Original Contribution

Effects of High- Versus Moderate-Intensity Training on Neuroplasticity and Functional Recovery After Focal Ischemia

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Background and Purpose—This study was designed to compare the effects of high-intensity interval training (HIT) and moderate-intensity aerobic training (MOD) on functional recovery and cerebral plasticity during the first 2 weeks after cerebral ischemia.

Methods—Rats were randomized as follows: control (n=15), SHAM (n=9), middle cerebral artery occlusion (n=13), middle cerebral artery occlusion at day 1 (n=7), MOD (n=13), and HIT (n=13). Incremental tests were performed at day 1 (D1) and 14 (D14) to identify the running speed associated with the lactate threshold (S_{LT}) and the maximal speed (S_{max}). Functional tests were performed at D1, D7, and D14. Microglia form, cytokines, $p75^{NTR}$ (pan-neurotrophin receptor p75), potassium–chloride cotransporter type 2, and sodium–potassium–chloride cotransporter type 1 expression were made at D15.

Results—HIT was more effective to improve the endurance performance than MOD and induced a fast recovery of the impaired forelimb grip force. The ionized calcium binding adaptor molecule 1 (Iba-1)–positive cells with amoeboid form and the pro- and anti-inflammatory cytokine expression were lower in HIT group, mainly in the ipsilesional hemisphere. A $p75^{NTR}$ overexpression is observed on the ipsilesional side together with a restored sodium–potassium–chloride cotransporter type 1/potassium–chloride cotransporter type 2 ratio on the contralesional side.

Conclusions—Low-volume HIT based on lactate threshold seems to be more effective after cerebral ischemia than work-matched MOD to improve aerobic fitness and grip strength and might promote cerebral plasticity.

Visual Overview—An online visual overview is available for this article. **(*Stroke*. 2017;48:00-00. DOI: 10.1161/STROKEAHA.117.017962.)**

Key Words: grip force ■ interval training ■ KCC2 ■ lactate threshold ■ microglia ■ $p75^{NTR}$

Ischemic stroke remains the leading cause of long-term physical disorders. Poststroke hemiparesis frequently leads to physical deconditioning that strongly reduces the quality of life and represents an important burden on the family and society. Growing evidence from animal and human experiments indicated that aerobic training induced beneficial effects at the cardiovascular, muscular, cerebral, and functional levels after cerebral ischemia.[1,2] Moderate-intensity aerobic training (MOD; for recommendations see Marsden et al[1]) is advised after stroke to improve the locomotor abilities, the peak oxygen uptake (VO_{2peak}), and the maximal running speed (S_{max}), which are strong indicators of quality of life. Early treadmill training in rodents could also promote functional recovery and cerebral plasticity by upregulating the neurotrophin levels, enhancing synaptogenesis, and limiting microglia-mediated proinflammatory cytokine release in the perilesional zones.[3,4]

However, beneficial effects of MOD on functional recovery, aerobic fitness, and quality of life remain frequently insufficient and controversial.[1,5] It is, thus, crucial to reconsider the current guidelines for exercise by defining a safe/effective dosage of training.[5] In this regard, authors recently showed that higher training intensities appeared promising for stroke patients.[6] Indeed, high-intensity interval training (HIT), known to be feasible and safe in moderate stroke patients,[7] could improve VO_{2peak}, running economy, and functional recovery, but it remains controversial.[6,7] No clear evidence indicated whether the HIT effectiveness is more efficient on aerobic fitness and neuroplasticity than MOD.[6,8] Given that HIT is a time-efficient strategy, we postulated that it might accentuate functional recovery in the acute phase of cerebral ischemia compared with MOD.

In light of these considerations, the present study was designed to compare the effects of work-matched HIT and MOD programs on functional outcomes and cerebral plasticity during the first 2 weeks after cerebral ischemia in rats. One of the key points of the endurance protocols relies on determining for each animal the training intensity from an underestimated

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submaximal physiological parameter, that is, the running speed associated with the lactate threshold (S_{LT}), which is relevant to distinguish high from moderate running speeds[9,10] and is highly sensitive to assess aerobic fitness.[10,11] In addition, the training effects on brain inflammation through microglia activation form was measured, as well as the related expression of pro- (IL [interleukin]-1β and IL-12p40) and anti-inflammatory (IL-10) cytokines, to determine the microglia function, which could be related to neurotrophin actions and synaptic plasticity.[9] Therefore, the $p75^{NTR}$ expression (pan-neurotrophin receptor p75), known to strongly influence the neurotrophin functions after cerebral ischemia,[12] was also assessed. The training-induced synaptic plasticity was observed through the expression of the potassium–chloride cotransporter (KCC2, a neuronal chloride extruder) and sodium–potassium–chloride cotransporter type 1 (NKCC1, an ubiquitously chloride importer) that are disturbed after cerebral ischemia and leads to alteration in the excitation/inhibition balance in brain.[13]

Material and Methods

Animals

Overall, 108 adult male Sprague–Dawley rats (250–270 g; JANVIER, France) were used, but 70 of them were included (see the online-only Data Supplement). Anesthesia and surgical procedures were performed according to the French law on animal care guidelines. Animal Care Committees of *Aix-Marseille Université* approved our protocol.

Each animal was randomly assigned to a group, making the impact of individuals less prominent: (1) control (n=15), no surgery was performed that enabled to verify the reliability of measurements on 14 days; (2) SHAM (n=9), animals underwent surgery without cerebral ischemia to ensure that it did not affect measurements; (3) middle cerebral artery occlusion (MCAO) (n=13), animals underwent middle cerebral artery occlusion–reperfusion (MCAO-r) that enable to assess the spontaneous functional recovery and to verify the variance of rat activity level (no training); (4) MCAO-D1 (n=7) in which animals were euthanized 1 day (D1) after MCAO-r to confirm that animals started training with a similar lesion severity; (5) HIT (n=13); and (6) MOD (n=13) in which animals underwent MCAO-r and performed HIT and MOD programs, respectively (see the online-only Data Supplement).

MCAO-r and Behavioral Tests

Rats were subjected to right MCAO-r for 2 hours (see the online-only Data Supplement). The elevated body swing test, the ladder-climbing test, and the forelimb grip force were performed before (PRE) and after the surgery at day 1, 7, and 14 (D1, D7, and D14, respectively) after MCAO-r (Figure I in the online-only Data Supplement).

Incremental Test

Incremental tests were performed on 1° inclined treadmill at D1 and D14. These tests started with 5 minutes of warm-up at 9 m/min to reduce stress. Then, running speed was increased by 3 m/min every 3 min until animals could not maintain the imposed speed. S_{max} was associated with the last reached stage. Each stage was separated by 20-s interval to perform blood sampling (0.2 μL) after partially cutting the distal area of the tail vein to determine S_{LT} (see the online-only Data Supplement).

Work-Matched HIT and MOD Programs on Treadmill

HIT and MOD programs included 10 sessions from D2 to D12 and 2 recovery days (D7 and D13) to reduce fatigue accumulation that may affect the incremental test performance (see the online-only Data Supplement).

Immunohistochemistry Analysis

Each animal was randomly assigned to either immunostaining analysis or Western blot at D15. Cresyl violet was used to measure the infarct volume and the percentage of tissue loss (% tissue loss). To investigate the changes of $p75^{NTR}$ and microglia form, immunostaining with antibodies against the $p75^{NTR}$ and ionized calcium binding adaptor molecule 1 (Iba-1) were made at D15 (see the online-only Data Supplement).

Western Blot Analysis

To detect IL-10, IL-1β, IL-12p40, $p75^{NTR}$, KCC2, and NKCC1 expression, the total protein extracted from each frozen hemisphere was used for Western blot (see the online-only Data Supplement).

Statistical Analysis

Statistical analysis was performed using SigmaStat software program (San Jose, CA). All data are presented as mean±SD (see the online-only Data Supplement).

Results

For the overall parameters, no difference was observed between control and SHAM groups from PRE to D14. Likewise, no significant difference was observed at D1 for functional outcomes, infarct volume, and endurance performance between MCAO, MCAO-D1, HIT, and MOD groups, indicating a similar lesion severity prior to training for each animal.

Endurance Programs

Running speed during MOD was lower than HIT during the first and second training weeks (−28.9% and −31.2%, respectively). Session duration of MOD group was higher than HIT group during the first and second weeks (+49.2%; +59.8%, respectively; Table I in the online-only Data Supplement).

