# Increased layer 5 Martinotti cell excitation reduces pyramidal cell population plasticity and improves motor execution

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**Abstract**

During motor activity and motor learning, pyramidal cells in the motor cortex receive inputs from local interneurons as well as deeper structures. Layer 5 pyramidal cells in the primary motor cortex then feed commands to spinal circuits for motor execution. The genetic ablation of layer 5 Chrna2 Martinotti cells, which selectively target pyramidal tract pyramidal cells, results in disturbed fine motor functions. Using calcium imaging combined with chemogenetics, we show that activation of layer 5 Chrna2 Martinotti cells during training increases pyramidal cell tuning, temporal patterning and assembly reconfiguration while not affecting motor learning success rates. However, in mice that had already learned a reach-and-grasp (prehension) task, Chrna2 Martinotti cell activation resulted in improved prehension and increased power in low theta and high gamma bands of local field potentials in the motor cortex. This work indicates that activation of Chrna2 Martinotti cells reduces pyramidal cell assembly plasticity during learning, facilitating preservation of already acquired motor skills.

Keywords: Martinotti alpha 2 cell; Assemblies; Motor function; Plasticity

## Introduction

Refined control of limb movement is crucial to perform essential behaviors such as building shelter, reaching for objects and exploring food. To initiate a voluntary movement, information is relayed from processed sensory information and command centers to circuits in the motor cortex, where the intended motor sequence is processed. The primary motor cortex (M1), which processes motor plans involving proximal and distal joints [1], delivers commands to spinal cord neurons to execute motor behaviors [2]. Motor plasticity continuously shapes and refines the neural circuits underlying motor control, ultimately influencing the execution of motor tasks in diverse contexts and conditions [3]. How the cortex is involved in motor learning and the execution of learned movements is still under debate. Most studies agree that cortical processing is necessary for the initiation of a learned motor sequence [4, 5, 6], but it remains unclear whether cortical processing is necessary for its execution. For example, a study used lesions and a lever pressing task to show that the rodent motor cortex is required for learning, but not for the execution of movements [7]. However, the use of optogenetic mid-movement activation of local inhibitory interneurons in the motor cortex could pause the execution of a learned movement, providing contradictory data [8]. Cortical excitatory and inhibitory interneurons serve as an interface between sensory inputs and motor commands and can thus provide entry points for the congregation of direct sensory input and processed information from the prefrontal cortex, to achieve the most purposeful movement at any given moment. Identifying the function and connectivity of specific interneuron populations within M1 is an important step towards disentangling the neuronal circuits that control and modulate motor function.

Martinotti cells (MCs) are inhibitory interneurons, mainly found in neocortical layer 3 and 5 [9], typically express the neuropeptide somatostatin (SST), and project to layer 1 where they form synapses onto the distal dendrites of cortical pyramidal cells [PCs; 10]. There are several studies investigating the connectivity and functional role of SST-expressing interneurons [11, 12, 13, 14, 15]. However, in the cortex, SST is expressed in a variety of diverse interneuron populations including large basket cells,

double-bouquet cells, long-range GABAergic projection cells, bitufted cells, fanning-out MCs and T-shaped MCs [9, 16, 17]. In fact, only around 50% of SST interneurons are MCs [18] and MCs are found in all cortical layers except for layer 1 [19]. Recently it has been shown that more specific genetic markers can subdivide SST interneurons and MCs into subtypes that reside in different cortical layers and exhibit different morphologies and axonal projection patterns [20, 21], resulting in different layer specific functional properties [22, 16]. Indeed, a motor learning study has suggested that each cortical layer operates under unique constraints reflective of its specialized function [23].

In mice, a subset of MCs (herein referred to as M$\alpha$2 cells) located in layer 5b, expressing the cholinergic receptor nicotinic alpha 2 subunit (Chrna2), send inhibitory projections selectively to thick-tufted type A pyramidal tract PC where they can reset and synchronize PC activity in a frequency-dependent manner [24, 21]. These findings suggest that M$\alpha$2 cells are important for coordinated pyramidal tract PC activity, which is involved in top-down control of subcortical structures. However, how M$\alpha$2 cells might influence PC activity to learn and execute purposeful motor behaviors is not understood. To probe the possible role of M$\alpha$2 cells in motor processing, we evaluated skilled forelimb motor learning and execution in a prehension task during chemogenetic activation of M$\alpha$2 cells, while simultaneously recording layer 5 PC activity using calcium imaging. We found that increased excitation of M$\alpha$2 cells reduced plasticity in PC assemblies during learning, although learning efficiency was unaffected. In contrast, motor performance of an already acquired skill was improved, suggesting that layer 5 Martinotti cells are required for cortical plasticity and motor execution.

## Materials and methods

### Animals

All animal procedures were approved by the local animal research ethical committee (Uppsala djurförsöksetiska nämnd) and followed the Swedish Animal Welfare Act (Svensk författningssamling (SFS) 2018:1192), The Swedish Animal Welfare Ordinance (SFS 2019:66) and the Regulations and general advice for laboratory animals (SJVFS 2019:9, Saknr L 150). Ethical permit number: 5.8.18-08463/2018, 5.8.18-08464/2018 and 5.8.18-07526/2023. Chrna2-Cre mice, produced and bred in our own facility [25], were crossed with either C57BL/6J (Taconic, Denmark), GT(ROSA)26Sortm14(CAG-tdTomato)Hze (RRID:IMSR_JAX:007914; madisen2009robust) or GT(ROSA)26Sortm1.1(CAG-EGFP)Fsh/Mmjax (RRID:MMRRC_032037-JAX; sousa2009characterization) mice and the offspring were genotyped in house for the presence of the Chrna2-Cre allele and/or the tdTomato allele and the EGFP allele. The following primers were used: Chrna2-Cre 5'-gacagccattttctcgcttc-3' (forward) and 5'-aggcaaattttggtgtacgg-3' (reverse); tdTomato 5'-ctgttcctgtacggcatgg-3' (forward) and 5'-ggcattaaagcagcgtatcc-3' (reverse); EGFP 5'-gacgtaaacggccacaagttc-3' (forward) and 5'-cttctcgttggggtctttgct-3' (reverse). The Chrna2-Cre, tdTomato and EGFP allele were kept heterozygous. Mice were housed with littermates in approximately 501 cm2 individually ventilated cages (up to 5 mice/cage) with bedding and enrichment (a carton house and paper tissues), kept in a 12-h light on/light off cycle (6 a.m.–6 p.m.), and maintained at 21 ± 2 °C with a humidity of 45-64%. Mice were provided food (diet pellets, Scanbur, Sweden) and tap water ad libitum, except for when food was restricted for behavioral testing (see single pellet prehension task and pasta handling task further down). After lens or electrode implant surgery, mice were housed individually to decrease risk of implant detachment. Mice were 6-25 weeks old at the start of experiments. Chrna2-Cre$^{tg/wt}$ and Chrna2-Cre$^{wt/wt}$ (Cre-negative littermates designated "controls") were used. Chrna2-Cre$^{tg/wt}$-tdTomato$^{lx/wt}$ mice (and Cre-negative littermates, Chrna2-Cre$^{wt/wt}$-tdTomato$^{lx/wt}$; controls) were only used for experiments with Cre-dependent caspase3-induced apoptosis. Female mice were used in the behavioral experiments, except for in the miniscope recordings in which male mice were used. Throughout the paper, control mice refer to Cre-negative littermates that have received the same treatment as Cre-positive mice.

### Viral injections

Mice were sedated with 1-4% Isoflurane (Baxter). Analgesia was given subcutaneously; 2 mg/kg bupivacaine (Marcain, AstraZeneca), 5 mg/kg carprofen (Norocarp vet, N-vet or Rimadyl Biovis vet) and 0.1 mg/kg buprenorphine (Vetergesic vet, Ceva). A midline incision was made in the scalp, muscles and periosteum were removed using 3% hydrogen peroxide (Sigma-Aldrich) and a hole (1 mm in diameter) was drilled at the site of injection (coordinates relative to bregma: AP 0.8 mm, ML 1.5 mm and DV -1.3 mm) with a hand-held drill. The injection coordinates targeted the forelimb area of the M1. The injection coordinates were chosen based on the Mouse Brain Atlas [26], as well as previous studies mapping the

motor cortex [27, 28]. Viral vectors (for more information see below for each experiment) were injected using a 10 µl Nanofil Hamilton syringe (WPI, USA) mounted on a stereotaxic frame. Ten minutes post injection the needle was slowly withdrawn from the brain and the wound was stitched with resorbable sutures (Vicryl rapide, Ethicon, 6-0). Mice were then left to recover for 2-4 weeks before any behavioral experiments were initiated. Viral transduction was confirmed by post-hoc histological analysis of brain sections.

For Cre-dependent caspase3-induced apoptosis, 14 female mice (7 Chrna2-Cre$^{tg/wt}$-tdTomato$^{lx/wt}$; 7 Chrna2-Cre$^{wt/wt}$-tdTomato$^{lx/wt}$) were injected bilaterally with 750 nl of AAV5-FLEX-TACASP3-TEVP (Addgene viral prep # 45580-AAV5, viral titer: 4.6 x 1012 VG/ml) at a speed of 100 nl/min and were used for behavioral experiments. Two additional mice were injected unilaterally in order to check the percentage of neuronal ablation comparing the injected to the non-injected side.

For chemogenetic activation during local field potential (LFP) recordings, 14 female mice (7 Chrna2-Cre$^{tg/wt}$; 7 Chrna2-Cre$^{wt/wt}$) were injected bilaterally with 300 nl of AAV9-hSyn-DIO-hM3Dq-mCherry (Addgene viral prep # 44361-AAV9, viral titer 2.3 x 1013 GC/ml) diluted in 300 nl sterile saline (9 mg/ml, Fresenius Kabi, Sweden) at a speed of 200 nl/min.

For repetitive chemogenetic activation during training of the single pellet prehension task, 21 mice (10 Chrna2-Cre$^{tg/wt}$; 11 Chrna2-Cre$^{wt/wt}$) were injected bilaterally with 300 nl of AAV9-hSyn-DIO-hM3Dq-mCherry diluted in 300 nl sterile saline (9 mg/ml, Fresenius Kabi, Sweden) at a speed of 200 nl/min.

