic patients undergoing rehabilitation therapy. Stroke 27, 1211–1214.

Deidda, G., Allegra, M., Cerri, C., Naskar, S., Bony, G., Zunino, G., et al., 2015. Early depolarizing GABA controls critical-period plasticity in the rat visual cortex. Nat. Neurosci. 18, 87–96.

Dennis, M., Forbes, J., Graham, C., Hackett, M.L., Hankey, G.J., House, A., Lewis, S., Lundström, E., Sandercock, P., Mead, G., 2019. Fluoxetine and fractures after stroke: exploratory analyses from the FOCUS trial. Stroke 50 (Nov. (11)), 3280–3282. https://doi.org/10.1161/STROKEAHA.119.026639. Epub 2019 Aug 20. PMID: 31426731.

Di Pino, G., Pellegrino, G., Assenza, G., Capone, F., Ferreri, F., Formica, D., et al., 2014. Modulation of brain plasticity in stroke: a novel model for neurorehabilitation. Nat. Rev. Neurol. 10, 597.

Gherardini, L., Gennaro, M., Pizzorusso, T., 2015. Perilesional treatment with chondroitinase ABC and motor training promote functional recovery after stroke in rats. Cereb. Cortex 25, 202–212.

Giorgi, A., Migliarini, S., Galbusera, A., Maddaloni, G., Mereu, M., Margiani, G., et al., 2017. Brain-wide mapping of endogenous serotonergic transmission via chemogenetic fMRI. Cell Rep. 21, 910–918.

Hakon, J., Quattromani, M.J., Sjölund, C., Tomasevic, G., Carey, L., Lee, J.M., et al., 2018. Multisensory stimulation improves functional recovery and resting-state functional connectivity in the mouse brain after stroke. Neuroimage Clin. 17, 717–730.

Hankey, G.J., Hackett, M.L., Almeida, O.P., Flicker, L., Mead, G.E., Dennis, M.S., et al., 2020. Safety and efficacy of fluoxetine on functional outcome after acute stroke (AFFINITY): a randomised, double-blind, placebo-controlled trial. Lancet Neurol. 19, 651–660.

Harrison, T.C., Silasi, G., Boyd, J.D., Murphy, T.H., 2013. Displacement of sensory maps and disorganization of motor cortex after targeted stroke in mice. Stroke 44, 2300–2306.

Hesse, S., Werner, C., Pohl, M., Rueckriem, S., Mehrholz, J., Lingnau, M.L., 2005. Computerized Arm Training Improves the Motor Control of the Severely Affected Arm After Stroke. Stroke 36, 1960–1966.

Hummel, F.C., Cohen, L.G., 2006. Non-invasive brain stimulation: a new strategy to improve neurorehabilitation after stroke? Lancet Neurol. 5, 708–712.

Jacobs, B.L., Fornal, C.A., 1997. Serotonin and motor activity. Curr. Opin. Neurobiol. 7, 820–825.

Johnstone, A., Levenstein, J.M., Hinson, E.L., Stagg, C.J., 2018. Neurochemical changes underpinning the development of adjunct therapies in recovery after stroke: A role for GABA? JCBFM 38 (9), 1564.

Klamroth-Marganska, V., Blanco, J., Campen, K., Curt, A., Dietz, V., Ettlin, T., et al., 2014. Three-dimensional, task-specific robot therapy of the arm after stroke: a multicentre, parallel-group randomised trial. Lancet Neurol. 13, 159–166.

Kleim, J.A., Jones, T.A., 2008. Principles of experience-dependent neural plasticity: implications for rehabilitation after brain damage. J. Speech Lang. Hear. Res. 51, S225.

Kwakkel, G., Kollen, B.J., Krebs, H.I., 2008. Effects of robot-assisted therapy on upper limb recovery after stroke: a systematic review. Neurorehabil. Neural Repair 22, 111–121.

Lai, S., Panarese, A., Spalletti, C., Alia, C., Ghionzoli, A., Caleo, M., et al., 2015. Quantitative kinematic characterization of reaching impairments in mice after a stroke. Neurorehabil. Neural Repair 29, 382–392.

Lambertsen, K.L., Finsen, B., Clausen, B.H., 2019. Post-stroke inflammation—target or tool for therapy? Acta Neuropathol. 137, 693–714.