Functional Tests

HIT induced a complete grip strength recovery without affecting other functional parameters. Indeed, grip force exerted by the affected forelimb decreased significantly between PRE and D1 for all injured groups ($P<0.001$) and was significantly lower in MCAO, HIT, and MOD groups than in control and SHAM groups ($P<0.001$). Grip force remained significantly decreased ($P<0.01$) at D7 and D14 for both MCAO and MOD groups, while it recovered in HIT group at D7 and D14 ($P<0.001$). Moreover, no difference was observed between control, SHAM, and HIT groups from D7 to D14 contrary to MCAO ($P<0.05$) and MOD ($P<0.001$) groups (Figure 1A). No difference was observed for both forelimbs force and for the nonaffected forelimb force between groups from PRE to D14 (data not shown). The A/N ratio significantly decreased at D1 compared with PRE for MCAO, HIT, and MOD groups ($P<0.001$) and was lower within all injured groups than control and SHAM groups ($P<0.001$). A/N ratio completely recovered only for HIT group from D7 to D14 compared with PRE ($P<0.001$). Likewise, A/N ratio of HIT group was significantly higher than MCAO and MOD groups from D7 to D14 ($P<0.01$) and remained similar to control and SHAM groups, contrary to MCAO and MOD groups ($P<0.01$; Figure 1B).

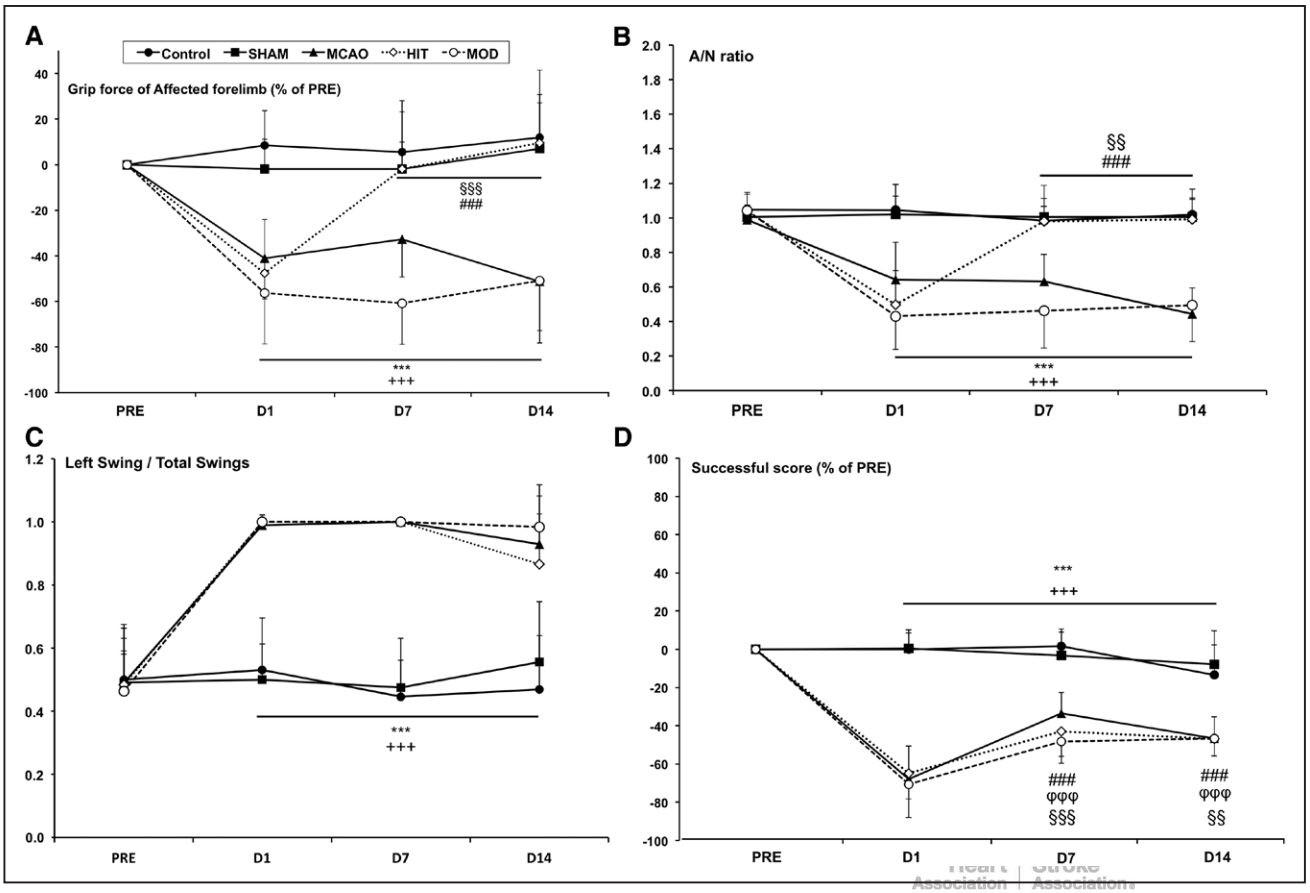

Figure 1. Forelimb grip force. **A**, Affected forelimb. **B**, A/N ratio. *Significant decrease from D1 to D14 in injured groups compared with PRE (except for high-intensity interval training [HIT] at D7 and D14). #Significant increase from D7 to D14 for HIT group compared with PRE. +Significant lower force in injured groups compared with noninjured groups from D1 to D14 (except for HIT at D7 and D14). §Significant higher force for HIT group compared with moderate-intensity aerobic training (MOD) and middle cerebral artery occlusion (MCAO) groups at D7 and D14. **C**, The elevated body swing test. *Left swings/total swing increase at D1, D7, and D14 compared with PRE for injured groups. +Higher left swings/total swing for MCAO, HIT, and MOD groups compared with control and SHAM groups from D1 to D14. **D**, Successful score (% of PRE). *Significant decrease at D1, D7, and D14 compared with PRE for injured groups. The successful score increased from D1 to D14 for MCAO (#), HIT (§), and MOD (φ). +Lower successful score for injured groups compared with control and SHAM groups.

The left swings/total swings ratio (elevated body swing test) significantly increased for MCAO, HIT, and MOD groups at D1, D7, and D14 compared with PRE (P<0.001; Figure 1C).

The successful score (ladder-climbing test) significantly decreased for MCAO, HIT, and MOD groups at D1, D7, and D14 compared with PRE (P<0.001), without difference between groups. Nevertheless, this score was significantly higher for MCAO, HIT, and MOD groups at D1 compared with D7 (P<0.001) and with D14 (P<0.001; P<0.01; and P<0.001, respectively; Figure 1D).

Incremental Test

HIT appeared to be more effective to recover aerobic fitness than MOD as indicated by changes in S_{max} and S_{LT}. The resting blood lactate concentration of MCAO group at D14 (4.4±1.4 mmol/L) was higher (P<0.001) compared with control (2.2±0.6 mmol/L), SHAM (2.0±0.6 mmol/L), MOD (3.1±1.3 mmol/L), and HIT (2.7±1.1 mmol/L) groups (Figure 2A).

S_{LT} of MCAO, HIT, and MOD groups at D1 were significantly lower than the one in control (P<0.001) and SHAM (P<0.001) groups (Figure 2B). S_{LT} significantly increased from D1 to D14 for HIT (20.4±2.4 m/min for D1 and 34.5±3.8 m/min for D14; P<0.001; Figure 2C) and MOD (21.8±3.3 m/min for D1 and 26.7±5.3 m/min for D14; P<0.01) groups contrary to MCAO (22.5±3.9 m/min for D1 and 22.5±4.5 m/min for D14), control (32.8±3.9 m/min for D1 and 30.8±4.1 m/min for D14), and SHAM (34.7±4.3 m/min for D1 and 33.7±3.6 m/min for D14) groups (Figure 2D). However, the S_{LT} of HIT group at D14 was higher than that of MOD and MCAO groups (P<0.001). The S_{LT} of MOD group was higher than the S_{LT} of MCAO group at D14 (P<0.01) but remained significantly lower than control and SHAM groups (P<0.01), contrary to HIT.