For calcium imaging of pyramidal cell activity, 8 mice (5 Chrna2-Cre$^{tg/wt}$; 3 Chrna2-Cre$^{wt/wt}$) were first injected bilaterally with 300 nl of AAV9-hSyn-DIO-hM3Dq-mCherry diluted in 300 nl sterile saline (9 mg/ml, Fresenius Kabi, Sweden) at a speed of 200 nl/min. One week post injection, the mice were injected unilaterally in the right hemisphere with 600 nl of AAV9-CamKII-GCaMP6f-WPRE-SV40 (Addgene viral prep # 100834-AAV9), at a speed of 200 nl/min. One week post-injection of AAV9-CamKII-GCaMP6f-WPRE-SV40, the mice underwent surgery for lens implantation (see Lens implantation below).

## Electrode array assembly and implantation

For LFP experiments, mice were injected with AAV9-hSyn-DIO-hM3Dq-mCherry (see viral injections above) bilaterally three weeks before electrode implantation surgery. Tungsten insulated wires of 35 $\mu$ m diameter (impedance 100-400 k $\Omega$, California Wires Company) were used to manufacture 2x5 arrays of 10 tungsten wire electrodes. The wires were assembled to a 16 channel custom made printed circuit board and fitted with an Omnetics connector (NPD-18-VV-GS). Electrode wires were spaced 200 $\mu$ m and the array was implanted into the right hemisphere of the M1. Surgery preparation was performed as described for viral injection, and four small craniotomies were done in a square at coordinates AP 0.70 mm, ML 1.25 mm; AP 0.70 mm, ML 2.25 mm; AP 1.0 mm, ML 1.25 mm; and AP 1.0 mm, ML 2.25 mm; to make a cranial window were the electrodes were slowly inserted at DV -1.30 mm. Four additional craniotomies were drilled for the placement of anchoring screws (AgnThos, MCS1x2), where the screw placed over the cerebellum served as reference. The electrode implant was fixed to the skull with dental cement (Tetric EvoFlow) around the anchor screws. After surgery, the mice were monitored until awake, housed individually and allowed to recover for one week before recordings.

## Lens implantation

The procedure was initially identical to the viral injections, but instead of injecting a virus, a prism lens (CLH type glass, lens diameter 1.0 mm, prism size 1.0 mm, total length of lens+prism 3.68 $\pm$ 0.156 mm, working distance 0.3 mm, enhanced aluminum and SiO2 protective coating, Go!Foton, USA) combined with a relay GRIN lens (Imaging Focusing Rod Lens, diameter 2.0 mm, working distance at infinity) for recordings of cortical layer 5 was implanted unilaterally using the stereotaxic frame. To implant the prism lenses, the tip of a dissection knife was inserted 0.3 mm into the cortex for three minutes before the lens was lowered into the cortex with the tip 0.3 mm lateral and -0.5 mm dorsoventral to the viral injection coordinates. The lens was fixed to the skull and anchor screws with cyanoacrylate glue (OptiBond, Kerr Dental). Silicon glue (Kwik-Sil, World Precision Instruments) was added to protect the lens from mechanical damage in between experiments. Mice were treated with Enrofloxacin 5 mg/kg (Baytril) for two days pre-surgery and seven days post-surgery. Two to three weeks post implantation the mice were again anesthetized, the scalp incised, and the baseplate (used to attach the miniscope during recordings) was mounted on top of the skull with cyanoacrylate glue.

## Drug preparation

The most commonly used chemogenetic agonist is clozapine-N-oxide (CNO), however, it has been suggested

that the main effect on the engineered receptors, after systemically administered CNO, is mediated by back-converted clozapine [CLZ; 29, 30]. Since the conversion rate might differ between individuals [31], we chose to use low dose CLZ for chemogenetic experiments. A stock solution was made from 1 mg of CLZ (Hello Bio, batch E0697-1-1) dissolved in 40 µl dimethyl sulfoxide (DMSO, Sigma-Aldrich). The stock solution was kept at room temperature for the entire set of experiments. The working solution given to the mice was made fresh daily; 2 µl of the stock solution were diluted in sterile saline to a final drug concentration of 1 µg/ml. The mice then received 0.01 mg/kg CLZ intraperitoneally (i.p.).

## Single pellet prehension task

The single pellet prehension protocol consisted of handling, habituation and test sessions. The handling and habituation were performed the same way throughout all experiments, while the number of test sessions varied based on the experimental design (see below for more information about each experimental group). During handling, each mouse was placed on the body and hands of the experimenter for five minutes during two consecutive days and sugar pellets (non-pareille, Dr.Oetker) were inserted in the home cages after handling. For habituation, after completed handling, mice were placed inside the test arena for ten minutes and allowed to freely behave with sugar pellets on the floor of the arena during two consecutive days. The arena consisted of an acrylic rectangular box of dimensions 22 cm x 9 cm x 20.5 cm with a vertical slit (0.5 cm wide) in one of the narrower walls. In front of the slit, an elevated (2cm) plate was attached to the outer wall of the arena. On the third day of habituation, mice were presented sugar pellets on a spoon, outside the slit of the arena, which they were allowed to retrieve with their tongues. Following habituation, the mice underwent ten minutes long test sessions every other day. All test sessions were performed after 15-20 hours of food restriction, starting at 5 p.m. the previous day. After the test session, the mice were returned to their homecage with free access to food and water. For all mice, the initial sessions were performed with spoon aided pellet delivery, to make it easier for mice to understand that they should reach for the pellet. The spoon was initially placed closer to the opening of the slit to increase success rate and motivation to perform the test. As the mice reached more, the spoon was gradually moved further away from the slit untill it was at the same distance as a fixed position on the plate outside the slit. After the fifth session, the pellet was presented on the fixed position on the plate, instead of on the spoon. The pellet was placed to the right of the slit opening, forcing the mice to reach with their left paw. The female experimenter was blinded regarding mouse genotype during the test. The behavior was recorded from the front using a high-speed camera (Panasonic high-speed camera, HC-V750) at 1920x1080 px fixed at 50 FPS.

For the caspase3 induced apoptosis group, after completing handling and habituation, 13 mice (7 Chrna2-Cre$^{tg/wt}$-tdTomato$^{lx/wt}$ and 6 Chrna2-Cre$^{wt/wt}$-tdTomato$^{lx/wt}$) were trained to take pellets from a spoon through the slit during four sessions, then trained to take pellets from the fixed position on the plate during three sessions. One mouse did not reach for pellets on the seventh session, and was therefore excluded. 12 mice (6 Chrna2-Cre$^{tg/wt}$-tdTomato$^{lx/wt}$ and 6 Chrna2-Cre$^{wt/wt}$-tdTomato$^{lx/wt}$) were selected and received viral vector injections (AAV5-FLEX-TACASP3-TEVP), followed by five more test sessions, starting at four weeks post-injection.

For the LFP group, 14 mice (7 Chrna2-Cre$^{tg/wt}$; 7 Chrna2-Cre$^{wt/wt}$) were used. After completing handling and habituation, the mice were trained during four sessions (session 1-4) to take pellets from a spoon through the slit, then trained for three sessions (session 5-7) to take pellets from the fixed plate through the slit. One mouse that did not reach for pellets in the seventh session was excluded. 13 mice (6 Chrna2-Cre$^{tg/wt}$ and 7 Chrna2-Cre$^{wt/wt}$) were selected and received viral vector injections (AAV9-hSyn-DIO-hM3Dq-mCherry), followed by six test sessions (session 8-13) with the headstage connected for LFP recordings four weeks post injections The LFP signal was recorded with a 16-channel Intan RHD 2132 headstage connected to an Intan RHD2132 amplifier board by a thin flexible wire. Mice were briefly anesthetized with isoflurane in order to connect the headstage, then allowed to fully recover and placed inside the arena. Afterwards, mice were disconnected from the headstage and returned to their homecage. In the first three sessions after viral injections (session 8-10) the mice received 0.9 % NaCl i.p. 45 minutes before the start of the recording. In the last three sessions (session 11-13) the mice received 0.01 mg/kg CLZ i.p. 45 minutes before the start of the recording.

For the group with repetitive M$\alpha$2 cell activation during learning, 21 mice (10 Chrna2-Cre$^{tg/wt}$; 11 Chrna2-Cre$^{wt/wt}$) were injected with viral vectors (AAV9-hSyn-DIO-hM3Dq-mCherry). Three to four weeks after viral injection, the handling and habituation was started. 45 minutes before the start of each session, the mice were injected with 0.01 mg/kg CLZ i.p. The first five sessions,

the mice reached through the slit for pellets placed on a spoon. After five minutes in the fifth session, the pellet was instead placed on a fixed position on the plate, and the mice were encouraged to reach for it for five more minutes. The following five sessions, the mice reached for the pellet in the fixed position on the plate during ten minute long sessions. Mice with no reaches in more than three sessions were excluded from the analysis, which resulted in exclusion of one Chrna2-Cre$^{tg/wt}$ mouse and three Chrna2-Cre$^{wt/wt}$ mice. Three Chrna2-Cre$^{tg/wt}$ mice were excluded due to no DREADD expression on post hoc tissue analysis.

For the miniscope group, 8 mice (5 Chrna2-Cre$^{tg/wt}$; 3 Chrna2-Cre$^{wt/wt}$) underwent viral injections and lens implant. 2 Chrna2-Cre$^{tg/wt}$ did not have any detectable expression of the calcium indicator GFP Calmodulin M13 peptide 6 fast (GCaMP6f; checked with the miniscope under anesthesia before baseplate fixation), so they were excluded before the baseplate fixation step. The handling was started two to five weeks after lens implantation, followed by one additional habituation step in which the mice were habituated to the miniscope. The mice were then sedated with 1-4% isoflurane for two to five minutes while the miniscope was mounted on the baseplate and thereafter placed in a circular arena (diameter 45 cm) for 45 minutes, then returned to their homecage for 45 minutes and then once again placed in the circular arena for 45 minutes. After this habituation step the mice were sedated with 1-4% isoflurane for two to five minutes while the miniscope was disconnected from the baseplate. The mice were then returned to their homecage. A few days later, the mice underwent habituation to the single pellet prehension task as previously described, followed by six test sessions. 45-60 minutes before each test session the mice received 0.01 mg/kg CLZ i.p.. The miniscope was mounted on the baseplate on session 1, 2, 5 and 6. Only on those sessions, approximately 20 minutes after CLZ administration, the mice were sedated with 1-4% isoflurane for two to five minutes while the miniscope was mounted on the baseplate. After 20 minutes recovery from the sedation, the mice were placed in the prehension arena for ten minutes. In the arena, the cellular activity was recorded with the miniscope on session 1 (naive), 2 (learning), 5 (training) and 6 (pellet placed on the fixed plate; retraining) and the behavioral activity was recorded with the video camera on session 1-6. A blinking LED light was used to synchronize the time stamps of the miniscope recordings with the behavioral videos. After the test, the mice were sedated with isoflurane in order to remove the miniscope from the baseplate. Session 1-5 were performed every other day, session 6 was performed on the same day as session 5 directly after session 5. Data was recorded using a data acquisition device (DAQ) and a computer with Miniscope controller software installed. Focus of the miniscope was adjusted manually (300 $\mu$ m range). Optimal laser power (around 30% of max brightness), imaging gain, and focal distance was selected for each mouse and conserved across all sessions. The calcium signal was acquired at a frame rate of $\approx$ 20 frames per seconds, with the timestamp of each frame registered onto a separate file.

