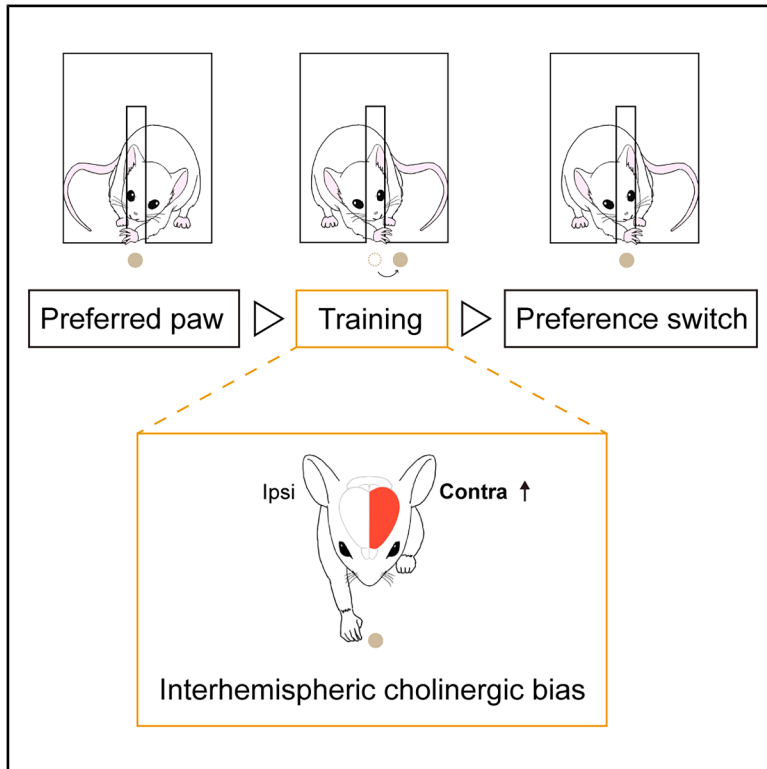


Paw switching with lateralized cholinergic modulation

Graphical abstract



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In brief

Okamoto et al. show that mouse paw preference can be switched through training and maintained over time. They reveal that acetylcholine signaling, particularly the balance of cholinergic activity between hemispheres, is critical for regulating this paw preference switch.

Highlights

- Mouse paw preference can be switched and maintained after training
- Contralateral cholinergic signaling is required for paw preference switching
- Paw use induces a transient interhemispheric imbalance in cholinergic activity
- Interhemispheric cholinergic balance regulates paw preference switching



Article

Paw switching with lateralized cholinergic modulation

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SUMMARY

Motor preferences, such as handedness, reflect asymmetries in brain function across vertebrates. Although generally stable, these hand or paw preferences can be reshaped through experience or training; however, the neural basis of such switching remains unclear. Here we show that experience-driven shifts in paw preference in mice arise from asymmetric cholinergic modulation between the two hemispheres. Using a behavioral paradigm designed to induce paw switching, we found that a subset of animals developed a stable change in preference following training. Selective activation or inhibition of cholinergic neurons on one side of the brain was sufficient to bias this switching behavior. During lateralized movements, an endogenous imbalance in cholinergic activity emerges between the hemispheres, suggesting that interhemispheric cholinergic asymmetry enables motor flexibility. These findings identify a previously unrecognized mechanism by which cholinergic signaling dynamically regulates lateralized motor control, providing insight into how hemispheric imbalances may shape persistent individual motor preferences.

INTRODUCTION

Motor skill lateralization arises from functional asymmetry between the two hemispheres of the brain.¹ Motor preferences, such as handedness, are a conserved behavioral expression of lateralization across vertebrates, including humans and rodents.^{2–4} In humans, hand preference is typically stable but can be modified through experience, as in historically enforced right-hand use among left-handed individuals.^{5–7} Such experience-dependent changes in lateralized motor behavior have been documented in humans.⁷ However, because experimental access to underlying neural circuits is necessarily limited in human studies, the mechanisms by which experience reshapes limb preference remain poorly understood.

Rodents similarly exhibit stable forelimb preference.^{8–10} As in other vertebrates, rodents display lateralized asymmetries across multiple behavioral domains, providing a broader context in which paw preference can be understood as one expression of hemispheric specialization. Paw preference in rodents has long been assessed using food-reaching paradigms, including the Collins test and its later adaptations, which established forelimb preference as a quantifiable expression of motor lateralization in mice and rats. Consistent with more recent studies on

behavioral asymmetries in rodents, these findings suggest that forelimb preference is stable enough to serve as an experimental trait, yet flexible enough to be shaped by training and task demands. Under specific conditions, rodents can even switch paw use.^{9,11} This behavioral flexibility makes rodents an ideal model for investigating the dynamics of lateralized motor control. Whether such switching represents a persistent change in forelimb preference or a transient, task-dependent adjustment in action selection remains unclear.

A potential contributor to such experience-dependent changes is cholinergic modulation of motor cortical plasticity during unilateral skill learning. In rodents, the refinement of dexterous forelimb movements relies on the plasticity of the primary motor cortex (M1) in the contralateral hemisphere,¹² and cholinergic input from the nucleus basalis (NB) in the basal forebrain is required for experience-dependent improvement in skilled reaching performance.^{13,14} More broadly, basal forebrain cholinergic signaling has been implicated in cortical plasticity, motor learning, and the acquisition of skilled forelimb actions in rodents. Notably, cholinergic projections from the NB are largely confined within each hemisphere,^{15,16} suggesting that interhemispheric asymmetries in cholinergic tone could differentially shape motor learning in the two hemispheres.



The key question, therefore, is whether experience-dependent paw switching can be induced in mice, and if so, whether hemispheric asymmetry in cholinergic signaling contributes to this process. To address this, we established a paw-switching paradigm in mice to test whether unilateral training induces a persistent shift in forelimb preference and whether this shift depends on asymmetric cholinergic signaling between the two hemispheres.

RESULTS

Sustained shift in paw preference following pellet-reach training

Although mice can adjust their paw use under biased task conditions,^{9,10} it remains unclear whether this behavioral shift persists once the bias is removed. To address this question, we developed a voluntary pellet-reaching task (Figure 1A).^{17,18} The first two days served as the before-training phase, during which individual baseline paw preference was assessed, given the substantial variability in lateralized paw use among mice. Paw use was clearly lateralized, with 54.2% of the mice classified as left-handed and 45.8% as right-handed ($n = 59$ mice; Figure 1B), consistent with previous reports.⁸ Fitting the data with a beta distribution ($\alpha = 0.010$ and $\beta = 0.024$) confirmed a strongly U-shaped distribution of paw preference. This individual lateralization remained stable across sessions in nearly all mice (91.5%, 54 of 59 mice, $R^2 = 0.91$, $p < 1.0 \times 10^{-4}$, linear regression; Figure 1C).

During the training phase, pellet positions were shifted to prevent retrieval with the preferred paw, thereby prompting use of the non-preferred paw. Non-preferred paw use progressively increased over the five-day training period. We used non-preferred paw use as the primary index of switching because increases in this measure provide a straightforward indication of the progression of paw switching during training. After training, both left- and right-handed mice exhibited greater use of the non-preferred paw, although a subset continued to favor the original preference ($n = 10$ left-handed: before -0.80 ± 0.09 , after 0.19 ± 0.16 , $n = 9$ right-handed mice: before -0.55 ± 0.12 , after 0.43 ± 0.16 ; Figure 1D). This trend was observed in both groups across the experimental phases. Although some directional differences were detected (group: $p = 0.73$, group $\times$ day: $*p = 9.7 \times 10^{-7}$, generalized linear mixed model (GLMM); Figure 1E), the present study was not intended to resolve left-right asymmetry in detail. We therefore pooled the data from both groups for the subsequent analyses focused on the overall switching process. Based on the rate of non-preferred paw use during the after-training phase, mice were retrospectively classified as switchers or non-switchers according to whether the non-preferred paw was used more frequently than the initially preferred paw. Notably, these groups diverged early during the training phase (switchers: $n = 14$, non-switchers: $n = 5$; Figure 1F). In the switched group, success rates improved over the training period, whereas the non-switched group showed little progress (switchers: $n = 14$, T1 vs. T2, $*p = 0.017$, T1 vs. T3, $*p = 1.8 \times 10^{-3}$, T1 vs. T4, $*p = 1.8 \times 10^{-3}$, T1 vs. T5, $*p = 4.9 \times 10^{-4}$, non-switchers: $n = 5$, $p = 0.81$, Friedman test followed by Wilcoxon signed-rank test; Figure 1G). These results suggest that the enhancement of motor

skills with the non-preferred paw can drive a stable switch in paw preference. Both newly acquired and pre-existing paw preferences persisted for at least two weeks following training ($n = 9$ mice, $R^2 = 0.93$, $p = 3.0 \times 10^{-4}$, linear regression; Figures 1H–1J).

Paw switch requires contralateral cholinergic system

We next examined the role of the cholinergic system originating from the NB, which is essential for the improvement of forelimb skills.^{13,19} This projection is predominantly ipsilateral,^{15,20,21} a feature we confirmed using retrograde tracing with cholera toxin subunit B (CTB) (NB contra: $0.21 \pm 0.21\%$, NB ipsi: $21.9 \pm 4.0\%$, horizontal limb of the diagonal band of Broca (HDB) contra: $0 \pm 0\%$, HDB ipsi: $1.7 \pm 1.2\%$, $n = 3$ mice, $*p = 0.045$, Friedman test; Figure S1) and anterograde labeling with an adeno-associated virus (AAV) vector (contra: $0.70 \pm 0.07\%$, ipsi: $20.9 \pm 1.7\%$, $n = 3$ mice, $**p = 7.1 \times 10^{-3}$, paired t test; Figures 2A–2E).