S_{max} at D1 was significantly lower in MCAO, HIT, and MOD groups than control and SHAM groups (P<0.001; Figure 2B). However, S_{max} significantly increased from D1 to D14 for HIT (26.1±3.5 m/min for D1 and 40.8±5.9 m/min for D14; P<0.001) and MOD (27.0±3.3 m/min for D1 and 35.7±4.5 m/min for D14; P<0.001) groups contrary to MCAO (27.5±4.9 m/min for D1 and 30.0±5.4 m/min for D14), control (40.0±4.5 m/min for D1 and 39.8±4.3 m/min for D14), and SHAM (43.2±4.5 m/min for D1 and 41.3±2.9 m/min for D14) groups (Figure 2D). Moreover, the S_{max} at D14 of HIT group was significantly higher than that of MOD (P<0.05)

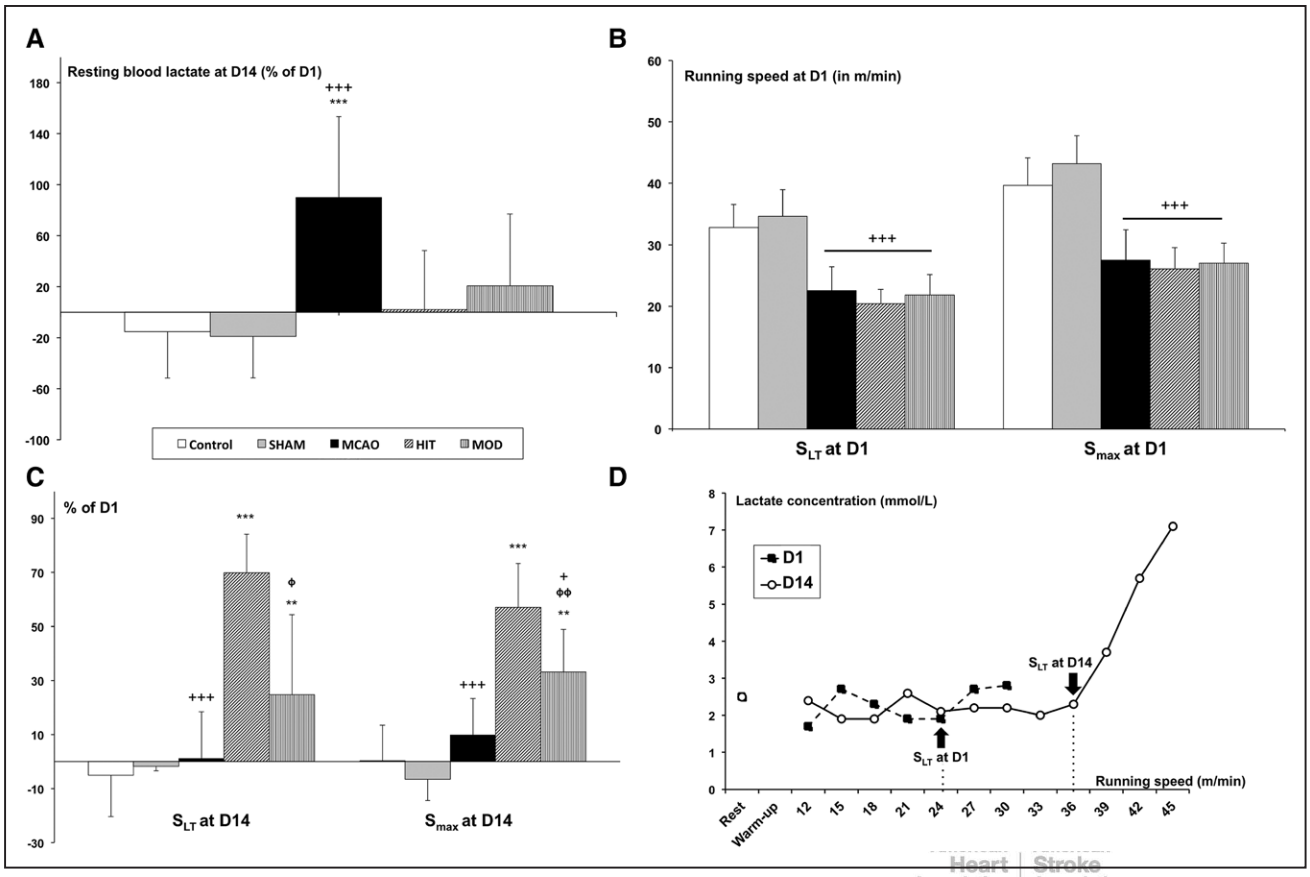

Figure 2. A, Resting blood lactate. *Resting lactatemia (mmol/L) increase between D1 and D14 for middle cerebral artery occlusion (MCAO) group (P<0.001). ⁺Higher resting lactatemia for MCAO group compared with control, SHAM, high-intensity interval training (HIT), and moderate-intensity aerobic training (MOD) groups at D14 (P<0.001). **B**, S_{LT} and S_{max} (m/min) at D1 after cerebral ischemia. ⁺Lower S_{LT} and S_{max} for MCAO, HIT, and MOD groups compared with control and SHAM (P<0.001). **C**, S_{LT} and S_{max} (m/min) after training. *S_{LT} and S_{max} increase from D1 to D14 for HIT and MOD groups (P<0.001 and P<0.01, respectively). ⁺Higher S_{LT} and S_{max} at D14 for HIT, control, and SHAM groups compared with MCAO and MOD groups (P<0.001 for S_{LT}; P<0.05 for S_{max} compared with MOD group). ᶲHigher S_{LT} and S_{max} at D14 for MOD group compared with MCAO group (P<0.05 for S_{LT} and P<0.05 for S_{max}). **D**, Example of lactatemia kinetic (raw data) during incremental test before and after HIT. At the D14 incremental test, S_{LT} was observed at a higher running speed (36 m/min) compared with D1 (24 m/min). Arrows indicate the lactate threshold. S_{LT} indicates speed associated with the lactate threshold; and S_{max}, maximal speed.

and MCAO (P<0.001) groups. The S_{max} of MOD group was higher than the S_{max} of MCAO group at D14 (P<0.01) but remained significantly lower than control and SHAM groups (P<0.05).

Immunohistochemistry

HIT promoted ramified microglia, p75 increase, restoration of NKCC1/KCC2 ratio, and downregulated pro- and anti-inflammatory cytokine expression, without affecting infarct volume and the percentage of tissue loss. Indeed, the number of amoeboid Iba-1⁺ cells for HIT group (20.6±5.2%) was significantly lower than that for MOD (74.9±29%; P<0.01) and MCAO (77.1±31.5%; P<0.001) groups within the perilesional site, as well as in the contralesional hemisphere (HIT, 11.3±3.1%; MOD, 54.3±22.2%; P<0.01; and MCAO, 46.9±29.9%; P<0.01; Figure 3).

For qualitative staining, the cells of damaged hemispheres expressed p75^{NTR} proteins in all lesioned groups contrary to SHAM group (Figure 4C).

No difference was observed between lesioned groups for infarct size and percentage of tissue loss (MCAO, −3.3±7.6%; HIT, −5.4±6.1%; MOD, −2.7±7.8% of the contralesional hemisphere).

Western Blotting

In the HIT group, IL-10 expression was significantly downregulated in the ipsilesional hemisphere when normalized to the IL-10 expression of MCAO group (0.75±0.09; P<0.01) contrary to MOD (1.03±0.23). IL-12p40 expression was significantly downregulated in the ipsilesional hemisphere after HIT (0.81±0.03; P<0.01). No difference was observed for IL-12p40 expression in MOD group (0.93±0.15). Likewise, no difference was observed for IL-1β between groups (0.85±0.23 for HIT and 0.93±0.12 for MOD).

The relative expression of p75^{NTR} protein within the ipsilesional hemisphere in HIT (4.4±2.7; P<0.01) and MOD (3.1±1.9; P<0.05) groups was significantly higher than that in SHAM, contrary to MCAO (2.8±2.6; Figure 4A). In the contralesional hemisphere, the p75^{NTR} expression was not different between groups (Figure 4B).