## Pasta Handling Test

The mice were tested in the pasta handling task, a quantitative assessment of skilled forelimb function, in order to measure forepaw dexterity [adapted from 28]. Mice were exposed to the pasta pieces (uncooked capellini pasta, 1.0 mm diameter, 2.6 cm length) in their home cage for five days to avoid neophobic reactions. Thereafter, the mice were habituated to eat inside the test arena (22 cm x 9 cm x 20.5 cm) for three days, without food restriction. After the habituation period, the mice were tested in the pasta handling test. The test consisted of sequential trials in which one pasta piece was delivered on the floor, in the center of the arena, for each trial. The behavior was recorded from the front using a high-speed camera (Panasonic high-speed camera, HC-V750) at 1920x1080 px fixed at 50 FPS. Test sessions were performed after 15-20 hours of food restriction, starting at 5 p.m. the previous day. Each session of ten minutes consisted of a maximum of five trials. To make it easier to see how the capellini strand would move while being consumed, each pasta piece received a food coloring mark on one half. The number of pasta drops and the time spent handling the pasta was then quantified by visual inspection. 9 mice (3 Chrna2-Cre$^{tg/wt}$-tdTomato$^{lx/wt}$; 6 Chrna2-Cre$^{wt/wt}$-tdTomato$^{lx/wt}$) bilaterally injected with AAV5-FLEX-TACASP3-TEVP were tested in the pasta handling task six weeks after viral injection and one week after the last pellet prehension task session.

## Hanging Wire Test

The coordination and strength of the forepaws was tested by the hanging wire test, in which mice suspend their body by holding on to a single wire stretched between two posts 40 cm above the ground. The mice were placed on the middle of the wire hanging from its forepaws for three minutes. A paper bed was placed between the two posts to avoid injury when the mice fell. The time hanging on

the wire and number of falls were quantified by visual inspection. 9 mice (3 Chrna2-Cre$^{tg/wt}$-tdTomato$^{lx/wt}$; 6 Chrna2-Cre$^{wt/wt}$-tdTomato$^{lx/wt}$) were used. Mice were injected with viral vectors (AAV5-FLEX-TACASP3-TEVP) bilaterally, then performed the single pellet prehension task, and after completion of the single pellet prehension task, they performed the pasta handling and finally the hanging wire test (six weeks after the viral vector injection).

## Automatic detection and classification of prehensions

For each pellet prehension task session, the positions of the pellet, both forepaws, thumb, index and middle fingers were tracked. The AI network used for tracking (see Software availability) was trained specifically for detection of the left paw and the three fingers at the prehension onset position (paw palm pointing inside, thumb pointing up and fingers pointing to the medial line), and a tracking likelihood threshold of 70% was achieved from prehension onset to grasp end. Therefore, prehensions were reliably detected as blocks where the tracking likelihood for the left paw and its three fingers was above 70%. Prehensions were classified as successful when the animal reached for the pellet, grasped the pellet and brought it to its mouth. Any other outcome (mice hitting the pellet but failing to grasp it; missing the pellet; grasping the pellet but dropping it before it reached the mouth; reaching for the pellet when no pellet was available) was considered a failed prehension.

## LFP electrophysiology analysis

Square voltage pulses were used to trigger LFP and miniscope recordings; and to drive a red LED placed at the top right corner of the behavioral camera field of view. Therefore, behavioral video recordings were synchronized with the corresponding electrophysiological recording by detecting the LED onset in the video. LFP data for each channel was filtered using a second-order Butterworth bandpass filter across the entire frequency range of 1-100 Hz, as well as within specific bands: theta (4-12 Hz), gamma (30-90 Hz), low theta (4-7 Hz), high theta (8-12 Hz), low gamma (30-40 Hz), and high gamma (60-90 Hz). This process was followed by the computation of the spectrogram, which offers a time-resolved representation of the frequency content in the data. The resulting spectrogram was normalized by dividing each power value by the cumulative mean, ensuring that the data reflected relative power rather than absolute magnitudes. Subsequently, average power values were extracted within the designated frequency bands by averaging over the frequency dimension, focusing specifically on the frequency bins that fell within the defined limits of each band.

## In-vivo calcium imaging analysis

The calcium signal in PCs was obtained using the viral vector with CamKII promoter driven expression of GCaMP6f. The increase in calcium levels corresponds to increased cell activity, and single spikes can only be indirectly estimated [32, 33, 34]. Miniscope videos were spatially downsampled 3x and motion-corrected, then cells signal was extracted using constrained nonnegative matrix factorization for microendoscopic data (CNMF-E) approach [35], and fluorescence traces were detrended by baseline subtraction (here referred as $\Delta$F detr.). Neuron identity was matched along multiple recordings through intersection over union metric and the Hungarian algorithm for optimal matching. Up to this step, all miniscope video analysis was done as implemented previously [36]. Prehension onset (time = 0 ms in all plots) was defined as the moment when the mouse's paw forms the grasp position, flipping the palm inside, thumb pointing up and fingers pointing to the medial line, then neurons fluorescence traces were sliced 1 s before and 1 s after forepaw prehension onset, and slices were grouped by prehension accuracy (successful or failed) and averaged separately for each session. Only neurons that displayed a significant change comparing the before onset and after onset phases (ANOVA or Kruskal-Wallis, according to normality of distribution, evaluated using Shapiro's test) were kept for further analysis. For L5 pyramidal cells fluorescence peak latency analysis, traces for successful or failed grasps were separately averaged and normalized for each neuron, and neurons were sorted according to peak latency at successful grasps from each session. For PC response peak width, the width in seconds of the maximum response peak was calculated at half the peak prominence.

For the assembly analysis, constraints were required to allow for detection of assemblies between sessions. This limited the analysis to the naive, learning and retraining sessions, and only PCs detected through those sessions whose identity could be matched along sessions were kept. Therefore, on average 84.8 $\pm$ 14.2 PCs (n = 4 mice; 2 ctrl, 133 and 68; 2 Chrna2-Cre+, 77 and 61) were kept. Assemblies were identified during the entire 10 minute recording of a session, such that assembly detection was not affected by prehension accuracy. Assemblies were detected using independent component analysis with circular shifts [ICA-CS; 37], as implemented previously [38]. After

removing neurons with no significant change comparing the before onset and after onset phases, only assemblies with 4 or more neurons and only neurons belonging to at least one assembly were kept for further assembly analysis. For calculating assembly area, neurons from the assembly were first sorted according to their cartesian angle relative to the center of the assembly, which is the average of neurons' (x,y) coordinates. Finally, the assembly area was calculated as the area of the polygon formed by the sorted neurons. Assemblies resilience was calculated by finding the assembly with highest neuronal composition intersection on the other sessions according to the following formula:

$$\frac{Size(Intersect_{Assembly1-Assembly2})}{Size(Assembly1)} + \frac{Size(Intersect_{Assembly1-Assembly2})}{Size(Assembly2)} * \left(\left(\frac{Size(Assembly2)}{Size(Assembly1)}\right) - 1\right) \quad (1)$$

such that resilience is within 0 (no intersection between assemblies) and 1 (exactly the same neurons forming both assemblies). To find assembly activity peaks, 1000 shuffles of activity of all neurons within an assembly on the time dimension were done, and activity peaks were considered as time samples where the sum of all neurons' real activity is larger than 95% of the sum of the shuffled neuronal activity, resulting in a rasterized activity (containing only 0 or 1 meaning inactive or active for each time point). Event histograms were calculated by slicing the assembly activity peaks in time windows of 2 s around each prehension (1 s before and 1 s after onset), where each bin corresponds to a one time sample of 50 ms, limited by the miniscope's 20 FPS recording speed. Assembly salience, a measurement of how relevant the activation (or silencing) of an assembly is within the prehension movement, was calculated as

$$\frac{ActivityPeaksInWindow}{TimeSamplesInWindow} - \frac{ActivityPeaksOutWindow)}{TimeSamplesOutWindow} \quad (2)$$

such that salience is within -100 (assembly is active every time sample out of the prehension window and not active on any time sample within the window, i.e., absent prehension-related activity and full activity outside the prehension window) to 100 (assembly active in all time samples within the prehension window and at no time sample outside the window, i.e., full prehension-related activity and absent activity outside the prehension window).

## Tissue preparation and immunohistochemistry

To fix the tissue for post-hoc analysis, mice were transcardially perfused with phosphate buffered saline (PBS, Fisher BioReagents, CAT 10051163) followed by 4% formaldehyde (VWR Chemicals BDH®, CAT 9713.1000). The brains were dissected out and post-fixed in 4% formaldehyde overnight at 4°C, washed three times for ten minutes in PBS and then kept in PBS at 4°C until sectioning (a few hours or days). Before they were sectioned, the brains were embedded in 4% agarose in PBS. Sections were cut at a thickness of 70 µm using a Vibratome (Leica VT1000S) and then mounted with ProLongTM Gold Antifade Mountant (ThermoFisher Scientific, CAT P10144) or Fluoroshield (Abcam) on glass slides (Superfrost®Plus, Thermo Scientific). For some mice, six weeks after the injection of AAV9-hSyn-DIO-hM3Dq-mCherry, CLZ 0.01 mg/kg was administered i.p. 135 minutes before transcardial perfusion. After sectioning these brains, the sections were washed in PBS and pre-blocked for one hour in 2% donkey serum (Biowest), 1% Bovine Serum Albumin (BSA, Sigma Aldrich), 0.1 % Triton®X-100 (Sigma Aldrich) and 0.05% Tween®20 (Sigma Aldrich) in PBS. The primary antibody, c-Fos(4)-G (goat polyclonal IgG, Santa Cruz Biotechnology, CAT K1314) or c-Fos (9F6) (Cell signaling, rabbit, #2250) and anti-mCherry (ThermoFisherScientific, 16D7, rat, M11217) were diluted 1:500 in 0.25% gelatin and 0.5 % Triton®X-100 in Tris-HCl buffered saline (TBS; 0.3% Tris, 0.8% NaCl and 0.02% KCl in mqH2O, pH 7.4) and added to the sections. After 48 hours of incubation at 4°C the sections were washed with TBS and the secondary antibodies, donkey anti-goat A647 (ThermoFisherScientific A21447), donkey anti-rabbit A647 (ThermoFisherScientific A31573) and donkey anti-rat A488 (ThermoFisherScientific A21208) were diluted 1:500 in 0.25% gelatin and 0.5 % Triton®X-100 in TBS, added to the sections and incubated for 90 minutes at room temperature. The sections were washed with 0.1% Tween in TBS and distilled water before they were mounted on glass slides.