We hypothesized that paw switching is associated with hemispheric asymmetry in cholinergic activity. To test this, we selectively ablated cholinergic neurons unilaterally in the NB contralateral to the training paw, using an immunotoxin (IT; anti-Tac(Fav)-PE38) system^{22,23} in choline acetyltransferase (ChAT)-Cre transgenic mice (Figures 2F–2I). This unilateral cholinergic ablation impaired paw switching compared with ChAT-Cre-negative littermates ($n = 7$ littermates, $n = 6$ ChAT-Cre mice, group: $p = 0.079$, group $\times$ day: $*p = 1.8 \times 10^{-6}$, GLMM; Figure 2J). When classified by initial paw preference, this effect appeared more pronounced in right-handed mice switching to left-paw use (Figure S2). The ablation group exhibited significantly lower non-preferred scores in the after-training phase ($n = 7$ littermates: -0.24 ± 0.28 , $n = 6$ ChAT-Cre mice: 0.44 ± 0.17 , $*p = 0.011$, Student's t test; Figure 2K), and their success rates during training appeared modestly reduced, although this effect was not statistically significant ($n = 7$ littermates, $n = 6$ ChAT-Cre mice, group, $**p = 2.0 \times 10^{-3}$, group $\times$ day, $p = 0.56$, two-way ANOVA; Figure 2L). Note that the extent of cholinergic cell ablation was not significantly correlated with the non-preferred score (Figure S3). In contrast, ablation performed after training did not affect switching ($n = 7$ controls, $n = 4$ ChAT-Cre mice, transition: group, $p = 0.27$, group $\times$ day, $p = 0.080$, GLMM; non-preferred score: $p = 0.95$, Student's t test; success rates: group, $p = 0.060$, group $\times$ day, $p = 0.82$, two-way ANOVA; Figure S4), consistent with previous findings¹³ that post-training cholinergic loss spares task performance. These findings indicate that cholinergic input is essential for enabling paw switching, and that its loss during the training phase disrupts motor learning performance.

Cholinergic axons from the NB innervate a wide range of brain areas within each hemisphere.^{24–26} We therefore tested whether the NB-to-M1 pathway is required for paw switching. Optogenetic inhibition of cholinergic axon terminals in M1 during the training phase significantly impaired paw switching compared with ChAT-Cre-negative littermates ($n = 8$ littermates, $n = 7$ ChAT-Cre mice, group: $p = 0.091$, group $\times$ day: $*p = 4.0 \times 10^{-7}$, GLMM; Figures 2M–2P). When classified by initial paw preference, a similar effect was observed in both right-to-left and left-to-right switching (Figure S5). Non-preferred scores in the after-training phase were reduced ($n = 8$ littermates: 0.38 ± 0.29 ,

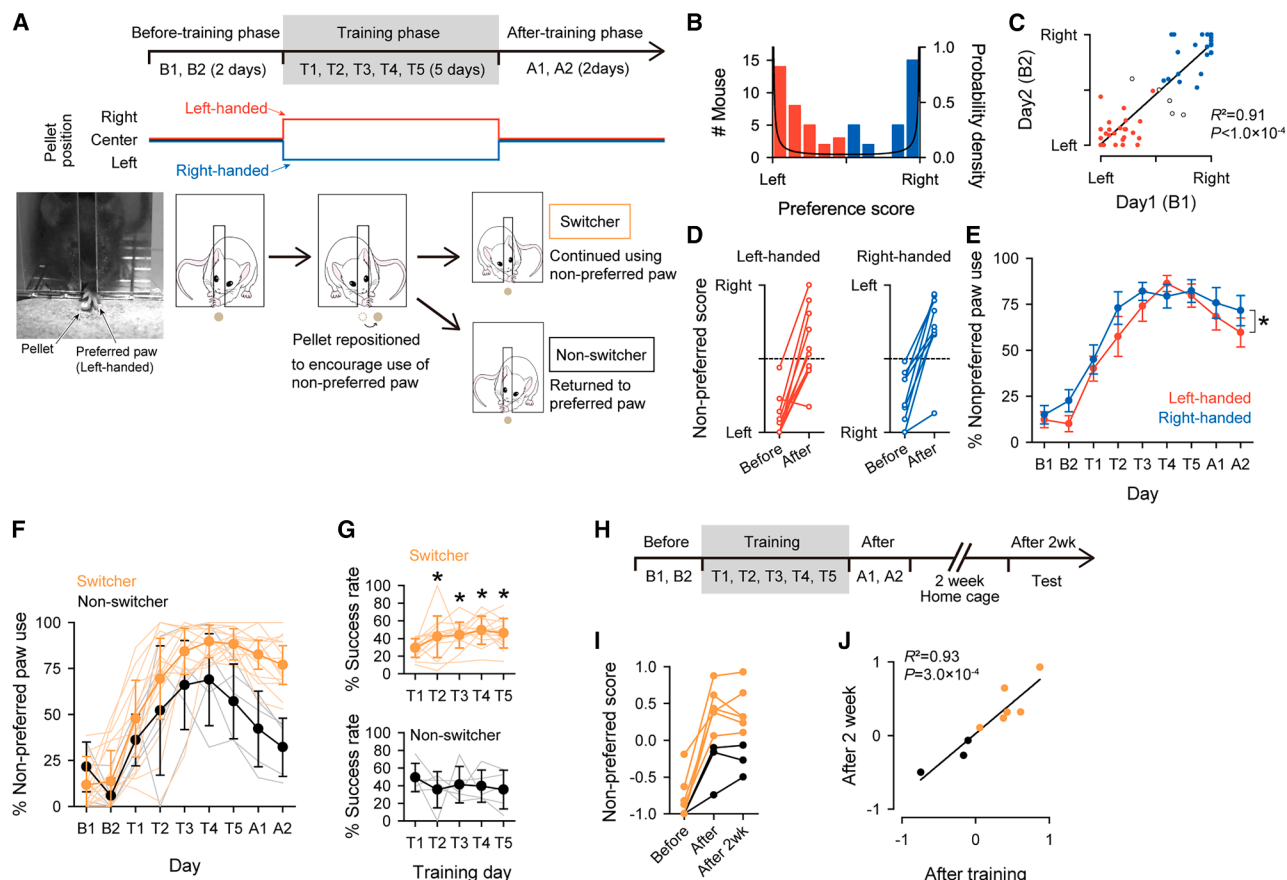


Figure 1. Sustained shift in paw preference following pellet-reach training

(A) Behavioral paradigm of the paw-switching experiment. The preferred paw of each mouse was determined over a two-day period by a pellet-reaching task (before-training phase). During the five-day training phase, the pellet was laterally displaced so that mice could reach it only with their non-preferred paw (training phase). Finally, the pellet was returned to the center for a two-day period (after-training phase).

(B) Distribution of left- and right-handed mice. $n = 59$ mice. Beta distribution fitting yielded $\alpha = 0.010$ and $\beta = 0.024$, indicating a strongly U-shaped distribution.

(C) Day 1 versus day 2 distribution of left- and right-handed mice shows stable individual lateralization ($n = 59$ mice, $R^2 = 0.91$, $p < 1.0 \times 10^{-4}$, linear regression).

(D) Non-preferred score, indicating the use rate of the non-preferred paw, increased in the after-training phase in both left- and right-handed mice ($n = 10$ left-handed and $n = 9$ right-handed mice).

(E) Transition in non-preferred paw use of left- and right-handed groups ($n = 10$ left-handed and $n = 9$ right-handed mice, group: $p = 0.73$, group $\times$ day: $*p = 9.7 \times 10^{-7}$, generalized linear mixed model (GLMM)).

(F) Transition of non-preferred hand use in the switchers and non-switchers.

(G) Success rates during training. Switchers: $n = 14$, $*p < 0.02$, non-switchers: $n = 5$, $p = 0.80$ (Friedman test followed by Wilcoxon signed-rank test).

(H) Behavioral paradigm including an after-two-week test.

(I) Non-preferred score at three time points.

(J) Non-preferred scores remained stable two weeks after training ($n = 9$, $R^2 = 0.93$, $p = 3.0 \times 10^{-4}$, linear regression).

$n = 7$ ChAT-Cre mice: -0.62 ± 0.26 , $*p = 0.025$, Student's t test; Figure 2Q), and success rates during training appeared similarly reduced, although this effect was not statistically significant ($n = 8$ littermates, $n = 7$ ChAT-Cre mice, group, $***p < 1.0 \times 10^{-3}$, group $\times$ day, $p = 0.62$, two-way ANOVA; Figure 2R). Together, these manipulations demonstrate that cholinergic input from the NB to M1 is indispensable for the acquisition of paw switching behavior.

Finally, we assessed whether unilateral manipulation of the NB in the opposite hemisphere affected paw switching. IT injection ipsilateral to the training paw did not impair paw switching, but instead facilitated it ($n = 7$ controls, $n = 5$ ChAT-Cre mice, transi-

tion: group, $p = 0.12$, group $\times$ day, $*p = 1.8 \times 10^{-5}$, GLMM; non-preferred score: $p = 0.18$, Student's t test; success rates: group, $p = 0.27$, group $\times$ day, $p = 0.64$, two-way ANOVA; Figure S6), suggesting that ipsilateral ablation, unrelated to the training circuit, may influence motor preference via interhemispheric mechanisms.