In the ipsilesional hemisphere, no difference was observed for NKCC1/KCC2 ratio between MCAO (2.20±1.46), MOD

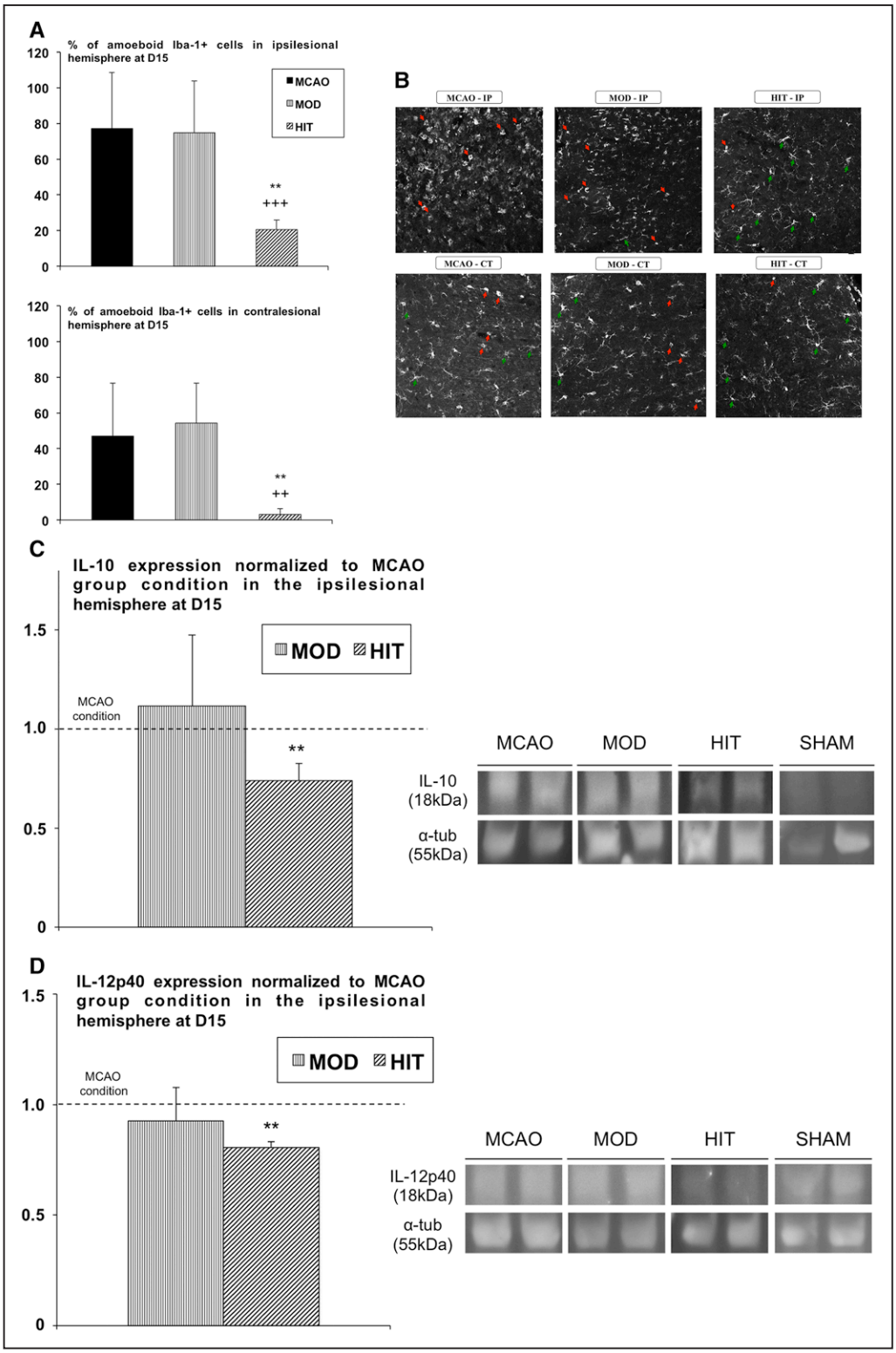

Figure 3. The impact of exercise training on microglial cells morphology and cytokine expression. **A**, Quantification of microglia morphology changes at peri-ischemic level in ipsilesional (**top**) and in contralesional (**bottom**) hemisphere at D15. In both hemispheres, the percent of amoeboid Iba-1^{+} cells is significantly lower in high-intensity interval training (HIT) group than in middle cerebral artery occlusion (MCAO) ($^{+}$, P<0.001 and P<0.01) and moderate-intensity aerobic training (MOD; *, P<0.01 and P<0.01) groups. **B**, Example of immunofluorescent staining with Iba-1 protein on MCAO, MOD, and HIT groups in ipsilesional and contralesional hemispheres. Green arrows indicate ramified reactive microglial cells, whereas red arrows indicate amoeboid microglial cells. **C**, IL-10 expression of HIT and MOD groups normalized to MCAO group condition in the ipsilesional hemisphere at D15 (**left**). *IL-10 expression decrease of HIT group (P<0.01). Representative immunoblot of IL-10 protein (and α-tubulin) in the ipsilesional hemisphere of injured and SHAM groups (**right**). **D**, IL-12p40 expression of HIT and MOD groups normalized to MCAO group condition in the ipsilesional hemisphere at D15 (**left**). *IL-12p40 expression decrease of HIT group (P<0.01). Representative immunoblot of IL-12p40 protein (and α-tubulin) in the ipsilesional hemisphere of injured and SHAM groups (**right**). CT indicates contralesional; Iba-1, ionized calcium binding adaptor molecule 1; and IP, ipsilesional.

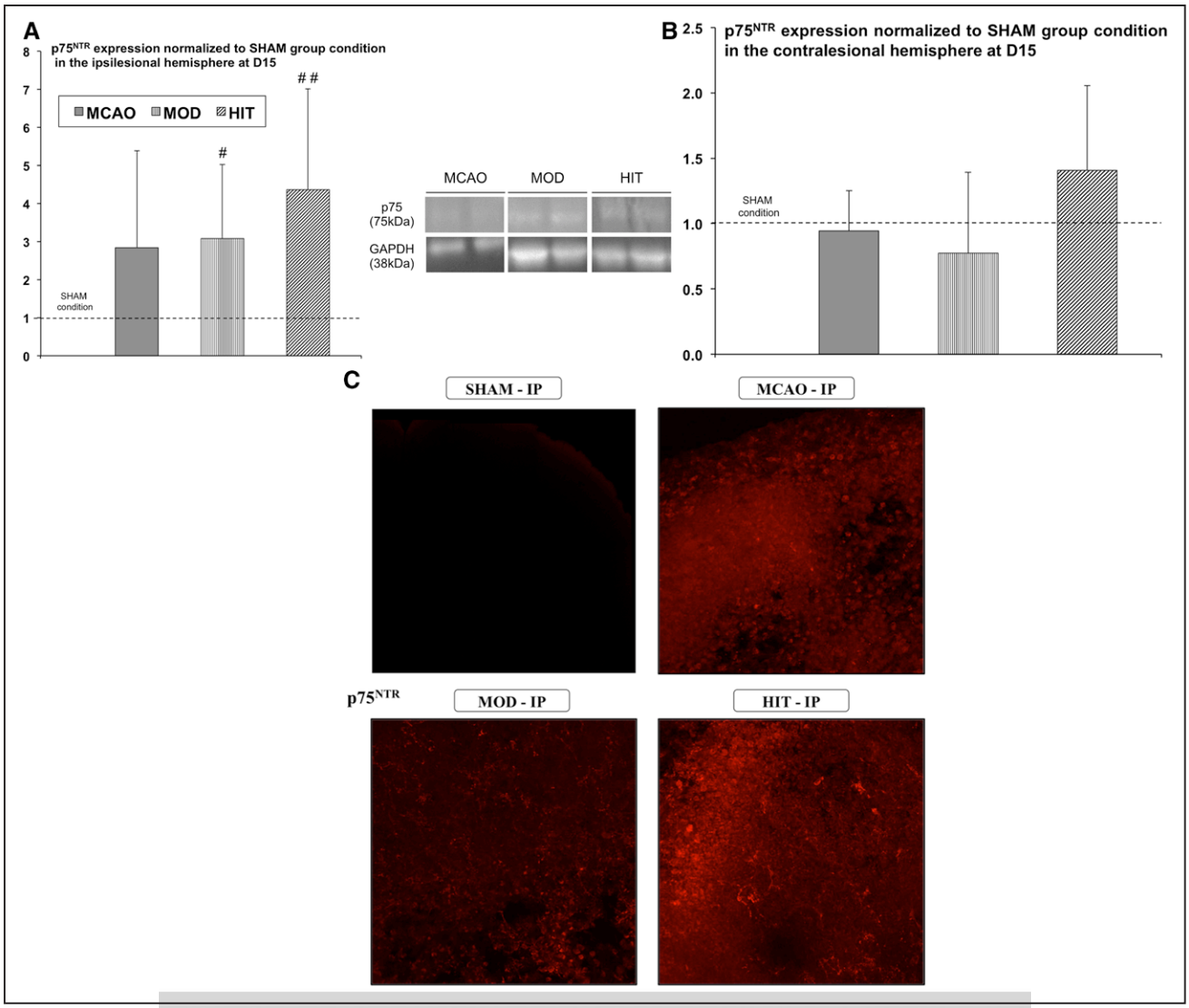

Figure 4. The impact of exercise training on p75^{NTR} expression. Expression of p75^{NTR} normalized to SHAM condition. **A**, # indicates a significant increase of p75^{NTR} expression in high-intensity interval training (HIT; $P<0.001$) and moderate-intensity continuous aerobic (MOD; $P<0.01$) groups compared with SHAM group in the ipsilesional hemisphere at D15. Representative immunoblot of p75^{NTR} protein (and GAPDH) in the ipsilesional hemisphere of injured groups. **B**, No difference in the contralesional hemisphere. **C**, Illustration of p75^{NTR} immunostaining in the ipsilesional hemisphere.