## Imaging and image post processing

Most wide field images were acquired on a Zeiss Axio Imager Z2 (Zeiss, Germany) with a 10x objective (effective NA 0.45), a colibri LED 7 and Zen blue software (Zeiss, Germany), using a multi-band bandpass filter (Zeiss, Germany) with the filter excitation wavelengths 370-400 nm,

450-488 nm, 540-570 nm, 614-647 nm, 720-750 nm and the filter emission wavelengths 412-438 nm, 501-527 nm, 582-601 nm, 662-700 nm, 770-800 nm for the fluorophores DAPI, Alexa 647 and GFP and a single-band bandpass filter (TxRed-4040C-ZHE-ZERO, Semrock) with the excitation wavelengths 540-552 nm and emission wavelengths 590-4095 nm for mCherry. The light source used for the different fluorophores was LED-module 385nm for DAPI, LED-module 630 nm for Alexa 647, LED-module 475 nm for GFP and LED-module 567 nm for mCherry. Some images were taken using an Olympus BX61WI fluorescence microscope (Olympus, Japan) with a 10x (effective NA 0.40) objective and a Volocity 4.1.0 software (Quorum Technologies) or a Zeiss LSM700 confocal microscope (BioVis facility, Uppsala University). Brightness and contrast were adjusted in the Fiji software [39], equally for the whole image and without obscuring any data.

## Statistical analysis

Differences were evaluated using mixed-models ANOVA, with post-hoc t-test for pairwise comparisons. Where the data showed non-normal distribution, evaluated using Shapiro's test, ANOVA-type statistics [40, mixed models] or Scheirer-Ray-Hare (independent factors) were used for multiple factors; Kruskal-Wallis and Friedman's tests with F-ratios [41] were used for independent and dependent single factors, respectively; and post-hoc Mann–Whitney U or Wilcoxon signed-rank tests for independent and paired factors, respectively. Post-hoc multiple comparisons were adjusted by Holm correction, and statistical significance was conditioned to a false positive risk lower than $\alpha = 0.05$. Error bars represent standard error of the mean (s.e.m) for all figures. For all violin plots, the filled area represents the entire data range, horizontal lines and triangle markers represent medians and means, respectively.

## Software availability

LFP recordings were done using the Open-ephys GUI [42]. Calculations were done using Scipy [43], Numpy [44] and SciScripts [45], all plots were produced using Matplotlib [46] and schematics were drawn using Inkscape [47]. Cell fluorescence signals were extracted and processed from miniscope videos using CaImAn [36] and OpenCV [48]. Tracking of the mouse forepaw and fingers was done using DeepLabCut [49]. All scripts used for data analysis, including the DeepLabCut trained network, are available online [git repository; 50].

# Results

## Increased M$\alpha$2 cell excitation affected calcium signal amplitudes in PCs during training and retraining

Considering the dual roles of the M1 as a motor control structure and a dynamic substrate that participates in motor learning [51], we were interested in how PCs were affected by modulation of M$\alpha$2 cells during motor learning. For this purpose, we turned to chemogenetic manipulation using designer receptors exclusively activated by designer drugs (DREADDs). In pilot studies, we found that increased excitation of M$\alpha$2 cells, but not increased inhibition, gave a measurable effect in a motor test (using modified Gq-coupled human M3 or Gi-coupled human M4 muscarinic receptors [hM3Dq/hM4Di; 52, 53, *preprint*]. We thus chose to continue with hM3Dq, and we first validated the effect of clozapine (CLZ) in Chrna2-Cre+ mice. To validate the clozapine (CLZ) induced activation of hM3DGq+ cells, we injected viral vectors carrying the modified Gq-coupled human M3 muscarinic receptor (hDM3Gq) and the fluorescent marker mCherry [54] under Cre-dependent expression and performed immunolabeling for c-FOS, a proto-oncogene expressed in most neurons after depolarization [55, 56, Supplemental Figure 1A-B]. In Chrna2-Cre+ mice (n=9, number of sections=346), on average 32 mCherry positive cells per hemisection were found, of which 76% were c-FOS positive. In control mice (n=5, number of sections=60), on average 5 mCherry positive cells per section were found, of which 0.8% were c-FOS positive (Supplemental Figure 1C). Throughout the manuscript, control mice refers to Cre-negative littermates that received the same treatment as the experimental Chrna2-Cre+ mice.

Next, a prehension task, in which mice are encouraged to reach for and grasp a sugar pellet, was used to evaluate fine motor functions and motor skill learning [57, 58, 59]. Most dendritic spines that correlate with learning are formed after the initial training session and are kept in the second training session [60]. Thus, we performed a set of recordings during the first "naive" session for collection of data in untrained mice, the second "learning" session and in the fifth "training" session to follow learning progression, and finally in a "retraining" session, during which a more challenging presentation of the pellet was introduced (Figure 1A-C). The contribution of increased M$\alpha$2 cell excitation on individual PCs was investigated with calcium imaging. We recorded emitted light in cortical layer 5 using an implanted optical lens and prism assembled to a miniscope [Figure 1A; 61, 62].

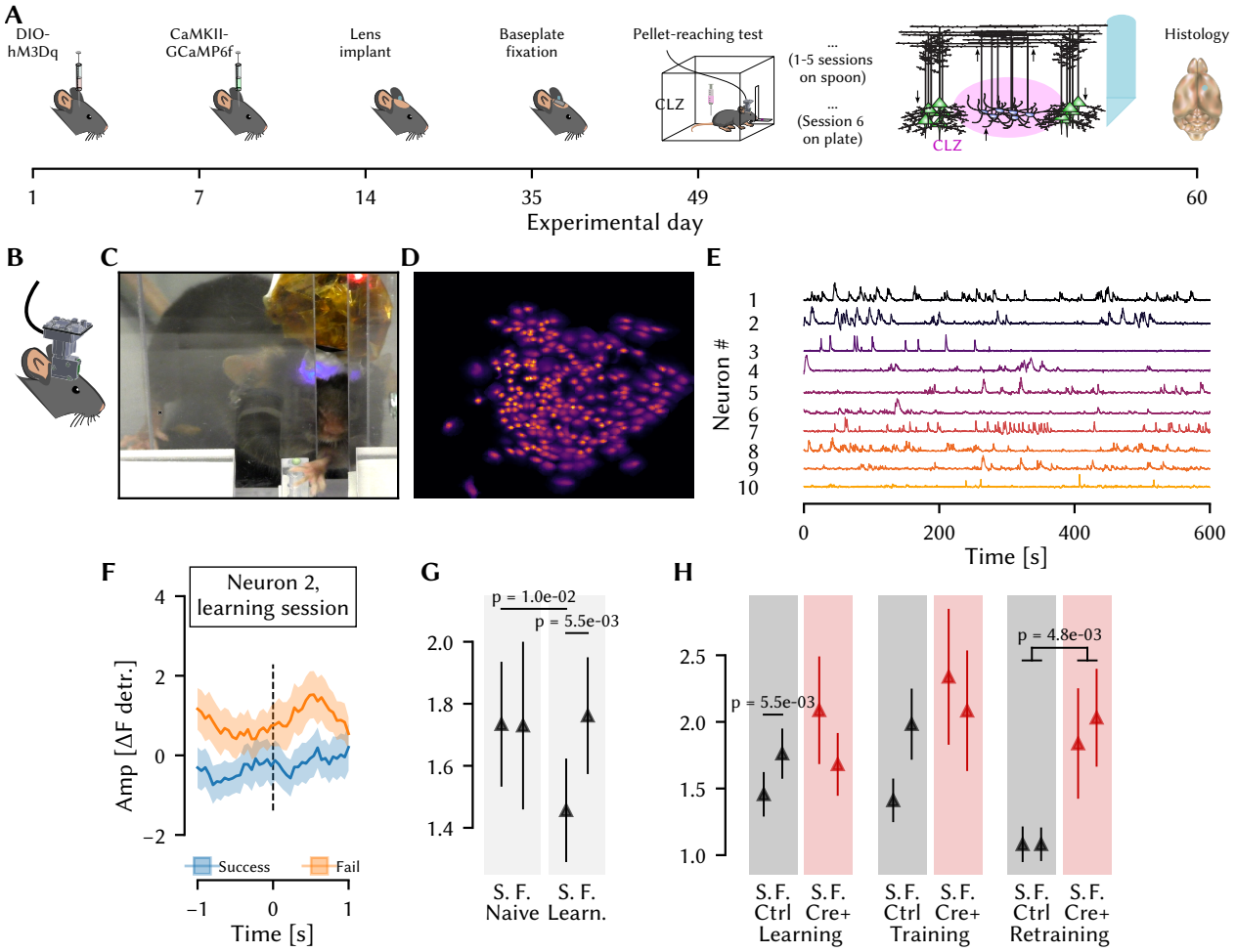


**Figure 1:** Mice with increased Mα2 cell excitability display increased PC activity after training and upon retraining. A) Timeline of experiments showing viral injections, miniscope lens implantation, base plate fixation and pellet-reaching task. GCaMP6f fluorescence and forepaw movements were recorded in naive, learning, trained and retrained sessions. B-C) Schematics (B) and photograph (C) of a mouse with the miniscope connected during prehension. D) Representative miniscope image of L5 pyramidal cells after cell detection. E) Ten representative example fluorescence traces from PCs. F) Representative traces showing the activity of one neuron -1 to 1 second around the prehension onset, averaging all successful (blue) and failed prehensions (orange). G-H) Average amplitude of PC activity represented as mean ± SEM, separated by session (naive, learning) and prehension accuracy (Success, successful prehensions; Fail, failed prehensions) for Control mice (G); and further including later sessions (learning, trained, retrained) and both genotypes (control, black; Cre+, red; H).