Task-evoked lateralization of cholinergic activity during paw use

To determine whether cholinergic activities in the two hemispheres are independent, we performed bilateral fiber photometry recordings. Cortical neurons were transduced with an AAV

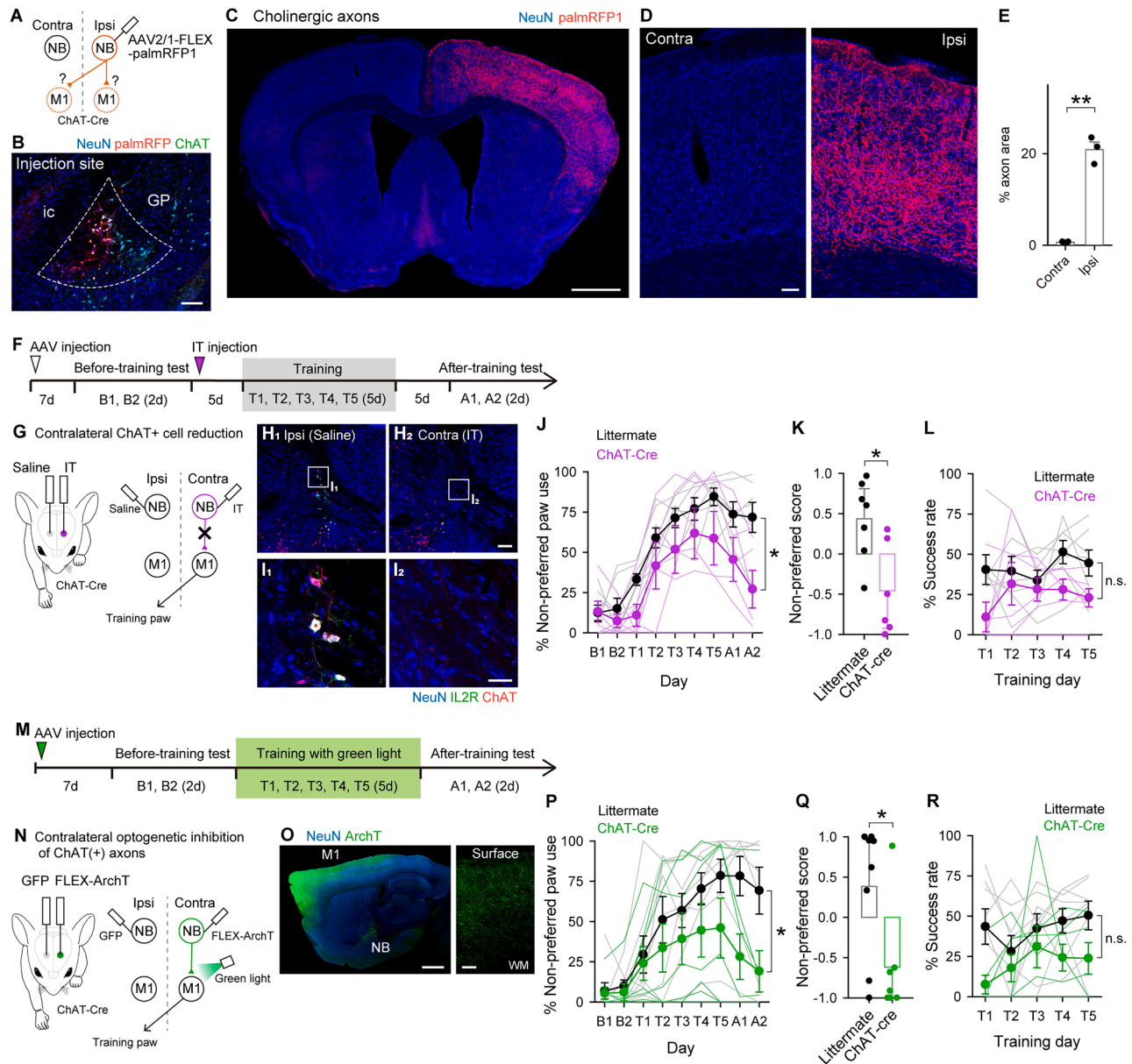


Figure 2. Paw switch requires contralateral cholinergic system

(A) Schematic of anterograde tracing using an AAV vector. AAV2/1-FLEX-palmRFP1 was unilaterally injected into the NB of ChAT-Cre mice.

(B) Representative image of the AAV injection site. Scale bars, 200 μ m.

(C and D) Representative images of RFP-labeled axons projected ipsilaterally to the cortex. Scale bars, 1 mm (C) and 200 μ m (D).

(E) Labeled axons were significantly more observed in the ipsilateral M1 forelimb area than in the contralateral area. ($n = 3$, $**p = 7.1 \times 10^{-3}$, paired t test).

(F and G) Schematic and behavioral paradigm for unilateral NB ablation. ChAT-Cre mice received bilateral NB injections of AAV2/1-CAGGS-FLEX-[IL2R-EGFP]-3'USS to induce selective expression of the human interleukin-2 receptor α -subunit (IL2R) in cholinergic neurons. After identification of the preferred paw in the before-training test, anti-Tac(Fv)-PE38, a recombinant immunotoxin (IT) targeting IL2R-expressing cells, was unilaterally injected into the NB contralateral to the subsequent training paw, while saline was injected into the opposite side.

(H and I) Representative images of the NB ipsilateral (Ipsi) and contralateral (Contra) to the training paw. Cholinergic neurons were ablated in Contra NB. Scale bars, 200 μ m (H) and 50 μ m (I).

(J–L) Behavioral effects of unilateral NB ablation. (J) Transition in non-preferred paw use in ChAT-Cre mice and littermate controls. ($n = 7$ littermates, $n = 6$ ChAT-Cre mice, group: $p = 0.079$, group $\times$ day: $*p = 1.8 \times 10^{-6}$, GLMM). (K) Non-preferred score on day 9, indicating the relative use of the non-preferred paw ($*p = 0.011$, Student's t test). (L) Success rate during training (group: $**p = 2.0 \times 10^{-3}$, group $\times$ day: $p = 0.56$, two-way ANOVA).

(M and N) Schematic and behavioral diagram for unilateral optogenetic inhibition of NB-origin axons in M1. Green light was continuously applied during the training session.

(legend continued on next page)

vector expressing gACh4h,²⁷ a genetically encoded fluorescent acetylcholine sensor, and signals were monitored bilaterally from the M1 as mice performed a voluntary pellet-reaching task (Figure 3A). Contrary to expectations based on the predominantly unilateral organization of NB projections (Figures 2A and 2B), cholinergic activity exhibited strong interhemispheric synchrony throughout the recording (Figure 3B). When signals were aligned to the pellet-reaching movement with the preferred paw, both hemispheres showed concurrent increases in activity, consistent with previous findings that acetylcholine levels rise during forelimb movement.²⁸ Notably, immediately after the reach, the hemisphere contralateral to the active paw displayed a transiently greater increase in signal intensity ($n = 96$ trials from 4 mice, $***p = 2.0 \times 10^{-4}$, cluster-based permutation test; ΔACh : ipsi 1.1 ± 0.2 a.u. $\cdot$ s, contra 1.5 ± 0.2 a.u. $\cdot$ s, $n = 4$ mice, Cohen's $d = 2.1$; Figures 3B–3E).

On the following day, when animals were trained to use the non-preferred paw, the hemisphere contralateral to the active paw, ipsilateral to the originally preferred paw, exhibited a stronger response than the opposite side ($n = 37$ trials from 4 mice, $***p = 8.0 \times 10^{-4}$, cluster-based permutation test; ΔACh : contra 1.4 ± 0.3 a.u. $\cdot$ s, ipsi 0.99 ± 0.28 a.u. $\cdot$ s, $n = 4$ mice, Cohen's $d = 1.4$, Figures 3F–3H). These results suggest that, while cholinergic activity in M1 is largely synchronized across hemispheres, it shows transient, task-locked lateralization contralateral to the engaged paw.

Opposing cholinergic imbalance disrupts paw switching

To experimentally induce asymmetry in cholinergic signaling, we unilaterally activated the NB using the DREADD system.^{29,30} We first combined bilateral fiber photometry recordings from the M1 with chemogenetic activation to evaluate the effect of hM3Dq on cortical cholinergic dynamics (Figures S7A and S7B). Following intraperitoneal administration of deschloroclozapine (DCZ), the gACh4h signals increased predominantly in the M1 ipsilateral to the unilateral virus injection side relative to the contralateral hemisphere ($n = 3$ mice, $*p = 0.037$, paired t test; Figure S7C).

We next applied this DREADD system to the pellet-reaching task by inducing unilateral hM3Dq expression in the NB (Figure 4). Animals were classified post hoc as the contralateral or ipsilateral activation group based on the injected hemisphere and paw preference. DCZ was administered each day intraperitoneally during the five-day training phase. Chemogenetic activation of the contralateral NB accelerated the transition of paw switching in ChAT-Cre mice ($n = 7$ littermates, $n = 7$ ChAT-Cre mice, group: $p = 0.51$, group $\times$ day: $*p = 8.8 \times 10^{-3}$, GLMM; Figures 4B–4D), although the final degree of paw switching was not significantly affected (littermate 0.39 ± 0.18 , ChAT-Cre 0.65 ± 0.19 , $p = 0.32$, Student's t test; Figure 4E). When classified by initial paw preference, this effect appeared more pronounced in left-handed mice switching to right-paw use (Figures S8A and S8B). Activated mice showed a modest, non-significant increase

in success rates compared with controls ($n = 7$ littermates, $n = 6$ ChAT-Cre mice, group, $*p = 0.027$, group $\times$ day, $p = 0.12$, two-way ANOVA; Figure 4F). Note that the extent of hM3Dq expression was not significantly correlated with the non-preferred score (Figures S9A and S9B).