(4.23±5.16), HIT (1.75±0.89), and SHAM (1.05±0.68; data not shown). The NKCC1/KCC2 ratio of HIT group (0.62±0.16) was significantly lower than MCAO (1.16±1.36; $P<0.05$) and MOD (2.42±1.38; $P<0.05$) groups in the contralesional hemisphere (Figure 5).

At D1 and D15, grip force of the left forepaw, infarct volume, and immunohistochemistry results were not correlated within groups (data not shown).

Discussion

For the first time, this study demonstrated that 2 weeks of HIT was more effective than a work-matched MOD program on multiscale measurements after cerebral ischemia. The use of lactate threshold enabled defining high (>25 m/min) and moderate (<20 m/min) running speed in an individualized manner for each animal with cerebral ischemia. Such intensity ranges were not in accordance to previous studies in which exercise intensity between 10 and 13 m/min was considered as intense for MCAO rats.[10] The difference might be explained by their use of empirical training intensities or maximal parameters (S_{max} or VO_{2peak}) that were not highly relevant to distinguish high from moderate intensities.[9–11] Indeed, the ability to prescribe the optimal training stimulus might be greater whether intensity was based on submaximal physiological parameter that could be reached by the majority of patients, contrary to maximal parameters.[11,14] In our study, rats could begin an early individualized intense program that seemed not to be deleterious for functional recovery and infarct volume.[15] It was in accordance to previous studies showing that treadmill training starting during the first 5 days induced beneficial effects on recovery (contrary to training that began within 24 hours postischemia in both rodent[2] and human[16]). It was also found in human that early constraint-induced movement resulted in less motor improvement at 90 days.[17] However, our results

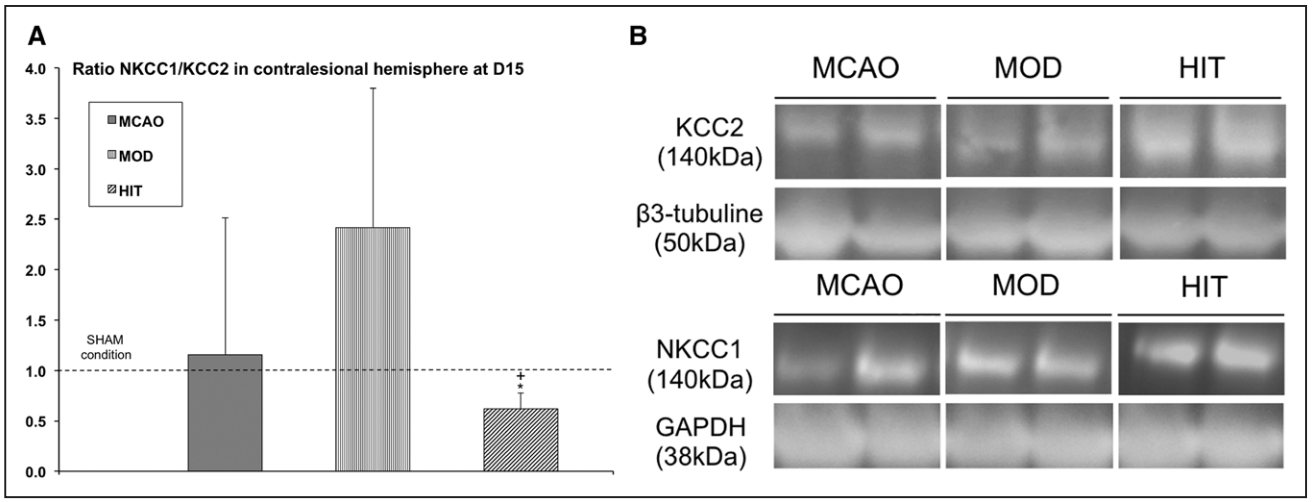

Figure 5. The impact of exercise training on NKCC1/KCC2 ratio in the contralesional hemisphere. **A**, The NKCC1/KCC2 ratio is significantly lower in high-intensity interval training (HIT) group than the one of moderate-intensity continuous aerobic (MOD; *) and MCAO (+) groups. **B**, Representative immunoblot of KCC2 and NKCC1 proteins (and GAPDH or β-tubulin). KCC2 indicates potassium–chloride cotransporter type 2, and NKCC1, sodium–potassium–chloride cotransporter type 1.

did not indicate that HIT need to be performed in moderate stroke patients during the first 2 weeks because the initiation of aerobic program should later be feasible and safe (during the subacute phase, ie, the first 3 months).[18,19] Moreover, HIT induced rapid physiological adaptations, although its session duration was shorter than work-matched MOD confirming that HIT is time efficient.[20] It was important given that lack of time remains a major barrier for patients to exercise participation. Interestingly, HIT could also elicit higher enjoyment than MOD, despite higher ratings of perceived exertion during intense series. After reporting an initial apprehension, the patient confidence progressively increased during HIT.[6] It suggested that higher intensities might not be considered as a major barrier for patients.

Our study revealed a maintained decrease of S_{LT} and S_{max} during the first 14 days after MCAO-r for nontrained animals. It was, thus, strongly argued that spontaneous aerobic fitness recovery was insufficient. It was in accordance with another study in which a decrease of S_{LT} was observed 2 days postischemia.[21] The decreased S_{LT} at D1 might be associated with sensorimotor alterations, such as interlimb coordination or strength deficits, given that neuromuscular disorders might disturb metabolic activity. Given that the S_{LT} was influenced by muscular typology composition and atrophy (observed from D7 after cerebral ischemia[10]), muscle typology changes might partially explain the decrease of S_{LT} at D14 but not at D1.

HIT was more effective than MOD to improve aerobic fitness as indicated by a superior shift of S_{LT} and S_{max} to higher intensities during incremental test. Other studies indicated that HIT induced higher ventilatory threshold improvements than MOD in cardiovascular patients.[22] It, thus, suggested that rats were able to exercise for longer durations at greater percentages of their S_{max}, reducing fatigue at a given intensity after HIT. Both programs are known to improve the maximal oxidative capacity in humans and animals by increasing VO_{2peak}, contributing to explain S_{max} improvement.[15] However, HIT further improved maximal running performance in our study because it was recently observed in rats with chronic heart failure.[23]

Our study also revealed a resting hyperlactatemia 14 days after the cerebral ischemia in nontrained rats. High lactate levels during the acute phase of stroke (<3 months) was also observed on 25% of stroke patients and seemed to have deleterious repercussions on functional recovery.[24] It might be suggested with caution that increase of cortisol and catecholamine blood levels induced by cerebral ischemia are known to be involved in blood lactate concentration accumulation, but also that anaerobic glycolysis promoted by cerebral hypoperfusion in affected neurons might facilitate an increase in blood lactate.[25] Given that HIT and MOD are known to increase the lactate transporter expression, the lactate clearance might be improved by training, preventing hyperlactatemia.

The decrease of affected forelimb grip force and its consequent strength asymmetry persisted until D14. However, HIT induced a rapid recovery of the affected forelimb grip force from D7 without acting on the other behavioral tests. The force improvement might be partially explained by a facilitation of fast motor units recruitment during HIT sessions because running speed was above the lactate threshold (contrary to MOD). The force improvement of forelimb flexor muscles might be possible because they were activated during locomotion (and not only extensor muscles) that might promote muscular changes. Given that only grip force was improved, HIT might be combined with skilled reaching training, known to improve limb function after stroke, to maximize recovery.

However, no functional improvements were observed after MOD, similarly to previous reports.[26] In humans, beneficial effects of MOD on motor functions and quality of life were not consistently observed.[18] In rodents, treadmill training did not have a significant effect on limb function.[2] It was also demonstrated that running at a low intensity (10.5 m/min) was not sufficient to observe a recovery.[27] We, thus, postulated that the intensity for MOD (−20% of S_{LT}) was too low to observe benefits.