To maximize the number of cells included in the analysis, PCs were identified for each session. Therefore, the number of PCs per animal varies for each session. On average, 101.8 ± 24.19 PCs (n = 6 mice; 3 Control, average of 210.5, 119, and 94.5; 3 Chrna2-Cre+, average of 107.5, 63 and 16) were detected. Analysis of calcium transients demonstrated that individual cell activities were readily identifiable and showed diversified activity profiles (Figure 1D-F). Our results showed an effect of session in PC amplitude on successful prehensions of controls (Supplemental Table 1A-C). PC activity amplitudes were decreased during successful prehensions in the learning session compared both to failed prehensions (Supplemental Table 1D) and to successful prehensions from the naive session (Supplemental Table 1E; Figure 1G). Furthermore, an overall effect of genotype on PC activity amplitude was found for the retraining session (Supplemental Table 1F), where PCs in Chrna2-Cre+ mice exhibited an overall increased activity compared to PCs in control mice (Figure 1H).

We next aligned cell activity to prehension onset (t = 0), and measured the average PC population activity across two distinct phases: a one-second window before prehension (motor planning) and another one-second window during prehension (motor execution). We found similar effects (Supplemental Table 1G-H; Supplemental Figure 2); reduced PC amplitudes for successful learning session attempts in control mice that was not observed upon increased excitation of Mα2 cells. Also, increased excitation of Mα2 cells in the retraining session resulted

in significantly larger PC amplitudes when compared to controls (see summary Figure 6A).

## Increased M$\alpha$2 cell excitation resulted in earlier PC firing during retraining

Changes in sequential activation of PCs are linked to learning [63]. Therefore, we next analyzed the temporal profiles of PCs, and identified the time point where they reached their maximum activity within the prehension window (Figure 2A-B). We compared the response peak latency between genotypes, sessions, and prehension accuracy while separating neurons based on the prehension phase where the activity peak is reached. For each session, the activity for each PC for all prehension types was averaged within the prehension window around the prehension onset and normalized (divided by its own maximum fluorescence). We sorted individual neurons based on their latency to peak during successful prehensions (Figure 2C). The PCs activity peak latency analysis showed that the temporal patterns were strongly affected by prehension accuracy (Supplemental Table 1I-K; Supplemental Figure 3; Figure 2H). In the failed prehension learning session, PCs in Chrna2-Cre+ mice had significantly delayed responses compared to PCs in control mice (Supplemental Table 1L; Figure 2I). In the successful prehension retraining session, PCs in Chrna2-Cre+ mice had significantly premature responses in the planning phase (before t=0) compared to PCs from control mice (Supplemental Table 1M; Figure 2J). Together, these results suggest that temporal patterns shifted according to prehension accuracy and session. Thus, increased M$\alpha$2 cell excitation caused delayed PC firing during motor learning and premature PC firing on task retraining (Figure 6A).

We also measured the width of the response peak, a measure of the temporal specificity of cell activation that is proposed to influence motor accuracy [64, 65]. Broader response peaks indicate less temporal specificity. We analyzed the response peak width for each genotype across all training sessions, separating prehensions by accuracy (Figure 2D-G). The width of PC activity peaks was most strongly influenced by the session followed by genotype (Supplemental Table 1N-R; Supplemental Figure 4; Figure 2K-L), where PCs in Chrna2-Cre+ mice displayed overall sharper peaks compared to PCs in control mice, particularly during the training session (Figure 6A).

## Increased M$\alpha$2 cell excitation resulted in PC assembly salience rigidity, area compaction, and increased resilience

We next hypothesized that examination of PCs Hebbian assemblies could provide insights into how heightened excitation of M$\alpha$2 cells modulate cortical processing. In the assembly analysis, we focused on the naive, learning, and retraining sessions, retaining PCs detected through those sessions whose identity could be matched across sessions. On average, 84.8 $\pm$ 14.2 PCs were kept for the analysis. Prehension accuracy did not influece assembly identification, since we based it on neuronal activity across the 10-minute session recording, rather than just during the prehension window (Figure 3A). Assemblies consisting of less than four neurons, and neurons that were not part of any assembly, were omitted from the analysis. We identified a total of 23 assemblies in controls and 19 assemblies in Chrna2-Cre+ mice, with an average number of 5.6 cells (SD 0.4) in each assembly for each session. The number of neurons within each assembly remained fairly consistent during learning and retraining (Figure 3D,E,H,I).

Motor training is associated with a selective increase in connectivity among task-related PCs [66]. On a PC population level, we investigated this through assembly salience, here defined as the activity of the assembly within the prehension window in relation to its own activity outside the prehension window (ranging from -100 to 100 (Figure 3B)). We found that our salience data varied from -2.04 to 24.62 and we found an overall effect of of training session on assembly salience (Supplemental Table 1S). In agreement, assemblies in control mice were significantly less salient in the successful retraining session compared to the successful naive and learning sessions (Supplemental Table 1T-U). In contrast, no differences in salience were found for failed prehension sessions in control mice, nor between any session type for Chrna2-Cre+ mice (Figure 3C; Figure 6B).

Functional imaging studies in humans show that brain activity varies regionally during different stages of motor learning [67]. Hence, we plotted the position of the individual PCs in each assembly and measured the spatial area which each assembly covered in the cortex. We found significantly smaller assembly areas in Chrna2-Cre+ mice compared to control mice in the retraining session (Supplemental Table 1V-W; Figure 3F,G,J,K), suggesting that upon increased M$\alpha$2 cell excitation assemblies were composed of PCs spatially closer together (Figure 6A-B).

To measure PC assembly plasticity, we investigated how much the assemblies had to change their neuronal

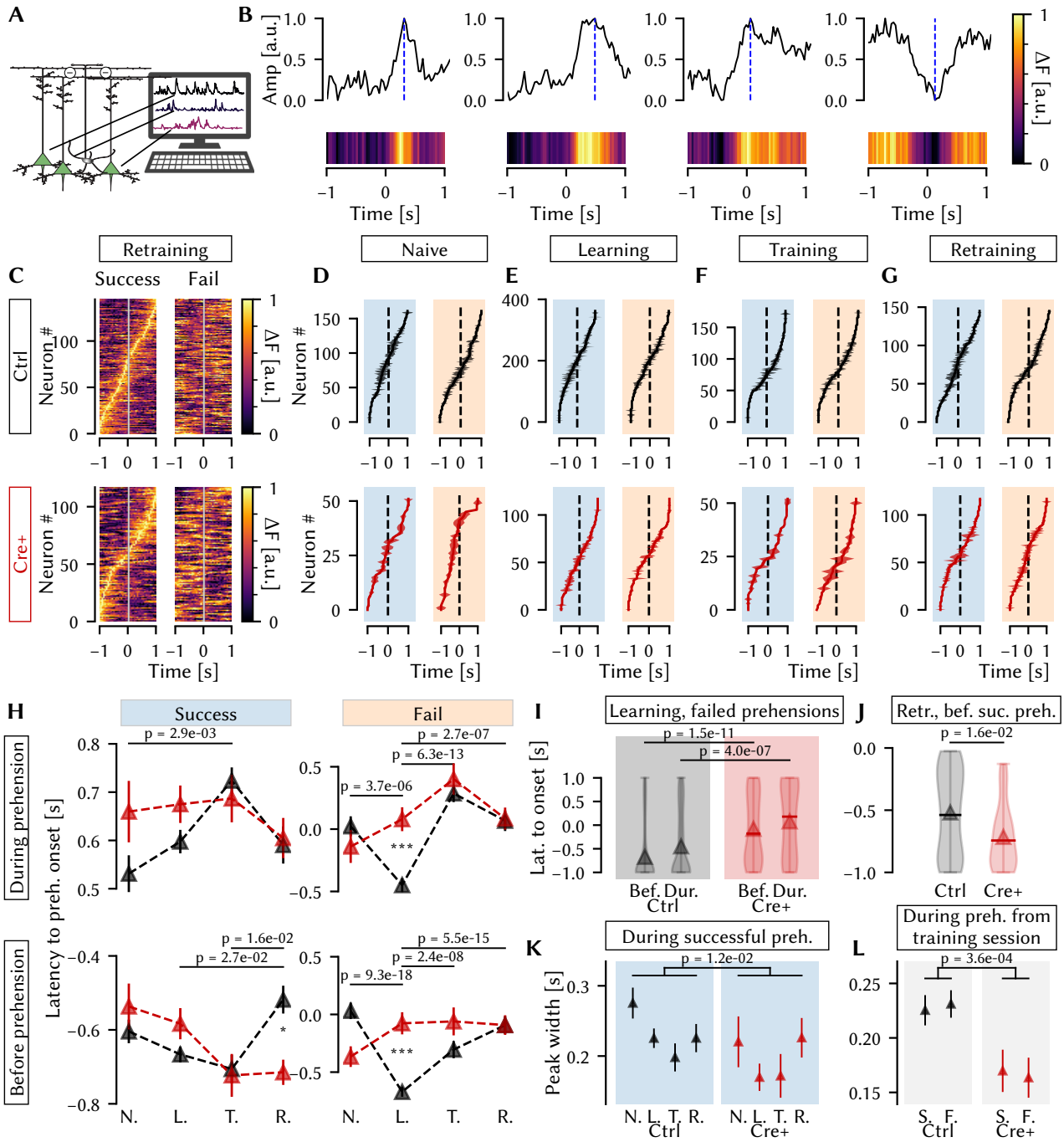


**Figure 2:** Enhanced excitation of Mα2 cells is associated with delayed PC activity during motor learning and premature PC activity on task retraining. A) Illustration of recorded calcium signals from GCaMP6f-expressing PCs. B) Representative examples of average fluorescence traces around prehension onset for four different neurons, showing their fluorescence curve (ΔF detr. over time) represented in a colormap and latency of detected activity peaks (dashed blue line). C-G) Normalized average fluorescence matrices (Neuron x Time x ΔF) for successful and failed prehensions, for control (top) and Chrna2-Cre+ (bottom) mice, showing temporal activation patterns for each neuron on the retraining session (C), and the peak latency with a shaded area indicating the peak width during naive, learning, trained, and retrained sessions for each neuron. Neurons were sorted according to their temporal profile for successful prehensions, and follow the same order for failed prehensions for each session. H) PC peak latency for both genotypes at each session before (bottom) or during (top) successful (left) and failed (right) prehensions. All significance bars refer to significant pairwise differences between sessions for controls; and asterisks represent significant pairwise differences between genotypes for a particular session. I-J) PC peak latency for both before and during failed prehensions from the learning session (I) and before successful prehensions from the relearning session (J). K-L) PCs response peak width during successful prehensions separated by genotype and session (K), and during prehensions from the training session separated by genotype and prehension accuracy (L). S., successful; F., failed; B., before; D., during prehension; N, naive; L, learning; T, training; R, retraining.