We then activated to the NB ipsilateral to the training paw (Figure 4G). This manipulation was intended to impose a cholinergic bias opposite to the task-evoked lateralization observed during paw use in Figure 3. Ipsilateral NB activation suppressed paw switching ($n = 13$ littermates, $n = 9$ ChAT-Cre mice, transition: group, $*p = 0.020$, group $\times$ day, $*p = 1.9 \times 10^{-7}$, GLMM; non-preferred score: littermate 0.47 ± 0.18 , ChAT-Cre 0.06 ± 0.23 , $*p = 0.045$, Student's t test; Figures 4H and 4I), without affecting overall task performance ($n = 13$ littermates, $n = 9$ ChAT-Cre mice, group, $p = 0.068$, group $\times$ day, $p = 0.92$, two-way ANOVA; Figure 4J). When classified by initial paw preference, a similar effect was observed in both right-to-left and left-to-right switching (Figures S8C and S8D). As in the contralateral activation group, the extent of hM3Dq expression was not significantly correlated with the non-preferred score (Figures S9C and S9D). This effect parallels that of ipsilateral IT ablation (Figure S6), suggesting that unbalanced cholinergic activation across hemispheres biases motor preference through interhemispheric interactions. Collectively, these findings indicate that improvements in task performance alone cannot account for the induction of paw switching; rather, an interhemispheric cholinergic dominance is required.

Paw choice reflects choice history more than immediate outcome

To examine whether paw switching during the training period is driven by reward-based strategy updating, we modeled trial-by-trial paw choice using Bayesian logistic regression.^{31–34} For each mouse, we predicted the paw used on trial t based on the choice and reach outcome on the preceding trial, with reach outcomes classified into four categories: no contact, failed attempt, success (imprecise grasp), and success (precise grasp) (Figure 5A). We first examined the conditional probability of repeating the same paw given each reach outcome. Mice showed a high probability of staying with the same paw across all outcome categories, including after trials with no contact with the pellet (no contact: 0.63 ± 0.04 , failed attempt: 0.76 ± 0.03 , success (imprecise grasp): 0.77 ± 0.04 , success (precise grasp): 0.80 ± 0.04 , $n = 18$ mice; Figure 5B). This suggests that paw choice is not strongly modulated by immediate reach outcome.

We next compared four models: a base model, which predicts the next paw using only the overall preference of each mouse; a choice history model (Model persev), based on the immediately preceding paw choice; a reinforcement learning model (Model RL), based on a win-stay/lose-shift rule using reach success as the reward signal; and a combined model (Model persev + RL)

(O) Representative images show ArchT expression in ipsilateral M1. WM, white matter. Scale bars, 1 mm (left) and 100 μ m (right).

(P–R) Behavioral outcomes of optogenetic inhibition. (P) Transition in non-preferred hand use ($n = 8$ littermates, $n = 7$ ChAT-Cre mice, group: $p = 0.091$, group $\times$ day: $*p = 4.0 \times 10^{-7}$, GLMM). (Q) Non-preferred score on day 9 ($*p = 0.025$, Student's t test). (R) Success rates during training (group: $***p < 1.0 \times 10^{-3}$, group $\times$ day: $p = 0.62$, two-way ANOVA).

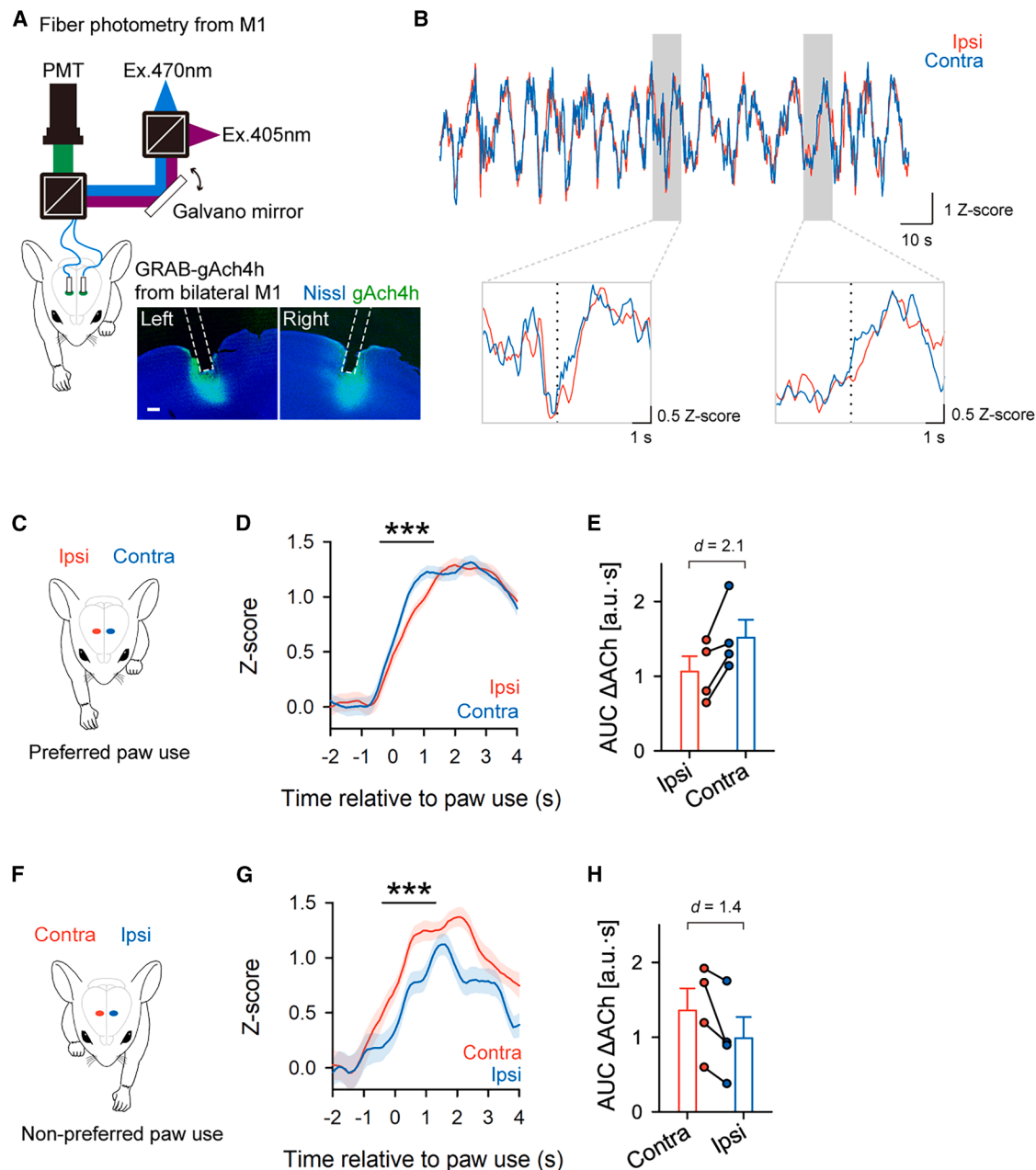


Figure 3. Task-evoked lateralization of cholinergic activity during paw use

(A) Schematic of bilateral fiber photometry targeting M1. Representative images showing fiber tracks and GRAB-gACh4h expression in M1. Scale bars, 200 μ m. (B) Top: representative traces of gACh4h signals from ipsilateral (red) and contralateral (blue) M1. Bottom: enlarged view of shaded region. Dashed lines indicate paw-use onset.

(C) Schematic of preferred paw use and corresponding ipsilateral and contralateral recordings.

(D) Averaged traces of gACh4h signals aligned to paw-use onset ($n = 96$ trials from 4 mice). Bar above indicates significant periods ($***p = 2.0 \times 10^{-4}$, cluster-based permutation test).

(E) Signal changes associated with paw use ($n = 4$ mice. Cohen's $d = 2.1$).

(F) Same as in (C), except that the active paw was switched from the preferred to the non-preferred paw, with ipsilateral and contralateral recordings correspondingly reversed. Red and blue continue to indicate the same sides as in (B–E).

(G) Same as (D), but for non-preferred paw use ($n = 37$ trials from 4 mice. $***p = 8.0 \times 10^{-4}$).

(H) Same as (E), but for non-preferred paw use ($n = 4$ mice. Cohen's $d = 1.4$).