Langhorne, P., Coupar, F., Pollock, A., 2009. Motor recovery after stroke: a systematic review. Lancet Neurol. 8, 741–754.

Lee, K.M., Coelho, M.A., Sern, K.R., Class, M.A., Bocz, M.D., Szumlinski, K.K., 2017. Anxiolytic effects of buspirone and MTEP in the porsolt forced swim test. Chronic Stress 1, 247054701771298.

Lesch, K.P., Waider, J., 2012. Serotonin in the Modulation of Neural Plasticity and Networks: Implications for Neurodevelopmental Disorders. Neuron 76, 175–191.

Lo, A.C., Guarino, P.D., Richards, L.G., Haselkorn, J.K., Wittenberg, G.F., Federman, D. G., et al., 2010. Robot-assisted therapy for long-term upper-limb impairment after stroke. N. Engl. J. Med. 362, 1772–1783.

Lundström, E., Isaksson, E., Näsman, P., Wester, P., Mårtensson, B., Norrving, B., et al., 2020. Safety and efficacy of fluoxetine on functional recovery after acute stroke (EFFECTS): a randomised, double-blind, placebo-controlled trial. Lancet Neurol. 19, 661–669.

Maya Vetencourt, J.F., Tiraboschi, E., Spolidoro, M., Castrén, E., Maffei, L., 2011. Serotonin triggers a transient epigenetic mechanism that reinstates adult visual cortex plasticity in rats. Eur. J. Neurosci. 33, 49–57.

Mehrholz, J., Pohl, M., Platz, T., Kugler, J., Elsner, B., 2018. Electromechanical and robot-assisted arm training for improving activities of daily living, arm function, and arm muscle strength after stroke. Cochrane Database Syst. Rev. Sep 03;9:CD006876. PMID: 26559225; PMCID: PMC6465047.

Micera, S., Caleo, M., Chisari, C., Hummel, F.C., Pedrocchi, A., 2020. Advanced neurotechnologies for the restoration of motor function. Neuron 105, 604–620.

Panarese, A., Colombo, R., Sterpi, I., Pisano, F., Micera, S., 2012. Tracking motor improvement at the subtask level during robot-aided neurorehabilitation of stroke patients. Neurorehabil. Neural Repair 26, 822–833.

Pariente, J., Loubinoux, I., Carel, C., Albucher, J.F., Leger, A., Manelfe, C., et al., 2001. Fluoxetine modulates motor performance and cerebral activation of patients recovering from stroke. Ann. Neurol. 50, 718–729.

Pasquini, M., Lai, S., Spalletti, C., Cracchiolo, M., Conti, S., Panarese, A., et al., 2018. A robotic system for adaptive training and function assessment of forelimb retraction in mice. IEEE Trans. Neural Syst. Rehabil. Eng. 26, 1803–1812.

Pratelli, M., Migliarini, S., Pelosi, B., Napolitano, F., Usiello, A., Pasqualetti, M., 2017. Perturbation of serotonin homeostasis during adulthood affects serotonergic neuronal circuitry. eNeuro 4. ENEURO.0376-16.2017.

Raffin, E., Hummel, F.C., 2018. Restoring motor functions after stroke: multiple approaches and opportunities. Neuroscientist 24, 400–416.

Raineteau, O., Schwab, M.E., 2001. Plasticity of Motor Systems after Incomplete Spinal Cord Injury.

Reinkensmeyer, D.J., Kahn, L.E., Averbuch, M., McKenna-Cole, A., Schmit, B.D., Rymer, W.Z., 2000. Understanding and treating arm movement impairment after chronic brain injury: progress with the ARM guide. J. Rehabil. Res. Dev. 37, 653–662.

Reinkensmeyer, D.J., Burdet, E., Casadio, M., Krakauer, J.W., Kwakkel, G., Lang, C.E., et al., 2016. Computational neurorehabilitation: modeling plasticity and learning to predict recovery. J. Neuroeng. Rehabil. 13, 42.

Rodgers, H., Bosomworth, H., Krebs, H.I., van Wijck, F., Howel, D., Wilson, N., et al., 2019. Robot assisted training for the upper limb after stroke (RATULS): a multicentre randomised controlled trial. Lancet 394, 51–62.