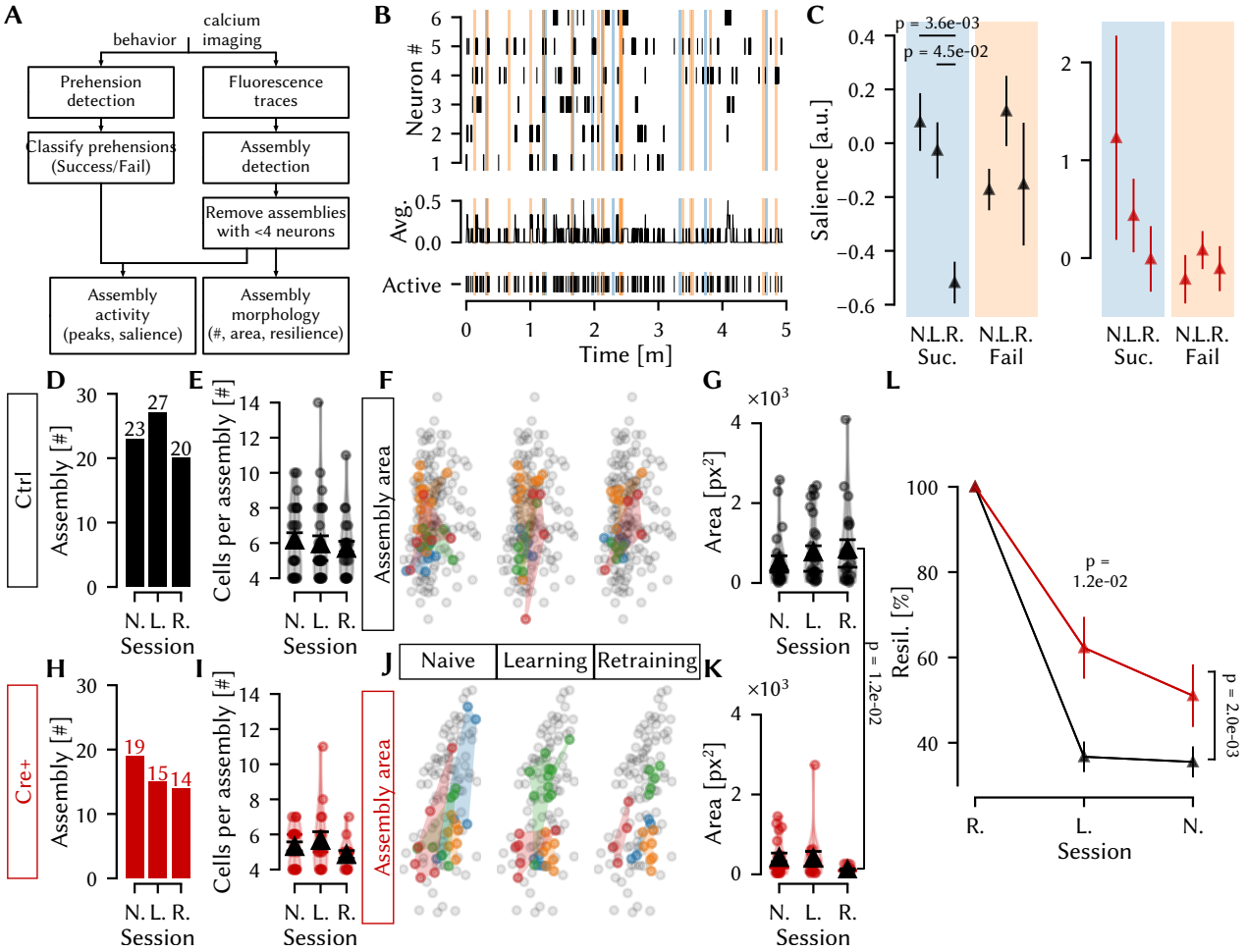


**Figure 3:** Mice with increased Mα2 cell excitability display assemblies with less prehension-related activity, decreased spatial area and increased resilience. A) Flowchart of the analysis pipeline. B) Activity of one representative assembly. Top, rasterized activity over the full recording time of the six neurons forming the assembly. Mid, the average of neuron activity. Bottom, the estimated rasterized activity of the assembly as a unit. Blue and orange shades show the successful and failed prehension windows, respectively. C) Salience of assemblies during the whole 2s window for each session, grouped by genotype and prehension accuracy. D-E) Number of assemblies and number of cells per assembly for control mice, detected at each session. F) Representative examples of 4 color-coded assemblies detected at session 6 and traced back to session 1. G) Area of detected assemblies at each session for control mice. H-K) Same as D-G, but for Cre+ animals. L) Resilience of assemblies for each genotype, for assemblies detected at retraining and followed back to the naive session. Suc., successful; Fail, failed; N, naive; T, trained; R, retrained.

composition to reach their final configuration in the retraining session, quantified here as assembly resilience. Resilience was calculated taking the assembly composition reported as the percentage of neuron match between sessions, with 100% being an assembly that did not incorporate or remove any neurons over time. We found that genotype and training session affected assembly resilience (Supplemental Table 1X), where assemblies in Chrna2-Cre+ mice displayed significantly increased resilience compared to assemblies in control mice in the learning session (n = 20 assemblies control and 14 assemblies Chrna2-Cre+; Supplemental Table 1Y; Figure 3L). Together, these results suggest that PC assemblies undergo less modifications on their neuronal composition upon increased Mα2 cell excitation (Figure 6A-B).

## Ablation of Mα2 cells affected fine motor functions

So far, we found that increased Mα2 cell excitation resulted in several effects on PC population and assembly plasticity in the motor cortex (Figure 6A-B). However, somewhat puzzling, we did not observe an effect of genotype in the prehension success ratio (Chrna2-Cre+, 6 mice; control, 8 mice) when mice were administered CLZ (before every session; Supplemental Table 2A; Supplemental figure 5). To investigate if and how Mα2 cells may impact motor execution, we next turned to a Caspase-based virus approach to ablate Mα2 cells in the forelimb area of the M1. After unilateral injections in Chrna2-Cre/tdTomato mice (n = 2; Figure 4A) we could validate deletion efficiency with a 80.4 % reduction of tdTomato

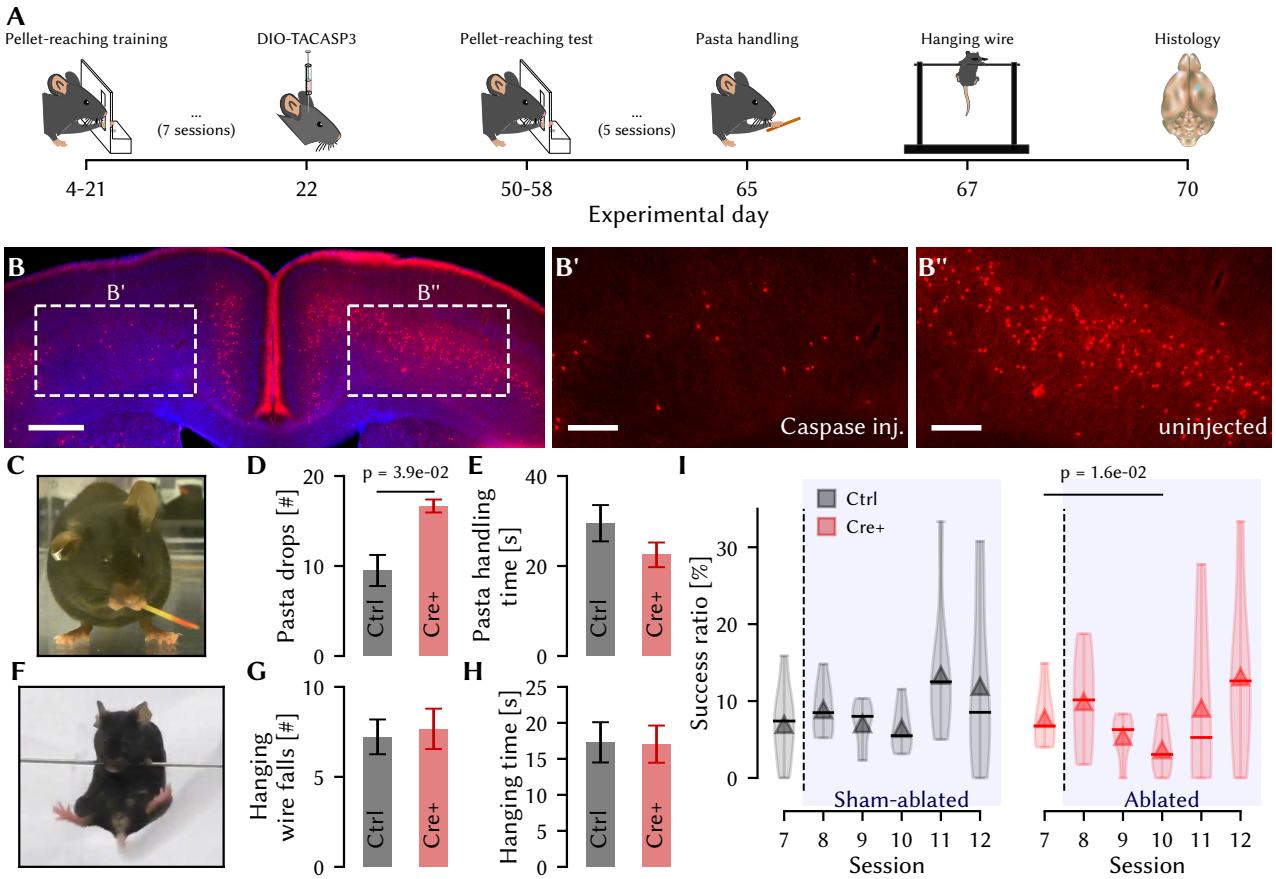


**Figure 4:** Apoptosis in M$\alpha$2 cells decreased pellet reaching and pasta handling performance. A) Timeline of the experiments. B) Unilateral injection of Cre-dependent TACASP3 decreased the amount of Chrna2-Cre+ cells in the motor cortex by 80.4%. Enlargement of the injected motor cortex (B'), and contralateral non-injected side (B"). Scalebars: 500µm (B) and 200µm (B' and B"). C) Animal performing the pasta handling task. D-E) Graph of results on pasta drops and time spent in the task. F) Animal performing the hanging wire task. G-H) Graph of results on falls and time spent in the task. I) Graph of results from the single pellet prehension task before and after ablation (blue shade). BL, baseline.