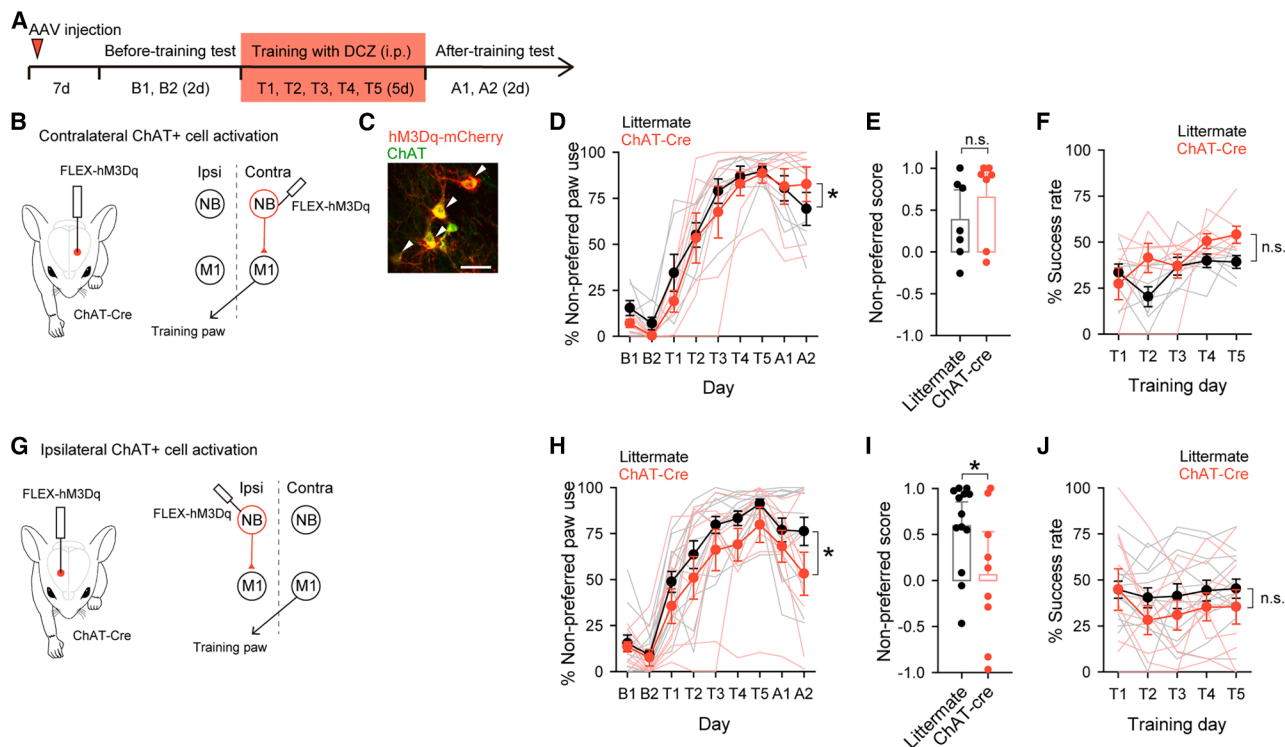


Figure 4. Opposing cholinergic imbalance disrupts paw switching

(A) Schematic of unilateral NB activation. (B) Behavioral paradigm for contralateral NB activation. (C) Representative image of hM3Dq-expressing cells in NB. Scale bars, 50 μ m. (D–F) Behavioral outcomes of contralateral NB activation. (D) Transition in non-preferred paw use ($n = 7$ littermates, $n = 7$ ChAT-Cre mice, group: $p = 0.51$, group $\times$ day: $*p = 8.8 \times 10^{-3}$, GLMM). (E) Non-preferred score on day 9, indicating the relative use of the non-preferred paw ($p = 0.32$, Student's t test). (F) Success rates during training (group: $*p = 0.027$, group $\times$ day: $p = 0.12$, two-way ANOVA). (G) Behavioral paradigm for ipsilateral NB activation. (H–J) Behavioral outcomes of ipsilateral NB activation. (H) Transition in non-preferred hand use ($n = 13$ littermates, $n = 9$ ChAT-Cre mice, group: $*p = 0.020$, group $\times$ day: $*p = 1.9 \times 10^{-7}$, GLMM). (I) Non-preferred score on day 9 ($*p = 0.045$, Student's t test). (J) Success rates during training (group: $p = 0.068$, group $\times$ day: $p = 0.92$, two-way ANOVA).

incorporating both predictors. We evaluated prediction accuracy of these models using the Widely Applicable Information Criterion (WAIC), measured as improvement from the base model. The choice history model substantially improved prediction, whereas the reinforcement learning model contributed minimally ($n = 18$ mice; Figure 5C). Among the four models, pairwise comparison of WAIC revealed that the choice history model significantly outperformed the reinforcement learning model (Model persev vs. RL: 343 ± 39 vs. 365 ± 37 , $n = 18$ mice, $*p = 0.020$, Wilcoxon signed-rank test with Bonferroni correction). The WAIC of the combined model did not significantly differ from that of the choice history model alone (Model persev vs. persev + RL: 343 ± 39 vs. 343 ± 39 , $p = 1.0$, Wilcoxon signed-rank test with Bonferroni correction). The reinforcement learning model did not significantly improve over the base model (base model vs. Model RL: 366 ± 37 vs. 365 ± 37 , $p = 0.12$, Wilcoxon signed-rank test with Bonferroni correction), indicating that reach outcome provided little additional predictive value for trial-by-trial paw choice. When both predictors were included in the combined model, the choice history weight (β persev)

was significantly larger than the reinforcement learning weight (β RL) (β persev vs. β RL: 1.26 ± 0.31 vs. 0.21 ± 0.07 , $n = 18$, $*p = 5.0 \times 10^{-3}$, Wilcoxon signed-rank test; Figure 5D).

We further extended the model to include up to 5 preceding trials. Choice history weights remained substantial across all lags (β persev lag 1: 0.54 ± 0.22 , lag 2: 0.84 ± 0.15 , lag 3: 0.84 ± 0.10 , lag 4: 0.54 ± 0.19 , lag 5: 0.51 ± 0.14), whereas reach outcome weights were weakly positive at lag 1 and decayed to near zero by lag 3 (β RL lag 1: 0.25 ± 0.07 , lag 2: 0.18 ± 0.07 , lag 3: -0.012 ± 0.057 , lag 4: -0.021 ± 0.044 , lag 5: -0.0029 ± 0.0718 ; Figure 5E). These results indicate that paw choice is predominantly driven by the history of previous choices rather than by reach outcome, and that this influence persists over multiple trials.

To test whether cholinergic manipulation altered this pattern, we applied the same analysis to mice with cholinergic cell reduction in Figures 2C–2I and mice with DREADD-mediated ipsilateral activation in Figures 4B–4F. In mice with cholinergic cell reduction, no significant differences were observed between control and manipulation groups ($n = 7$ littermates, $n = 6$

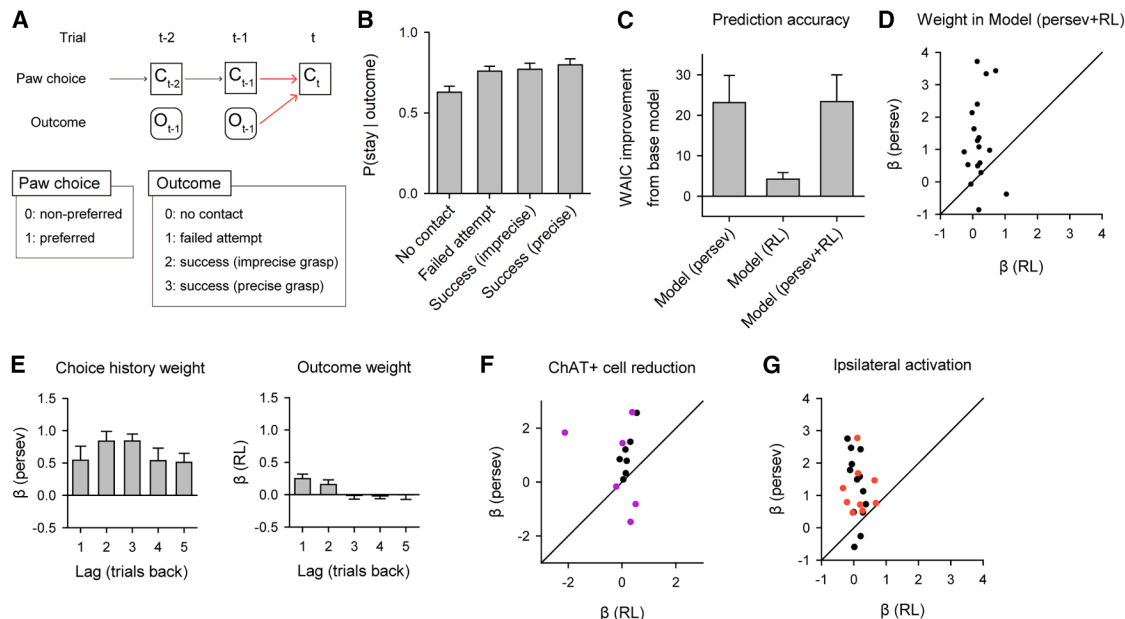


Figure 5. Paw choice reflects choice history more than immediate outcome

(A) Schematic of the Bayesian logistic regression model. Paw choice on trial t (C_t) was predicted from the history of previous paw choices ($C_{t-1}, \dots$) and reach outcomes ($O_{t-1}, \dots$). Paw choice was coded as preferred (1) or non-preferred (0). Reach outcomes were classified into four levels (0–3).

(B) Conditional probability of repeating the same paw on the next trial as a function of outcome on the immediately preceding trial (1-back). Mice showed high stay probability (>0.6) regardless of reach outcome. Data from mice are presented in Figure 1 ($n = 18$ mice).

(C) Model comparison of prediction accuracy for paw choice (C_t) using the Widely Applicable Information Criterion (WAIC). WAIC improvement from the base model for three models: Model (persev; choice history only), Model (RL; win-stay/lose-shift only), and Model (persev + RL; combined). Higher values indicate better prediction of trial-by-trial paw choice. Data from mice are presented in Figure 1 ($n = 18$ mice).

(D) Estimated weights (β) in the combined model (Persev + RL) for each mouse. Each dot represents one mouse. Data from mice are presented in Figure 1 ($n = 18$ mice, $p = 5.0 \times 10^{-3}$, Wilcoxon signed-rank test).