The amoeboid Iba-1$^+$ cells, known to secrete proinflammatory agents and free radicals, was higher in MCAO and MOD animals within both hemispheres (which was not always observed after MCAO-r[28]) than in HIT animals. It reflected an inflammatory state, even after MOD. We postulated that MOD exercise intensity might not be sufficient to promote significant changes in microglia morphology, indicating that running intensity under the lactate threshold was unlikely the most effective strategy to reduce inflammation. Conversely, the Iba-1$^+$ cells after HIT mainly showed ramified form within both hemispheres. This was in accordance with previous studies showing that physical training might induce microglia morphological changes.[29] However, the ramified form could exert either detrimental activity (M1 phenotype), characterized by proinflammatory cytokine release, or beneficial activity (M2 phenotype) by secreting anti-inflammatory cytokines and neurotrophins. In the present study, HIT, but not MOD, might decrease neuroinflammation in the ipsilesional hemisphere by downregulating both pro- and anti-inflammatory cytokine expression. It was in accordance with findings revealing that aerobic training might be beneficial for neuroprotection by reducing the proinflammatory cytokine expression in healthy animals[30] and in mice with neurodegenerative diseases.[29] However, our results disagreed with a study on healthy mice in which vigorous training on treadmill enhanced anti-inflammatory cytokine IL-10 release.[30] The different training protocols and rodent models (healthy versus cerebral ischemia) might explain controversial findings. Alternatively, it might also be possible that microglia progressively returned to resting state after HIT, resulting in a downregulation of IL-10 level at D15. Therefore, the time course of cytokines during aerobic training remains to be investigated. Nevertheless, the absence of an IL-10 upregulation after MOD was consistent with the observed amoeboid Iba-1$^+$ cells, which was in accordance with a previous study.[30] Finally, results should be interpreted with caution because of the sample size for immunohistochemistry analysis of injured groups. However, we observed similar outcomes in each group without excessive variability that might be caused by the individualization of training programs and by a strict control of behavioral outcomes after MCAO-r. Several studies, focused on brain structures with larger sample size, are required to clarify the effects of exercise intensity on cytokine expression and microglia function after cerebral ischemia. It might be possible that a decrease of a proinflammatory state promoted an adaptive neuroplasticity even if the influence of neuroinflammation on neuroplasticity remains to be explored.[31]

To determine whether HIT or MOD might influence brain plasticity, we quantified the p75^{NTR} level, which strongly contribute to mediate the neurotrophin cellular functions during embryonic development and after central nervous system lesion.[12] Our study was the first to measure the effectiveness of different training intensities on p75^{NTR} expression within both hemispheres after severe cerebral ischemia. We found that HIT induced an increase of p75^{NTR} at ipsilesional level. However, p75^{NTR} expression could be associated with beneficial or detrimental functions complicating result interpretation. It was, thus, difficult to establish whether the increase of p75^{NTR} expression was associated with cellular death processes or with beneficial neuroplasticity. Nevertheless, several results allowed us to suggest that the p75^{NTR} expression might be beneficial. First, aerobic training stimulated endogenous BDNF (brain-derived neurotrophin factors)/tropomyosin receptor kinase B expression within both hemispheres that is known to influence p75^{NTR} expression.[4,32] Moreover, BDNF suppression, by injecting the ectodomain of tropomyosin receptor kinase B (TrkB) inhibited the increase of p75^{NTR} expression after axotomia, reducing the neuronal survival.[33] Only one study found on aged rats that after 8 weeks of endurance training, the p75^{NTR} level increased in parallel with an enhancement of BDNF expression.[34] Authors postulated that p75^{NTR} increase might promote survival of damaged neurons, trigger apoptosis for cleaning debris, and induce beneficial environment for axonal regrowth and inflammatory prevention.[12] In addition, it was demonstrated in healthy rats that high-intensity training could induce higher cerebral concentrations of BDNF and glial cell-derived neurotrophic factor compared with MOD, which was related to neuroprotection.[4,35] To reinforce our hypothesis on the p75^{NTR} role, it was demonstrated that aerobic training could facilitate the conversion of the proBDNF to the mature BDNF in the peri-ischemic regions, which was associated with functional improvements.[4,29,36] Indeed, the decrease of proBDNF expression was associated with lower cell death and synaptic depression. On the other hand, the increase of mature BDNF expression could promote synaptic plasticity and rescue neuronal loss.[37] In light of these findings, we suggested that the increase of p75^{NTR} expression after HIT might reflect a beneficial role of neurotrophin expression. Moreover, circulating BDNF levels was not measured because it does not mirror brain BDNF levels after stroke. It, thus, complicates interpretation and might be less relevant than brain BDNF.

Finally, to link neurotrophin action to brain plasticity, we studied the Cl^- homeostasis through the KCC2 and NKCC1 expression, proteins known to be sensitive to neurotrophin levels. Indeed, BDNF could promote KCC2 expression after central nervous system trauma. To our knowledge, no study determined the role of different aerobic trainings on the Cl^- cotransporters after cerebral ischemia, despite their crucial role in the central nervous system function.[38] Only 1 study indicated that these chloride cotransporters were sensitive to aerobic training because such physical activity could affect the spinal KCC2 and NKCC1 expression after spinal cord injury, in parallel with functional recovery improvement.[39] We, thus, postulated that HIT could influence the Cl^- homeostasis by changing the KCC2 and NKCC1 expression after cerebral ischemia that might optimize the equilibrium between excitation/inhibition in brain cells. It also appeared interesting to postulate that the contralesional hemisphere was sensitive to brain plasticity, as indicated by the decrease of NKCC1/KCC2 ratio after HIT, together with changes in reactive gliosis and inflammation.[40]

Conclusions

This study provided new promising insights into the effectiveness of low-volume HIT on the physiological

determinants of aerobic fitness and grip strength. It also seemed that HIT might promote neurotrophin action, synaptic plasticity compared with work-matched MOD. According to human studies, results needed to be interpreted with caution because risk factors and comorbidities were not taken into account in the present study that might change the effects of these endurance programs. This study needed to be considered as an initial assessment of the effects of these training protocols. Nonetheless, HIT is known to be feasible in moderate stroke patients, but its effectiveness compared with MOD needs to be assessed. In animals, it was recommended to deepen the neuroplasticity mechanisms induced by HIT without forgetting its outcomes on functional recovery. It seems now critical to bring evidence on the effects of detraining for the different aerobic programs that remains poorly investigated but important for improving the long-term recovery.

Disclosures

None.

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Effects of High- Versus Moderate-Intensity Training on Neuroplasticity and Functional Recovery After Focal Ischemia

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SUPPLEMENTAL MATERIAL

Effects of high vs moderate-intensity training on neuroplasticity and functional recovery after focal ischemia

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Supplemental Methods:

Animals

Food and water were available *ad libitum* (maintained at 22°C with a 12 h light/dark cycle). As in clinical trial, all acquisitions and analysis were done blind. Experimenters performing behavioral and incremental tests could only see the scar on the neck without distinguishing SHAM and injured rats.

Animals were excluded when one of the following points was observed: 1) weight exceeded 270g before inducing cerebral ischemia, 2) animals not able to run on treadmill during the familiarization or after the surgery, 3) prostrated animals (extreme physical weakness and absence of movement in the cage) and not able to run on treadmill, 4) more than 20% of their baseline weight lost, 5) absence of sensorimotor deficits and 6) death.

Based on these exclusion criteria, 38 rats on 84 subjected to MCAO-r were excluded because of death within the first 48h (n=28), absence of sensorimotor deficits (n=4) and more than 20% of their baseline weight and/or prostrated (n=3) and not able to run on treadmill (n=3).

Animals *(second mark in the manuscript)*

See below Supplemental figure I (page 6).

MCAO-r surgery and behavioral tests

Surgery was described elsewhere[1]. Central temperature (maintained at 37-38°C), age, weight, sex and ischemia duration were rigorously controlled, in order to ensure a reproducible lesion size between animals. Anesthesia was induced and maintained with 5% and 2.5-3% of isoflurane respectively (Anesteo, Villetelle, France). A 0.2 ml of 0.5% bupivacaine was injected subcutaneously along the prospective incision site. The right external, internal and common carotid arteries (ECA, ICA and CCA) were exposed. After a partial arteriotomy on ECA, a 0-4 monofilament nylon suture (silicon-coated tip length and diameter: 5 mm and 0.39±0.02 mm respectively; Redland, CA, USA) was inserted into the ICA via the ECA and approximately pushed 20 mm away from the carotid bifurcation. Blood flow was thus blocked at the MCA (middle cerebral artery) origin during 120 min. Then, the monofilament was removed to allow reperfusion. Finally, the skin was sutured. Moreover, we used only males in this study as observed in several other studies[2–6]. Some authors indicated that the response to training might be different between male and female but need to be

considered in further studies[7]. Moreover, $GABA_A$ receptors might be influenced by menstrual cycle and thus the inhibitory effect of GABA might be modulated[8–10].