Siepmann, T., Penzlin, A.I., Kepplinger, J., BM-W, Illigens, Weidner, K., Reichmann, H., et al., 2015. Selective serotonin reuptake inhibitors to improve outcome in acute ischemic stroke: possible mechanisms and clinical evidence. Brain Behav. 5 n/a-n/a.

Sonde, L., Lökk, J., 2007. Effects of amphetamine and/or l-dopa and physiotherapy after stroke? A blinded randomized study. Acta Neurol. Scand. 115, 55–59.

Spalletti, C., Lai, S., Mainardi, M., Panarese, A., Ghionzoli, A., Alia, C., et al., 2014. A robotic system for quantitative assessment and poststroke training of forelimb retraction in mice. Neurorehabil. Neural Repair 28, 188–196.

Spalletti, C., Alia, C., Lai, S., Panarese, A., Conti, S., Micera, S., et al., 2017. Combining robotic training and inactivation of the healthy hemisphere restores pre-stroke motor patterns in mice. Elife 6, 1–31.

Starkey, M.L., Bleul, C., Zörner, B., Lindau, N.T., Mueggler, T., Rudin, M., et al., 2012. Back seat driving: hindlimb corticospinal neurons assume forelimb control following ischaemic stroke. Brain 135, 3265–3281.

Straudi, S., Fregni, F., Martinuzzi, C., Pavarelli, C., Salvioli, S., Basaglia, N., 2016. tDCS and robotics on upper limb stroke rehabilitation: effect modification by stroke duration and type of stroke. Biomed Res. Int. 2016, 1–8.

Sun, Y., Zehr, E.P., 2019. Training-induced neural plasticity and strength are amplified after stroke. Exerc. Sport Sci. Rev. 47, 223–229.

Takeoka, A., Vollenweider, I., Courtine, G., Arber, S., 2014. Muscle spindle feedback directs locomotor recovery and circuit reorganization after spinal cord injury. Cell 159, 1626–1639.

Testa, G., Olimpico, F., Pancrazi, L., Borello, U., Cattaneo, A., Caleo, M., et al., 2019. Cortical Seizures in FoxG1 +/− mice are accompanied by akt/s6 overactivation, excitation/inhibition imbalance and impaired synaptic transmission. Int. J. Mol. Sci. 20, 5–14.

Tran, D.A., Pajaro-Blazquez, M., Daneault, J.-F., Gallegos, J.G., Pons, J., Fregni, F., et al., 2016. Combining dopaminergic facilitation with robot-assisted upper limb therapy in stroke survivors. Am. J. Phys. Med. Rehabil. 95, 459–474.

Veerbeek, J.M., Langbroek-Amersfoort, A.C., van Wegen, E.E.H., Meskers, C.G.M., Kwakkel, G., 2017. Effects of robot-assisted therapy for the upper limb after stroke. Neurorehabil. Neural Repair 31, 107–121.

Vetencourt, J.F.M., Sale, A., Viegi, A., Baroncelli, L., De Pasquale, R.F., O'Leary, O., et al., 2008. The antidepressant fluoxetine restores plasticity in the adult visual cortex. Science (80-) 320, 385–388.

Wahl, A.S., Omlor, W., Rubio, J.C., Chen, J.L., Zheng, H., Schroter, A., et al., 2014. Asynchronous therapy restores motor control by rewiring of the rat corticospinal tract after stroke. Science (80-) 344, 1250–1255.

Ward, N.S., 2017. Restoring brain function after stroke - bridging the gap between animals and humans. Nat. Rev. Neurol. 13, 244–255.

Zeiler, S.R., Krakauer, J.W., 2013. The interaction between training and plasticity in the poststroke brain. Curr. Opin. Neurol. 26, 609–616.

Zeiler, S.R., Gibson, E.M., Hoesch, R.E., Li, M.Y., Worley, P.F., O'Brien, R.J., et al., 2013. Medial premotor cortex shows a reduction in inhibitory markers and mediates recovery in a mouse model of focal stroke. Stroke 44, 483–489.

Zittel, S., Weiller, C., Liepert, J., 2008. Citalopram improves dexterity in chronic stroke patients. Neurorehabil. Neural Repair 22 (May-Jun (3)), 311–314. https://doi.org/10.1177/1545968307312173. Epub 2008 Jan 24. PMID: 18219053.