(E) Weights (β) by trial lag in the combined model extended to include up to 5 preceding trials. Left: choice history weight (β persev). Right: reach outcome weight (β RL). Data from mice are presented in Figure 1 ($n = 18$).

(F) Weights (β) in the combined model for mice with cholinergic cell reduction in Figure 2. Black: littermate controls ($n = 7$); magenta: ChAT-Cre ($n = 6$). No significant difference was observed between groups (β persev: $p = 0.73$; β RL: $p = 0.84$, Wilcoxon rank-sum test).

(G) Same as (F) for mice with DREADD-mediated ipsilateral activation in Figure 4. Black: littermate controls ($n = 13$); red: ChAT-Cre ($n = 9$). No significant difference was observed between groups (β persev: $p = 0.74$; β RL: $p = 0.84$, Wilcoxon rank-sum test).

ChAT-Cre, β persev: 1.04 ± 0.31 vs. 0.56 ± 0.66 , $p = 0.73$; β RL: 0.19 ± 0.08 vs. -0.19 ± 0.40 , $p = 0.84$, Wilcoxon rank-sum test; Figure 5F). Similarly, in mice with DREADD-mediated ipsilateral activation, no significant differences were found ($n = 13$ littermates, $n = 9$ ChAT-Cre, β persev: 1.26 ± 0.29 vs. 1.15 ± 0.25 , $p = 0.74$; β RL: 0.10 ± 0.05 vs. 0.16 ± 0.11 , $p = 0.84$; Wilcoxon rank-sum test; Figure 5G). These findings indicate that the manipulations did not shift the behavioral strategy toward trial-by-trial reward-based updating.

DISCUSSION

In this study, we investigated the role of interhemispheric cholinergic modulation in paw preference switching. We established a behavioral paradigm in which mice exhibited training-induced changes in paw preference, revealing that this process requires cholinergic input from the NB, which exerts hemispheric-dominant control over M1. Although acetylcholine dynamics in M1 were largely synchronized bilaterally, a transient lateralized enhancement contralateral to the reaching paw emerged during

task execution. Notably, manipulation of the hemisphere not directly engaged in the trained paw movement also influenced switching, suggesting that lateralized motor behavior is governed by the interhemispheric balance of cholinergic activity.

Although forelimb preference in rodents is well-documented, how it can be modified through training remains unclear. Using our developed behavioral paradigm, we demonstrated that repeated training induces a robust and persistent switch in paw use, with the newly acquired preference maintained for at least two weeks (Figures 1H–1J). Consistent with the individually determined nature of paw preference, the paradigm revealed substantial inter-individual variability. Many mice adopted and maintained the trained paw, whereas others either retained their original preference or reverted after transient switching (Figure 1F). Such heterogeneity likely reflects intrinsic differences in the flexibility of lateralized motor circuits. Bayesian logistic regression indicated that trial-by-trial paw choice was more strongly influenced by perseveration than by reinforcement learning-like updating (Figure 5). This may suggest that paw choice has a substantial habit-like component, reflected in the stabilization of repeated action

selection. However, this interpretation applies primarily to trial-by-trial choice and does not exclude the possibility that reward-driven learning contributes to the gradual shift in paw use across training sessions.

The switching paradigm revealed a directional asymmetry (Figure 1E), despite previous reports suggesting the absence of a marked population-level left-right bias in rodents (consistent with our observation in Figure 1B). Specifically, switching appeared to occur more readily from right to left, whereas left-to-right switching was less readily induced. One possible concern is that relatively novel experimental conditions may have affected the initial assessment of paw preference.³⁵ However, animals showing inconsistent paw preference during the two before-training sessions were excluded from the subsequent analyses. Thus, the directional asymmetry observed here is unlikely to be attributable solely to unstable baseline classification and may instead represent an asymmetry that becomes apparent under this retraining paradigm. A similar direction-dependent tendency was also observed in the cholinergic manipulation experiments (Figures S2 and S8). In particular, the effect of cholinergic ablation was apparent in initially right-handed mice undergoing right-to-left switching, whereas a comparable effect was not evident in initially left-handed mice (Figure S2). Thus, the present study does not determine whether cholinergic mechanisms operate symmetrically across right-to-left and left-to-right switching. The basis of this directional asymmetry remains to be clarified in future studies.

Our findings may also have relevance to the literature on handedness shift or relearning in humans. While handedness itself has been extensively studied in humans, direct investigations of handedness shift or relearning remain limited. Human studies suggest that manual preference can be modified by training, environmental pressure, or injury, but that such changes are constrained.⁷ In this respect, our mouse switching paradigm may provide a useful model for studying how latent lateral bias and neural asymmetry influence hand-use plasticity.³⁶

Unilateral skill enhancement is required for paw-switching, a process that depends on cholinergic signaling,^{13,14,28} and hemispheric dominance of projections from the NB.^{15,16,21} Consistent with this, unilateral disruption of cholinergic input prevented paw switching (Figure 2). However, motor improvement alone was insufficient to robustly induce switching, as shown in Figure 4. Chemogenetic activation of cholinergic input contralateral to the training paw increased success rates and moderately affected the time course of switching, but did not result in a clear shift in paw preference (Figures 4B–4F), whereas ipsilateral activation suppressed switching without impairing performance (Figures 4G–4J). These findings suggest that cholinergic signaling contributes not only to the facilitation of motor learning but also to the regulation of interhemispheric balance associated with lateralized motor output, although the downstream circuit mechanisms remain to be determined.

Basal forebrain cholinergic activity has been implicated in a range of functions, including arousal, attention, and learning.^{13,37–39} Given this diversity, the gACh4h signals recorded here may reflect multiple components beyond motor-specific modulation, potentially accounting for the high degree of interhemispheric synchrony observed (Figure 3B). Nevertheless,

anatomical evidence indicates that cholinergic projections from the NB to M1 are largely confined within hemispheres (Figures 2A–2E and S1),^{15,16} and that these M1-projecting cholinergic neurons receive input exclusively from the striatum, which itself exhibits lateralized connectivity.²⁵ Therefore, the task-locked contralateral enhancements observed during single-paw reaching may represent a motor-related component of this broader cholinergic activity (Figures 3C–3F). Repeated experience may initially amplify subtle cholinergic asymmetries, leading to the stabilization of lateralized motor output over time. It also remains possible that asymmetry in cholinergic activity influences not only training-induced switching but also other aspects of paw preference. However, because the present study focused specifically on skill enhancement during switching, the broader contribution of acetylcholine to paw preference remains unclear.

Finally, our findings suggest that interhemispheric asymmetry in cholinergic activity contributes to the regulation of motor lateralization. Although cholinergic projections from the NB to M1 are predominantly unilateral (Figures 2A–2E and S1), non-cholinergic neurons within the NB have been reported to form reciprocal connections across hemispheres,^{40,41} and cortical areas are extensively interconnected through commissural fibers.^{42–44} These interhemispheric connections may permit competitive interactions between the hemispheres, thereby facilitating lateralized motor execution.⁴⁵ Therefore, even subtle imbalances in cholinergic activity during unilateral training may gradually bias motor output and consolidate asymmetric motor behaviors at the individual level. However, the NB-derived cholinergic influence examined here is likely to represent only one component of the mechanisms underlying motor asymmetry. More generally, lateralization may be formed through the combined influence of multiple factors, including cholinergic modulation, other synaptic inputs, and interhemispheric circuit interactions.

Limitations of the study

Although this study provides evidence that cholinergic signaling bias contributes to training-induced changes in paw use, several limitations should be noted. First, the paw-switching paradigm requires animals to acquire a new behavioral strategy while changing paw use, and therefore cannot fully dissociate cholinergic contributions to motor learning or reward-related processes from those to paw switching or paw preference. Second, the directional asymmetry observed here remains unresolved. Paw switching and the effects of cholinergic manipulation both showed direction-dependent tendencies, but the basis of this asymmetry remains unclear. Thus, the present study does not determine whether cholinergic mechanisms operate symmetrically across right-to-left and left-to-right switching. Finally, although the ablation and chemogenetic experiments suggest that interhemispheric cholinergic balance contributes to paw switching, the downstream cortical and interhemispheric circuits mediating this effect remain unresolved.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Hiroyuki Hioki (h-hioki@juntendo.ac.jp).

Materials availability

All unique/stable reagents generated in this study are available from the [lead contact](#) with a completed materials transfer agreement.