positive cells comparing the injected to the uninjected side in the same mouse (Figure 4B). We used a pasta handling test to measure forepaw dexterity, and found that bilaterally injected Chrna2-Cre+ mice dropped the pasta significantly more times compared to control mice (n = 3 Chrna2-Cre+, 6 controls; Supplemental Table 2B), whereas they had similar pasta handling times (Figure 4C-E). To evaluate parameters such as grip strength and endurance [68], we used a hanging wire test, in which the two groups of mice performed equal, both in number of falls and time spent on the wire (Figure 4F-H). In the prehension task, we did not find an interaction of genotype, although we found a significant drop in prehension performance for Chrna2-Cre+ mice, comparing the last session before ablation (n = 6 mice, baseline, 599 prehensions, success ratio of 7.74%) with the third session after M$\alpha$2 cell ablation (session 10, 509 prehensions, success ratio of 3.68%; Supplemental Table 2C). No significant differences were found in control mice (n = 8 mice, baseline and session 10, 989 and 743 prehensions, success ratios of 6.96 and 6.44, respectively). Thus, ablation of M$\alpha$2 cells reduced dexterity in the pasta handling task and affected prehension task success, suggesting that M$\alpha$2 cells do have a role in orchestrating precise forelimb movements (Figure 6C).

## Increased M$\alpha$2 cell excitation resulted in improved execution of a learned motor task

To investigate the potential influence of increased M2 cell excitability on already learned motor skills, we hypothesized that our previous experiments where repetitive CLZ administration was initiated from the start of training might have obscured such effects. To test this, we trained mice for seven sessions prior to injecting them with hM3Dq and then evaluated their performance in a prehension task. In the first three post-injection sessions, saline was administered to control for injection effects, while CLZ was administered in the following three sessions to measure the potential impact (Figure 5A). We found

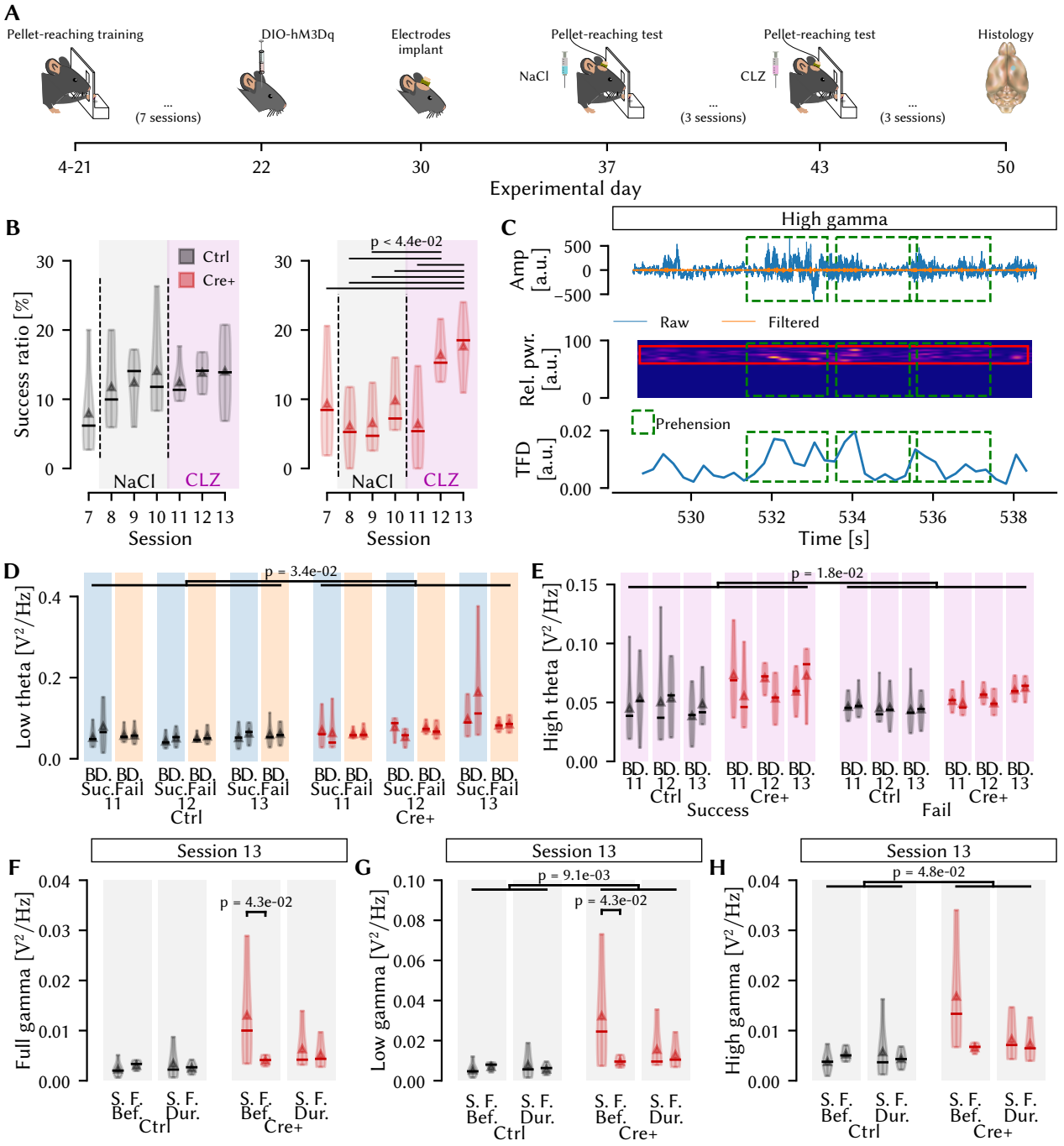


**Figure 5:** Mice with increased Mα2 cell excitability display increased prehension success rate, increased LFP low theta and gamma power. A) Timeline of the experiments. Animals were trained to reach for the pellet and after the 7th session, the hM3Dq was injected and the LFP electrodes implanted. B) Prehension success ratio for control (Left, black) and Chrna2-Cre+ (Right, red) mice in the sessions with saline injection (gray shade) and clozapine injection (purple shade). C) Example of time-frequency domain power processing, with three prehensions highlighted (green dashed squares). Top, raw (blue) and high gamma-filtered (orange) LFP traces. Mid, spectrogram from the filtered signal, with highlights for the high gamma band (red square). Bottom, the time-frequency domain power over time, calculated as the average of the previously highlighted spectrogram region. D-H) Relative power for low theta, high theta, full gamma, low gamma and high gamma, respectively. D and E shows the full dataset due to the observed overall effects, while for F-H only isolated effects for session 13 were found. Shades: blue and orange (successful and failed prehensions); magenta (sessions under CZP treatment). Abbreviations: B. and D. or Bef. and Dur. (before and during prehension); S. and F. or Suc. and Fail (successful and failed prehensions).

that saline administration did not alter success rates relative to the final training session in control mice. However, CLZ treatment significantly increased the success rate in Chrna2-Cre+ mice compared to saline treatment (Supplemental Table 2D), which was not observed in control mice (Supplemental Table 2E; Figure 5B). These results

suggest that enhancing M2 cell excitability improved the execution of an already learned fine motor task (Figure 6C).

Low and high sub-bands within theta and gamma frequencies are associated with different functional roles in attentional control, sensory integration, and fine motor control. To gain deeper insight into how M2 cell modulation affects motor planning and execution, we recorded local field potentials (LFPs) in the same mice during task performance in the CLZ-treated sessions (sessions 11–13). This allowed us to correlate neural activity patterns with specific phases of the task. We separated successful and failed prehensions and analyzed one-second time windows before (planning phase) and during (execution phase) task onset. Using spectrograms, we calculated power in various frequency bands across the LFP signal, spanning 1–100 Hz, with specific focus on the theta (4–12 Hz) and gamma (30–90 Hz) ranges. The theta band was further divided into low (4–7 Hz) and high (8–12 Hz) frequencies, and gamma was divided into low (30–40 Hz) and high (60–90 Hz) frequencies (Figure 5C).

LFP analysis provided a detailed view of M2 cell involvement, showing significantly increased power in the full theta band and the low theta band in Chrna2-Cre+ mice compared to controls (Supplemental Table 2F-H; Figure 5D). Notably, in the high theta band, we observed significantly higher power in successful than failed prehensions (Supplemental Table 2I; Figure 5E). Session 13 showed similar trends, with higher power in successful than failed prehensions across gamma bands (Supplemental Table 2J-K; Figure 5F–H). For the low gamma band, Chrna2-Cre+ mice displayed significantly higher power than controls, especially in successful prehensions (Supplemental Table 2L-M; Figure 5G). High gamma power was also elevated in Chrna2-Cre+ mice compared to controls (Supplemental Table 2N-O; Figure 5H).

Collectively, these findings suggest that M2 cell activation plays a role in increasing low theta and gamma power during motor planning and execution phases of a learned task, supporting the role of M2 cells in fine-tuning motor performance (Figure 6C).

## Discussion

Here we investigated how M$\alpha$2 cells impact PC population dynamics during motor learning and execution. We found that, upon increased M$\alpha$2 cell excitation, parameters of plasticity at the cell, population and assembly level, all indicate decreased plasticity (Figure 6A-B). Moreover, M$\alpha$2 cells are involved in skilled motor execution, as evidenced by altered fine motor function both after loss of M$\alpha$2 cells and during increased M$\alpha$2 cell excitation. Whereas increased M$\alpha$2 cell excitation during training did not affect prehension accuracy, it did improve the success ratio when chemogenetic activation was initiated in mice that had already learned a task (Figure 6C). Thus, our data suggest that increased M$\alpha$2 cell excitation reduces PC plasticity and facilitates the preservation of already acquired motor skills.