MCAO-r surgery and behavioral tests *(second mark in the manuscript)*

All rats were familiarized before the experiment only with the ladder-climbing test (2 series per day, 3 days per week during 2 weeks) to ensure that rats understood they have to climb the ladder by gripping the rung. Familiarization is not required for Elevated body swing test and Forelimb grip force as already observed[11,12].

EBST. Animals were suspended by the base of the tail. A swing was recorded each time the animal raised to either its left or right side (more than 10 degrees). Ten swings were recorded. The number of swings made to the left side was divided by the total number of swings (left swings/total swings ratio).

Ladder-climbing test. Rats climbed up an inclined ladder (Inclination: 45° angle; rungs of equal 10 mm spacing). The affected paw grip was recorded with a numerical camcorder (MV 830i; Canon, Courbevoie, France; 100-Hz) and analyzed with SimiMotion software (Unterschleissheim, Germany). If the grip on the rung was correct, the score was 2 points. If the rung was completely missed by the affected forelimb, the score was 0 point. If the paw slipped on the rung, incompletely gripped it, or touched intermediate rungs during the swing phase, the score was 1 point. For each testing session, the successful score was normalized by the maximal score, depending on the number of performed steps (20 steps minimum).

Forelimb grip force. The grip forces exerted by both forelimbs and by each forelimb separately were quantified by using a grip force tester (Grip Strength Tester bio-GT3, Bioseb, Vitrolles, France). The rat was held by the base of its tail and by its body above the bar. When its forelimbs, or each forelimb separately, grasped the middle of the bar, the experimenter pulled the rat following the axis of the sensor (horizontally) until the grasp was released. The affected forelimb force was then normalized by the non-affected forelimb force to calculate the affected/non-affected ratio (A/N). The time interval between each trial was fixed to 1 min to avoid fatigue accumulation. The 2 maximal forces (in grams) were averaged for each session (in each condition) and expressed in percentage of PRE values.

Incremental test

Blood lactate concentration (mmol/L) was measured with a portable device (Lactate Scout+, EKF diagnostics®, France), reliable for animal models. Lactate threshold is detected at the previous stage when 1) the inflection point of blood lactate concentration was observed and/or 2) an increase of 1 mmol/L between two blood lactate values was measured[13]. All rats were familiarized before the experiment with running on a motor-driven treadmill (5min per day, 3 days per week during 2 weeks, at low speeds, 10–15 m/min). Such step is important to detect which rats were not able to run despite familiarization, as it was largely recognized[14–16]. The rats not completing this familiarization procedure were thus excluded from the study (n=3).

Work-matched HIT and MOD programs on treadmill

In accordance with stroke rehabilitation recommendations, 1) training started early after ischemia, 2) workload was progressively increased throughout the protocol and 3) running intensity was individualized[17,18].

To justify our training protocols, we used the physiological determinants of endurance performance (S_{LT} and S_{max}) in order to both improve the exercise intensity prescription and assess the effectiveness of aerobic programs[19]. To clarify the level of translational relevance to stroke patients, we already indicated that the feasibility of relative moderate and high intensities was already observed for moderate stroke patients[19–22].

For MOD, several reviews indicated the following exercise recommendations for moderate stroke patients[22–24]:

- Intensity should be fixed around 40-70% of the maximum heart rate reserve.
- Frequency: 3-5 sessions/week and the session duration is 20-90min (average 47.78 ± 25.8min).

These parameters were physiologically similar to the exercise parameters for MOD group in our study: 5 sessions/week, duration around 40min (table 1) and intensity below the lactic threshold (LT), which is considered as moderate intensity (40-70% of VO_2peak). In accordance with several authors, the use of LT is likely more relevant for clinical perspectives because the majority of patients could reach this physiological threshold[19,25].

For HIT, two studies revealed that 4x4min treadmill running interrupted by 3min of active recovery at 85-95% of peak heart rate (during 4-6 weeks; 5 sessions/week) was feasible for individuals with moderate stroke[21,26]. In our study, the running intensity during HIT series remained above the S_{LT} and below the S_{max}. It is an important physiological characteristic of HIT[27]. Therefore, intensity is always in the "aerobic range" that can be reached by animals, and thus, below the maximum tolerated. It seems that such intensities in rats could be applied to moderate stroke patients (80-95% of the difference between S_{LT} and S_{max}). Patients could reach these intensities because most of them could exceed the speed associated with ventilatory threshold (observable at an intensity similar to lactic threshold during an incremental test). Interestingly, it was revealed that most stroke patients reported initial feelings of apprehension, which were followed by increased confidence and enjoyment of HIT[20].

Other arguments could increase the translational relevance of our training protocols to human stroke in accordance with the STAIR study of Fisher et al. (2009)[28]:

- These training protocols can be performed on treadmill or cycle-ergometer in humans[18,23]. Cycle is recommended for stroke patients with postural disturbances to reduce the risk of falls/injuries. Likewise, it is prescribed for cognitive deficits and for limiting the influence of lower limb dysfunctions on aerobic performance by fixing the feet to the pedals[18,22,23,29]. HIT might thus be preferentially performed on cycle-ergometer. Nevertheless, we chose to use treadmill running because it allows a complete control of the exercise workload, permitting thereby the comparison between rats and humans[30].
- Young rats were used as in most preclinical studies on trained rodents (less mortality, focus on cerebral ischemia effects, lower cost, comparison of results with other training studies is closer) but that could also represent a limitation. Indeed, cerebral ischemia in older rats, with or without comorbidities (hypertension, systemic atherosclerosis, type 2 diabetes…), could exhibit exacerbated cellular changes compared to younger rats and could

be closer to human stroke conditions[31,32]. Contrary to young rats with cerebral ischemia, comorbidities likely influence recovery of patients and on their abilities to perform relative intense exercises.

- We were focused on the first 15 days after ischemia that is the minimum required by Fischer et al. (2009)[28]. This time period is already known to influence the recovery process[33]. The delayed effects of training need now to be deepened in further studies.

Overall, this study needs to be considered as an initial assessment of the effects of these training protocols before investigating them in males and females, aged rats with and without comorbidities and on longer time period after cerebral ischemia.

For both programs, each session began by 5min of warm-up at 70% of the S_{LT}. When necessary for rats to run, their tails were stimulated using a soft bristle brush (and not electrical stimulation)[15].

HIT. Sessions were composed of 4x4min treadmill running interrupted by 3min of active recovery between each intense series (i.e. running speed between series is fixed at the S_{LT}; 4x3min). From the 1st to 5th sessions (week-1), running speed was fixed at 80% of the difference between S_{max} and S_{LT} in order to ensure that running speed was above the S_{LT} without exceeding the S_{max}. Then, running speed was increased to 95% of the S_{max} - S_{LT} variation from the 6th to 10th sessions (week-2).

MOD. Running speed was fixed at 80% of the S_{LT} to avoid lactate accumulation. The session duration depended of HIT workload (exercise + recovery) to match the total amount energy expenditure (isocaloric sessions) between groups as follows:

Mass (kg) x Intensity (m/min) x Duration (min) x Treadmill inclination (°) x 9.8 (J/kg.m).

The total workload was the same between groups because the energy expenditure needs to be equivalent between exercise types in order to only compare the influence of intensity. Therefore, experimenters trained individually each rat.

Immunohistochemistry analysis

Animals (n=6 per group) were perfused transcardially (25 ml/min) with 250-300 ml of cold phosphate buffer saline (PBS 1X), after intraperitoneal ketamine-xylazine injection (100mg/kg–10mg/kg respectively), followed by paraformaldehyde 3% (antigenfix). Brain tissue was removed, post-fixed for 2h at 4˚C in antigenfix, cryoprotected 24 h at 4˚C in 30% sucrose and then snap-frozen with isopentane and stored at -80˚C Coronal sections of rat brain (slide thickness: 100 µm; Bregma +5.64mm to -6.84mm) were performed before mounting onto Superfrost Plus glass slides. Sections were then permeabilized and blocked in PBS 1X with 0.3% Triton X-100 and 5% normal goat serum (NGS) for 1 h at room temperature and incubated with primary antibodies diluted in PBS with 5% NGS and 0.1% Triton X-100 at 4°C overnight using rabbit anti-Iba-1 antibody (1:500, Life Science) or mouse anti-p75^{NTR} (1:1000, Santa Cruz). After rinsing three times in PBS, slices were incubated with the corresponding secondary antibody diluted in PBS (1:500, Alexa Fluor 488 or 543, Molecular probes) for 1 h at room temperature and finally counterstained for 5 min with Hoechst 33258 (10 µg/ml in PBS, Sigma-Aldrich). Then coverslips were applied onto Superfrost Plus glass slides with Fluoromount G mounting medium. For quantitative analysis images were acquired with an Olympus Fluorview-500 confocal microscope (40×; 1.0 NA). We choose fields at the peri-lesional site both from ipsi- and contralesional hemispheres. Images were then acquired

consecutively for Iba-1 and Hoechst staining and 25µm stack were acquired at 2.5µm step images. The Iba-1 positive cells (Iba-1+) morphology was manually assessed using the ImageJ software after making the images projection. Amoeboid Iba-1+ cells were expressed in percentage of total Iba-1+ cells. Acquisition parameters were the same for all experimental conditions. We add that it is not possible to distinguish resting microglia and circulating macrophage/monocyte cells with Iba-1 staining[34].