Data and code availability

- The source data have been deposited at Zenodo at <https://doi.org/10.5281/zenodo.19659219> and are publicly available as of the date of publication.
- The original code has been deposited at Zenodo at <https://doi.org/10.5281/zenodo.19659219> and is publicly available as of the date of publication.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, K.O. and H.H.; methodology, K.O., Y.R.T., and H.H.; investigation, K.O.; resources, S.K., S.X., G.L., K.K., Y.L., and H.H.; visualization, K.O.; writing – original draft, K.O. and H.H.; writing – review and editing, K.O., Y.R.T., S.K., S.X., G.L., K.K., Y.L., M.K., and H.H.; project administration, K.O. and H.H.; funding acquisition, K.O. and H.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE MANUSCRIPT PREPARATION PROCESS

During the preparation of this work, the authors used ChatGPT (OpenAI) or Claude (Anthropic) to check grammar, enhance clarity, and assist with code drafting related to the Bayesian logistic regression analysis. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-ChAT (Goat)	Millipore	Cat# AB144P; RRID: AB_2079751
Anti-NeuN (Mouse)	Millipore	Cat# MAB377; RRID: AB_2298772
Anti-ChAT (Rabbit)	Millipore	Cat# AB143; RRID: 2079760
Anti-CTb (Goat)	List Biological Laboratories	Cat# 703; RRID: AB_10013220
Anti-mRFP (Rabbit)	Hiroyuki Hioki ⁴⁶	RRID: AB_2336889
Anit-GFP (chicken)	Aves Labs	Cat# GFP-1020; RRID: AB_10000240
Alexa Fluor 647 donkey anti-goat	Abcam	Cat# ab150131; RRID: AB_2732857
Alexa Fluor 405 donkey anti-mouse	Abcam	Cat# ab175658; RRID: AB_2687445
Alexa Fluor 568 donkey anti-goat	Thermo Fisher Scientific	Cat# A11057; RRID: 2534104
Alexa Fluor 647 donkey anti-rabbit	Thermo Fisher Scientific	Cat# A31573; RRID: 2536183
Alexa Fluor 647 donkey anti-chicken	Thermo Fisher Scientific	Cat# A21469; RRID: AB_253572
Alexa Fluor 568 donkey anti-rabbit	Thermo Fisher Scientific	Cat# A10042; RRID: AB_2534017
anti-Tac(Fav)-PE38	Kazuto Kobayashi ²²	N/A
Bacterial and virus strains		
AAV2/1-CAGGS-FLEX-[JL2R-EGFP]-3'USS	Kazuto Kobayashi ²²	N/A
pAAV-FLEX-[ArchT-EGFP]	addgene	#28307
pAAV hSyn FLEX-FRT hM3Dq-mCherry	addgene	#161575
AAV2/1-SynTetOff-FLEX-[palmRFP1]	Hiroyuki Hioki ⁴⁷	N/A
pAAV-hSyn-Ach4h	Yulong Li ²⁷	N/A
Chemicals, peptides, and recombinant proteins		
NeuroTrace 435/455 Blue	Thermo Fisher Scientific	Cat# N21479
Cholera toxin subunit b	List Biological Laboratories	Cat# 103C
Deschloroclozapine	MedChemExpress	Cat# HY-42110
Experimental models: organisms/strains		
Mouse: C57BL/6JmsSlc	Nihon SLC	N/A
Mouse: STOCK Tg(Chat-cre)60Gsat/Mmcd	MMRRC	RRID: MMRRC_017258-UCD
Software and algorithms		
MATLAB	MathWorks	RRID:SCR_001622
Python	Python Software	RRID:SCR_008394
LabVIEW	National Instruments	RRID:SCR_014325
SigmaPlot	HULINKS	RRID:SCR_003210

METHOD DETAILS

Animals

All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Committees for Animal Care and Use and Recombinant DNA Study at Juntendo University (2024231, 2024232, 2024213, and 2024213). C57BL/6J mice (male and female; 6–10 weeks old) were obtained from Nihon SLC. ChAT-Cre(BAC) mice (male and female; 6–16 weeks old) were obtained from The Mutant Mouse Resource and Research Center (MMRRC) and maintained by continuous breeding. Animals were housed under a 12-h light/dark cycle with *ad libitum* access to food and water. All procedures were designed to minimize animal suffering and reduce the number of animals used. This study was conducted in compliance with the ARRIVE guidelines.

Pellet reaching task

Mice were food-restricted to maintain 85–90% of their free-feeding weight before the start of training. The behavioral chamber was an acrylic box (115 mm tall × 55 mm deep × 38 mm wide) with a transparent front wall and opaque walls on the remaining three sides. A vertical slit (5 mm wide × 57.5 mm high) was located at the center of the front wall. A food pellet was positioned on a cork plate, 5–7 mm from the slit.^{17,18} During training, the pellet was placed 5 mm to the left or right of the slit center, according to each mouse's initial paw preference. On the day prior to behavioral testing, each mouse was habituated to the chamber for 10 min.

The behavioral procedure consisted of three phases. In the before-training phase (2 days), the preferred paw of each mouse was determined using a pellet-reaching task. During the training phase (5 days), the pellet was repositioned laterally so that the mouse could reach it only with its non-preferred paw. In the after-training phase (2 days), the pellet was returned to the center position in front of the slit. Each daily behavioral session lasted 10 min. For the immunotoxin (IT) experiments, an additional 5-day interval was introduced between each phase to allow for cellular reduction.

Behavioral performance for one or two mice was recorded frontally using a monochrome camera (BU205M; Toshiba Teli) at 100 Hz. The number of reaching attempts with the right and left paws, as well as the success rate, were manually assessed. In the before-training phase, the paw with the greater number of reaching attempts was defined as the preferred paw. Mice that switched their preferred paw between sessions B1 and B2 were excluded from subsequent analyses (from Figure 1D onward).

The percentage of non-preferred paw use was calculated as:

$$\text{Non - preferred use (\%)} = \frac{N_{np}}{N_{np} + N_p} \times 100$$

The non-preferred score was calculated as:

$$\text{Non - preferred score} = \frac{N_{np} - N_p}{N_{np} + N_p}$$

where N_{np} and N_p represent the number of reaching attempts with the non-preferred and preferred paw, respectively. The success rate was calculated as the number of successful pellet retrievals divided by the total number of attempts. As shown Figure 1, mice were categorized as switched when their non-preferred score in session A2 was positive and as non-switched when it was negative.

Production and purification of AAV vectors

AAV vector particles were produced and purified as described previously.^{48–50} HEK293T cells were incubated in 10 CELLSTAR cell culture dishes (145 mm; #639160, Greiner Bio-One). Each genomic plasmid and two helper plasmids, pBSISK-R2C1⁴⁷ and pHelper (28060929; Stratagene) were co-transfected into HEK293T cells using polyethylenimine (23966; Polysciences). Virus particles were harvested from both the cell lysate and the supernatant, followed by ultracentrifugation with OptiPrep (Axis-Shield). The purified solution was ultrafiltrated and concentrated in Dulbecco's phosphate-buffered saline (PBS; 14249–95; Nacalai Tesque) containing 0.001% Pluronic F-68 (24040032; Thermo Fisher Scientific) using an Amicon Ultra-15 (NMWL 30K; Merck Millipore). Following the ultrafiltration, each virus solution was concentrated to a final volume of 100 μ L. The virus titer (gc/mL) was quantified using qPCR with Fast SYBR Green Master Mix (#4385612, Applied Biosystems). The final titers were 8.4×10^{11} gc/mL for AAV2/1-CAGGS-FLEX-[IL2R-EGFP]-3'USS,^{22,23} 1.4×10^{15} gc/mL for AAV2/1-CAG-FLEX-[ArchT-EGFP],⁵¹ 8.4×10^{13} gc/mL for AAV2/1-CAG-FLEX-[hM3Dq-mCherry],³⁰ 7.7×10^{14} gc/mL for AAV2/1-hSyn-gACh4h,²⁷ and 3.6×10^{14} gc/mL for AAV2/1-SynTetOff-FLEX-[palmRFP1].⁴⁷ The virus solutions were stored in aliquots at -80°C until use.

Stereotaxic surgical procedures

Mice were deeply anesthetized by intraperitoneal injection of a mixture of medetomidine (0.3 mg/kg; Nippon Zenyaku Kogyo), midazolam (4 mg/kg; Sandoz), and butorphanol (5 mg/kg; Meiji Seika Pharma) and placed in a stereotaxic apparatus. We injected 0.2 μ L of the solution into the NB (0.5 mm posterior to the bregma, ± 1.8 mm lateral to the midline, 4.5 mm ventral to the brain surface) and/or the M1 forelimb area (0.5 mm anterior to the bregma, ± 1.7 mm lateral to the midline, 0.8 mm ventral to the brain surface) by pressure through a glass micropipette attached to Picospritzer III (Parker Hannifin).

For retrograde tracing, cholera toxin subunit b (CTb; #103C, List Biological Laboratories) was used at a concentration of 2% (w/v).

For IT-mediated cell reduction,^{22,23,52} an IT solution (5 μ g/ μ L anti-Tac(Fav)-PE38 in PBS containing 0.1% bovine serum albumin) was injected into the NB of animals previously injected with AAV2/1-CAGGS-FLEX-[IL2R-EGFP]-3'USS, after a delay of more than one week.

For optogenetic manipulation, a 1-mm-diameter cranial window was opened over the M1 injection site. A green light-emitting diode (LED) device of TeleOpto system (TeleLDP-530nm-3.4mm; Bio Research Center) was implanted and secured to the skull with dental cement. A 2-g dummy receiver (TeleDummy, Bio Research Center) was attached to the LED device.

For fiber photometry, optic fiber cannulas (400 μ m diameter, 0.5 NA, R-FOC-BL400C-50NA; RWD) were bilaterally implanted into the M1 forelimb area following viral injection. Fiber ferrules were fixed to the skull with a quick-setting adhesive (LOCTITE 454; Henkel) and coated with a black surfacer (GS-03; Gianotes).

After surgery, mice received an intraperitoneal injection of atipamezole (Antisedan; 1.5 mg/kg; Orion) and recovered from anesthesia within approximately 15 min.

Optogenetics manipulation

The TeleOpt system (Bio Research Center) was used for optogenetic manipulations. Before the behavioral experiments, the wireless receiver (TeleR-2-C; Bio Research Center) was replaced with a dummy and connected to the LED device. Green light (approximately 2 mW) was continuously delivered bilaterally throughout the 10-min training sessions. After the behavioral experiment, the wireless receiver was again replaced with the dummy unit.