Lateral inhibition, a proposed function of inhibitory circuits in general and MCs in particular, can sharpen excitatory responses in sensory systems [69, 15]. Through inhibitory interneuron connections to adjacent excitatory PCs, it is believed that this mechanism helps establish a winner-take-all strategy, facilitating the selective excitation of the desired target. The winner-take-all concept implies that early activated PCs are more likely to silence neighboring cells, and thus become more influential "leader" PCs. It was previously shown that motor learning induces a shift in temporal activation of layer L2/3 PCs, with more PCs firing earlier post training than pre training [11]. In our PC calcium signal data, we found that robust patterns of temporal activation were strongly related to whether a prehension attempt was successful or failed, suggesting that PC temporal activation patterns are more influential than amplitudes. Specifically, the temporal profile of PCs in successful prehensions showed an early peak in the before prehension phase and a late peak in the during prehension phase. It has been demonstrated that SST cortical interneurons enable and maintain sequential activation of layer 2/3 PCs during motor skill learning, and that increased activity of SST interneurons is required for maintaining previously acquired layer 2/3 PC temporal activation patterns [11]. However, subtypes of SST interneurons show layer-specific and opposite activity patterns during whisking [16]. Hence, simultaneous modulation of SST interneuron activity across several layers may counteract or conceal layer-specific differences in functionality. Our data, based on a more homogenous subpopulation of SST interneurons and layer 5 dynamics, showed that increased excitation of M$\alpha$2 cells resulted in altered temporal dynamics, where more PCs reached their peak activation prematurely in the motor planning phase. This was particularly apparent in relearned successful prehensions, suggesting that M$\alpha$2 cells participate in the control of layer 5 PC sequential activation for agile movements and the selection of more influential "leader" PCs during retraining events.

Temporal properties vary between interneuron populations and similarly, PCs are likely to be varyingly inhibited depending on the timing, synaptic weights and

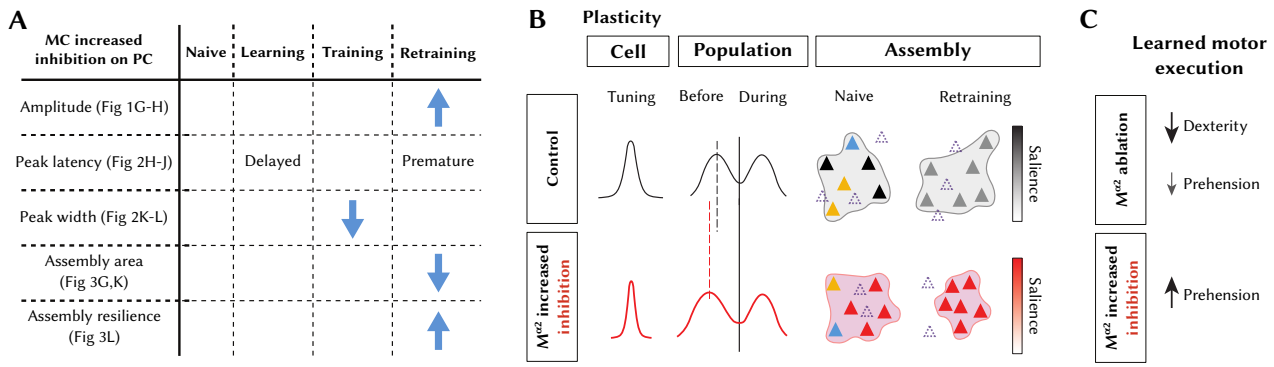


**Figure 6:** Summary of findings. A) Table summarizing the effects of increased M$\alpha$2 cells on PCs. B) Schematic outline illustrating plasticity parameters. Increased excitation of M$\alpha$2 cells resulted in more narrow tuning of layer 5 PCs, a temporal shift of the average population activity both in the motor planning and execution phases, enhanced resilience, reduced physical assembly coverage and rigid salience upon retraining. Dashed lines mark PCs that are part of the retrained assembly but not the naive assembly, and vice versa. Blue and yellow depict PCs in the naive assembly that are part of another assembly in the retraining session. Salience values are represented by the degree of color in PCs and assembly areas. C) M$\alpha$2 cells influence fine motor movements, where caspase induced ablation of M$\alpha$2 cells resulted in decreased accuracy and dexterity, whereas increased excitation of M$\alpha$2 cells resulted in increased accuracy.

the position of the interneuron contact [70]. Notably, a hypothesis postulates a central role of a local inhibitory drive for tuning of circuits that influence motor accuracy [64, 65]. Accordingly, a strong inhibitory drive would decrease the directional tuning width and lead to more accurate movements, whereas reduced inhibition would lead to the opposite. Our experimental data on motor execution adhere to and support this theory, since we found that M$\alpha$2 cell ablation, i.e. less inhibition, resulted in decreased accuracy, whereas heightened excitation of M$\alpha$2 cells, i.e. more inhibition, resulted in increased accuracy and more narrow tuning of PC activity (Figure 6).

Hebbian learning postulates that when neurons fire synchronously, they reinforce synaptic efficiency and form an assembly. The original theory has been extended to include all cells with above-chance interactions, leading to variable definitions of neural assemblies [71]. We here adhere to the proposition that assemblies are formed by neurons that together increase their average firing rates for a specific period [72, 37], and that they can harbor varying degrees of temporal precision, scale and internal structure [73]. According to the extended theory, assemblies dynamically form, reconfigure, and fire simultaneously, with a reduced number of cells triggering the entire assembly [74]. The theory also proposes that one single neuron could participate in multiple neural assemblies, leading to distributed information coding. When PCs in our data set were sorted into assemblies, we found that the average number of cells composing the assemblies were similar regardless of M$\alpha$2 cell activation state. Notably though, the area of each assembly, which remained constant during learning and retraining sessions in control mice, were significantly smaller during retraining in mice with increased M$\alpha$2 cell activity, demonstrating that M$\alpha$2 cell activity can affect PC cell assembly internal structure. In learning and retraining sessions, increased M$\alpha$2 cell excitation resulted in an increased resilience in PC assemblies, suggesting that M$\alpha$2 cells contribute to PC assembly plasticity. Accordingly, during retraining, the assembly salience decreased in control mice, but not in Chrna2-Cre+ mice, further supporting that increased M$\alpha$2 cell excitation decreases plasticity and network reorganization (Figure 6A-B).

In the motor cortex, different motor tasks induce dendritic calcium spikes on different apical branches of individual PCs, which reduces the chance of acquired skills to be disrupted by the formation of new motor programs. Intriguingly, after SST interneuron ablation, the maintenance of synaptic potentiation acquired during previously learned tasks is lost, resulting in decreased performance [3]. We found, after ablation of M$\alpha$2 cells, an increased number of drops in the pasta handling task and reduced prehension accuracy. Thus, M$\alpha$2 cells, as part of the SST population, also seem important for preserving and refining already acquired fine motor skills. Further, it has been shown that increased SST interneuron activity helps maintain previously acquired motor skills when executing a slightly different motor task, whereas decreased SST interneuron activity is important for improved motor performance and learning of the initial motor task [11, 75]. Also, the initial learning phase involves weak cortical activation of SST interneurons, and even though individual SST interneurons show diverse activity profiles during movement phases in a lever-press task, the average activ-

ity of SST interneurons increases as learning progresses [75]. We found that increased excitation of M$\alpha$2 cells during execution of a learned motor task, the single-pellet prehension task, improved the success ratio. However, in contrast to what has been shown for SST interneurons in layer 2/3 [11], repetitive increased excitation of M$\alpha$2 cells during training did not affect motor performance. Hence, it seems that increased excitation of M$\alpha$2 cells, resulting in decreased PC assembly reconfiguration and plasticity, mainly influences the execution of already acquired motor skills and that decreased motor learning during SST interneuron activation is likely mediated by other SST interneuron subpopulations. It has been suggested that layer specific network activity takes place during learning of a motor task, where the L2/3 network seem to represent coordination of signals during learning, whereas L5a may participate in networks for well-learned movements [76].

The network of inhibitory interneurons is essential for generating gamma rhythms [77]. Previous studies have highlighted the critical roles of parvalbumin and somatostatin interneurons in sustaining gamma activity in both the cortex and hippocampus [78, 79]. Research involving local field potentials (LFPs) in monkeys and humans has shown an increase in high gamma power (70–170 Hz) alongside a decrease in beta power (8–30 Hz) prior to and during forelimb movements [80, 81, 82]. In rats, high gamma power was also observed during upward movements in a reaching task, measured through epidural field potentials (EFPs) and LFPs [83], where both demonstrated an increase in high gamma power during upward reaches. Additionally, significant power increases in the high gamma band (70–170 Hz) were noted just before movement onset for upward, left, and right reaches across different tasks [83]. Our results pinpoint a role for M$\alpha$2 cells in gamma and theta oscillations during motor performance, particularly in the context of successful versus failed trials. The elevated high theta power in the success trials compared to failed trials, and increased power in low and high gamma oscillations in Chrna2-Cre+ mice compared to controls, suggests that M$\alpha$2 cells may be important for motor planning and execution. Overall, these findings reveal a complex interplay between M$\alpha$2 activity, oscillatory brain dynamics, and motor function, prompting further investigation into how these mechanisms impact behavioral outcomes.

In conclusion, we have identified a role for M$\alpha$2 cells in PC population dynamics, motor learning and execution. We find that activation of layer-5-specific Martinotti cells improves the execution of an already learned task, whereas learning efficiency of the task is unaffected, functionally separating these two aspects of motor activity. Even so, M$\alpha$2 cells are involved in temporal shaping of PC population dynamics and impact several aspects of assembly plasticity during motor learning, training and retraining. Further studies are warranted to address the activity patterns of M$\alpha$2 cells themselves during motor learning and execution, and also whether modulation of M$\alpha$2 cell activity can affect other types of learning and skills emanating from other cortical areas.

## Conflict of Interest Statement

The authors declare no conflict of interest.

## Author Contributions

Methodology (AV, JW, BC, RL), Data curation (AV, JW, BC, KH), Validation (AV, JW, BC), Software (GN, TM), Formal analysis (TM, AV, KH, GN), Visualization (TM, BC, AV, KH, KK), Original draft preparation (KK, AV, TM, BC), Conceptualization (KK, AV, RL), Writing- Reviewing and Editing (KK, AV, TM, BC).


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## Data Availability Statement

The datasets generated and/or analyzed in the current study are available on request.

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