To measure the infarct volume and % tissue loss, one out of every four slides is stained (6 on 26 slides per brain) to have a representative cerebral injury. Sections were rinsed in distilled water for 5 min, incubated 3 min in a cresyl violet bath and then dehydrated through a sequence of ethanol baths (70, 95, and 100%). They were finally cleaned in Xylene during 2 min and medium mounted with coverslip using Permount (Fair Lawn, NJ, USA). For each section, the infarct size and the total size of each hemisphere were calculated with ImageJ software and expressed in percentage of the ipsilesional hemisphere. Likewise, volume of the ipsilesional hemisphere was calculated with ImageJ as a percentage of the volume of the contralesional hemisphere to determine the percentage of tissue loss following cerebral ischemia.

Western blot analysis

Animals were decapitated after ketamine/xylazine injection (n=4 per group). Hemispheres were quickly dissected out and separated, flash-frozen in liquid nitrogen and stored at -80 °C until processed. Brain tissue was homogenized in RIPA lysis buffer (50 mM Tris-HCl pH 8; 150 mM NaCl; SDS 0.1%; Deoxycholic Acid 0.5%; 1% triton X-100), containing complete Protease/Phosphatase Inhibitor Tablet (Thermofisher) and loaded with Laemmli X-3 loading buffer. The sample proteins were separated in SDS–PAGE gel (4% stacking gel and 15% resolving; Criterion gel, Bio-Rad) by electrophoresis under constant voltage (75V in stacking gel and 100V in revolving gel). The proteins on gel were then transferred to nitrocellulose membrane. The membranes were washed with Tris-buffered saline with 0.1% of Tween (TBST) and blocked in TBST with 5% of bovine serum albumin (BSA). Membranes were exposed overnight at 4°C with the appropriate primary antibody diluted in blocking solution (TBST/2.5% BSA): mouse anti-IL-10 (1:1000, Santa Cruz), rabbit anti-IL-1β (1:1,000; Abcam, Cambridge, UK), goat anti-IL-12p40 (1:1,000; Abcam, Cambridge, UK), mouse anti-$p75^{NTR}$ (1:1000, Santa Cruz), rabbit anti-KCC2 (1:5000, home product[35]) and mouse anti-NKCC1 (1:2000, T4 antibody, DSHB, University of Iowa, USA). Then membranes were washed twice with TBST and secondary antibodies (1:1000, Alexa Fluor 543 anti-rabbit, Alexa Fluor 488 anti-mouse, Alexa Fluor 546 anti-goat, Molecular probes) were applied 2h at room temperature before fluorescence detection. We measured signal intensities with the image analysis software G box (Syngene). After rinsing three times in TBST and membranes were incubated with the corresponding primary antibody for normalization with rabbit anti-β3-tubulin (1:10000, Covance) or anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:500, Millipore) or anti-α-tubulin (1:1000, Millipore). The membranes were exposed to secondary antibodies (see above) before signal detection. Quantification was performed using Gel Plot Analysis plugin (ImageJ). The relative expression levels are normalized to SHAM condition for $p75^{NTR}$, KCC2 and NKCC1 and to MCAO condition for IL-10, IL-1β and IL-12p40.

Statistical analysis

Behavioral test results and incremental exercises parameters were compared by two-way repeated measures ANOVA tests (groups x time). Immunostaining analysis as well as lesion size results were compared by one-way analysis of variance (groups). Student's t-test was conducted for Western blotting data. Post-hoc comparisons were performed with Student-Newman-Keuls multiple post-test comparisons. Results were considered significant when $p<0.05$. Finally, we used Pearson's test to calculate correlations.

Supplemental Figure:

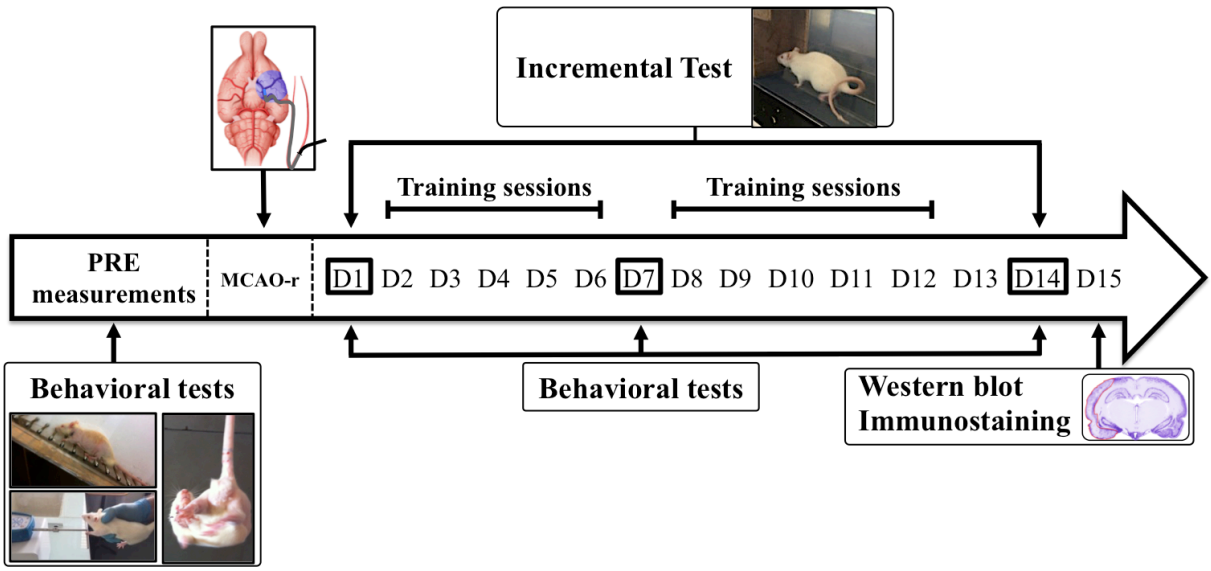

Figure I: Experimental protocol illustrating the timeline for the behavioral tests, incremental tests, brain sample and training sessions. D7 and D13 are the recovery days.

Supplemental table:

	HIT		MOD	
	Week-1	Week-2	Week-1	Week-2
Work speed (m/min)	24.5±2.6	25.4±2.7	17.5±2.6	17.5±2.6
Recovery speed (m/min)	20.2±2.4	20.2±2.4	None	None
Session duration (min)	28	28	41.8±9.5	44.8±12.3
Total Workload (joules)	1458±210	1654±342	1458±210	1654±342

Table I: *Exercise intensity, duration and energy expenditure during HIT and MOD*. The total workload was the same between groups because the energy expenditure needs to be equivalent between exercise types.

Stroke online supplement preclinical checklist

Stroke Online Supplement

Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

Methodological and Reporting Aspects	Description of Procedures
Experimental groups and study timeline	☒ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. ☒ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated. ☒ An overall study timeline is provided.
Inclusion and exclusion criteria	☒ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.
Randomization	☒ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. ☒ Type and methods of randomization have been described. ☒ Methods used for allocation concealment have been reported.
Blinding	☒ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. ☒ Blinding procedures have been described with regard to masking of group assignment during outcome assessment.
Sample size and power calculations	☒ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.
Data reporting and statistical methods	☒ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. ☒ Baseline data on assessed outcome(s) for all experimental groups have been reported. ☒ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. ☒ Statistical methods used have been reported. ☒ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.
Experimental details, ethics, and funding statements	☒ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. ☒ Different sex animals have been used. If not, the reason/justification is provided. ☒ Statements on approval by ethics boards and ethical conduct of studies have been provided. ☒ Statements on funding and conflicts of interests have been provided.

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