Fiber photometry

Fiber photometry recordings were performed using bifurcated fiber-optic bundles (400 μ m diameter, 0.39 NA; BFYL4LF01, Thorlabs) attached to the optic fiber ferrules and covered with black sleeves (Lymyth). Recordings were conducted using a Thorlabs fiber photometry system, which delivered excitation light (470 nm for the active signal and 405 nm for the isosbestic reference) through the patch cord as interleaved LED pulses at 40 Hz. The two optical paths alternated at 20 Hz using a galvanometric mirror (GVS001, Thorlabs), resulting in an acquisition rate of 10 Hz per channel. Light was reflected through a dichroic mirror (DMLP490R, Thorlabs) and detected using a photomultiplier tube (PMT1001/M, Thorlabs). Excitation power was adjusted at $\sim$ 50–90 μ W at the patch cord tip. Signals were acquired with a data acquisition interface (USB-6003, National Instruments) and custom-written LabVIEW software (National Instruments). Fiber photometry recordings began immediately after the start of behavioral video acquisition. Synchronization between the two systems was achieved by capturing a 40-Hz-blinking infrared LED within the video frame.

During the recordings, mice performed a pellet-reaching task for 10 min. Photometry data were collected across two sessions with the preferred paw, during which the pellet was placed at the center of the slit. On a separate day, recordings of the non-preferred paws were obtained by laterally shifting the pellet position.

The photometry signals were analyzed using custom MATLAB scripts (MathWorks). Raw fluorescence traces from the 470 nm and 405 nm channels were median-filtered; the 470-nm active channel was normalized by subtracting the 405-nm reference signal, low-pass filtered, and converted to z-scores. Processed signals were time-locked to successful paw-reaching events for event-related analysis.

Dread manipulation

Deschloroclozapine (HY-42110, MedChemExpress) was dissolved in saline containing 0.1% dimethyl sulfoxide (DMSO) to a final concentration of 2 μ g/mL and intraperitoneally administered at a 0.1 mL per mouse, 10–20 min before each behavioral session during the training phase.²⁹ Animals received unilateral injections of AAV2/1-CAG-FLEX-[hM3Dq-mCherry] into NB 1–2 weeks before the start of the behavioral testing.

Histology

Mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (200 mg/kg; Somnopentyl, Kyoritsu Seiyaku) and perfused transcardially with 20 mL of PBS (pH 7.4, 20°C–25°C), followed by perfusion for 3 min with 4% paraformaldehyde (1.04005.1000; Millipore) in 0.1 M phosphate buffer (PB; pH 7.4, 20°C–25°C). The brains were removed and post fixed overnight at 4°C in the same fixative. After cryoprotection in 30% sucrose in 0.1 M PB, the brains were cut into 40- μ m-thick parasagittal or coronal sections on a freezing microtome (REM-710; Yamato Kohki Industrial). Sections were collected in six bottles containing 0.02% sodium azide in PBS and stored at 4°C until use for free-floating immunostaining.

All following incubations were performed at 20°C–25°C and followed by rinsing twice with PBS containing 0.3% (v/v) Triton X-100 (PBS-X) for 10 min. Free-floating sections (40- μ m thick) were incubated for 16 h with 1:1000-diluted goat polyclonal antibody against ChAT (AB144P, Millipore), 1:1000-diluted mouse monoclonal antibody against NeuN (MAB377, Millipore), 1:500-diluted rabbit polyclonal antibody against ChAT (AB143, Millipore), 1:1000-diluted goat polyclonal antibody against CTb, 1:1000-diluted affinity-purified rabbit antibody against mRFP,⁴⁶ or 1:100-diluted chicken polyclonal antibody against GFP (GFP-1020, Aves Labs) in PBS-X containing 1% (v/v) donkey serum (S30-100ML; Merck) and 0.12% (w/v) λ -carrageenan (035-09693; Wako Chemicals) (PBS-XCD). The sections and slices were incubated for 4 h with 5 μ g/mL Alexa Fluor (AF) 647-conjugated donkey anti-goat IgG (ab150131; Abcam), AF405-conjugated donkey anti-mouse IgG (ab175658; Abcam), AF568-conjugated donkey anti-goat IgG (A11057; Thermo Fisher Scientific), AF647-conjugated donkey anti-rabbit IgG (A31573; Thermo Fisher Scientific), AF647-conjugated donkey anti-chicken IgG (A21469; Thermo Fisher Scientific), or AF568-conjugated donkey anti-rabbit IgG (A10042; Thermo Fisher Scientific) in PBS-XCD, followed by incubation for 30 min with 1:300-diluted NeuroTrace 435/455 Blue Fluorescent Nissl stain (N21479; Thermo Fisher Scientific) in PBS-X.

The sections were imaged using a confocal laser scanning microscope (TCS SP8; Leica Microsystems) equipped with 10 $\times$ (HCX PL APO 10x/0.40 CS, NA = 0.40; Leica Microsystems), 16 $\times$ multi-immersion (HC FLUOTAR 16x/0.60 IMM CORR VISIR, NA = 0.60; Leica Microsystems), and 25 $\times$ water-immersion (HC FLUOTAR L 25x/0.95 W VISIR, NA = 0.95; Leica Microsystems) objective lenses. NeuroTrace Blue, EGFP or AF488, AF568, mRFP1, or mCherry, and AF647 were excited using 405-, 488-, 552-, and

638-nm laser lines, respectively. Emission signals were collected through 410–503, 495–550, 570–650, and 660–750-nm emission prism windows, and detected in photon-counting mode using a hybrid detector (HyD; Leica Microsystems).

Bayesian logistic regression

Bayesian logistic regression in Figure 5 was performed following the framework of Beron et al.³¹ For each mouse, paw choice on trial t (C_t ; coded as 0 = non-preferred, 1 = preferred) was predicted from the preceding trial's paw choice and reach outcome. Reach outcomes (O_{t-1}) were classified into four categories: no contact (0), failed attempt (1), success with imprecise grasp (2), and success with precise grasp (3). Four models were compared: (1) a base model with only an intercept term (β_0) representing each mouse's overall paw preference; (2) a choice history model (Model persev) including the previous paw choice as a predictor; (3) a reinforcement learning model (Model RL) using a win-stay/lose-shift signal ($WSLS_{t-1}$); and (4) a combined model (Model persev+RL) incorporating both predictors. All models used a logistic link function:

$$\text{logit}(p) = \beta_0 + \beta_{\text{persev}} \times C_{t-1} + \beta_{\text{RL}} \times WSLS_{t-1}$$

where p is the probability of choosing the preferred paw on trial t , and $WSLS = (2 \times C_{t-1} - 1) \times (2 \times S_{t-1} - 1)$, and S_{t-1} indicates reach success (1 if $O_{t-1} \geq 2$, 0 otherwise). Terms were included or excluded depending on the model.

For each model and mouse, posterior distributions of the coefficients were estimated using Markov chain Monte Carlo sampling (slice sampling; MATLAB Statistics and Machine Learning Toolbox). Weakly informative priors were placed on all coefficients (Normal(0, 2)). Four independent chains were run, each with 2,000 burn-in samples followed by 5,000 sampling iterations with a thinning factor of 2, yielding 10,000 posterior samples per model. Convergence was assessed using the Gelman-Rubin statistic ($\hat{R} < 1.1$ for all parameters in all mice). Model comparison was performed using the Widely Applicable Information Criterion (WAIC), computed from pointwise log-likelihoods across posterior samples. To examine the temporal extent of choice history and reach outcome influences, the combined model was extended to include up to 5 preceding trials, with independent coefficients estimated for each lag. Session boundaries were respected such that trial pairs did not cross sessions. Generative AI tools (Claude, Anthropic) were used solely to assist with code drafting for this analysis. All outputs were reviewed, validated, and finalized by the authors.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are presented as mean $\pm$ standard error of the mean (SEM). The exact values of n are provided in the results section and the corresponding figure legends. No formal power analysis was performed to predetermine sample size. Sample sizes were based on variability observed in related experiments and on commonly used group sizes in the field, while also minimizing animal use. Statistical analyses were conducted using MATLAB (MathWorks) and SigmaPlot (HULINKS). Two-way analysis of variance (ANOVA) was used to compare daily success rate changes between groups, after confirming normality (Shapiro-Wilk test) and homogeneity of variance (Brown-Forsythe test). Hand use rates violated the normality assumption and were therefore analyzed using a generalized linear mixed model (GLMM) with a binomial distribution and logit link, including Group $\times$ Day as fixed effects and a by-subject random intercept. For time-course plots, statistical annotations indicate the group $\times$ day interaction. Per-day group comparisons were Holm-corrected. Friedman test was applied to compare CTb+ cells (Figure S1). Friedman test followed by Wilcoxon signed-rank test was applied for within-group changes across days (Figure 1G). Student's t -tests were used for independent pairwise group comparisons. Paired t test was used for pairwise group comparisons (Figure S3C). Linear regression was used to compare distributions across two days (Figures 1C and 1J). Cluster-based permutation tests were used to evaluate temporal differences in bilateral activity (Figures 3C and 3E). Cohen's d was calculated to estimate the effect sizes of differences in bilateral activity (Figures 3D and 3F). Wilcoxon signed-rank post-hoc tests were used for pairwise model comparisons and for comparing coefficient magnitudes within the combined model (Figure 5D). Wilcoxon rank-sum tests were used for group comparisons of model coefficients (Figures 5F and 5G). All tests were two-sided, and statistical significance was set at $p < 0.05$.