Ablation of the inferior olive prevents H-reflex down-conditioning in rats

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Chen XY, Wang Y, Chen Y, Chen L, Wolpaw JR. Ablation of the inferior olive prevents H-reflex down-conditioning in rats. J Neurophysiol 115: 1630–1636, 2016. First published January 20, 2016; doi:10.1152/jn.01069.2015.—We evaluated the role of the inferior olive (IO) in acquisition of the spinal cord plasticity that underlies H-reflex down-conditioning, a simple motor skill. The IO was chemically ablated before a 50-day exposure to an operant conditioning protocol that rewarded a smaller soleus H-reflex. In normal rats, down-conditioning succeeds (i.e., H-reflex size decreases at least 20%) in 80% of animals. Down-conditioning failed in every IO-ablated rat (P < 0.001 vs. normal rats). IO ablation itself had no long-term effect on H-reflex size. These results indicate that the IO is essential for acquisition of a down-conditioned H-reflex. With previous data, they support the hypothesis that IO and cortical inputs to cerebellum enable the cerebellum to guide sensorimotor cortex plasticity that produces and maintains the spinal cord plasticity that underlies the down-conditioned H-reflex. They help to further define H-reflex conditioning as a model for understanding motor learning and as a new approach to enhancing functional recovery after trauma or disease.

METHODS

Subjects were 12 young adult male Sprague-Dawley rats weighing 375 (±68 SD) g (range 269–537 g) at the beginning of study. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, 2011) and had been approved by the Institutional Animal Care and Use Committee of the Wadsworth Center. The protocols for implantation of the nerve-stimulating cuff and EMG recording electrodes, M-response and H-reflex elicitation, H-reflex conditioning, and data collection and analysis in freely moving rats have been fully described previously (Chen and Wolpaw 1995, 2002, 2005; Wolpaw and Chen 2006; Wolpaw and Herchenroder 1990) and are reviewed here. The procedures for IO ablation and histological evaluation are described in detail below.

IO ablation and postablation animal care and well-being. We used a simple, well-developed pharmacological ablation method to make a selective lesion of the IO: intraperitoneal (ip) injection of 3-acetylpypyridine (3-AP), followed several hours later by ip injection of nicotinamide. This method has been used in numerous studies (e.g., Balaban 1985; Gasbarri et al. 2003; Llinas et al. 1975; O’Hearn and Molliver 1997; Saxon and White 2006; Seoane et al. 2005; Watanabe et al. 1997). Histological analysis indicates that its effects are largely confined to the IO; other CNS regions show no or only minimal effects (Seoane et al. 2005). The toxicity of 3-AP is believed to result from its action as an antimetabolite of niacinamide; the reason for its areal specificity is less clear (Balaban 1985; Seoane et al. 2005).

Rats were injected with 3-AP (70 mg/kg ip) followed by nicotinamide (300 mg/kg ip) 3.5 h later. During the following days, they were carefully watched and checked 4–6 times/day, 7 days/wk. They displayed no signs of pain or distress. In the first 24 h after the injections, exploratory behavior was attenuated. In subsequent days, they displayed postural and locomotor signs similar to those seen after cerebellar nuclear ablation in our previous studies (Chen and Wolpaw...
holding the trunk close to the ground, limb rigidity, intermittent hopping locomotion, occasional oscillatory head movements). These signs disappeared within 14 days. Rats that ate poorly in the first few postablation days were fed manually with water-soaked rat chow and a high-calorie dietary supplement (Nutri-Cal) until they resumed normal eating (i.e., within 5–10 days). Body weight decreased ~10% in the first postablation week and recovered to its preablation level by 2–3 wk after ablation. Every rat gained weight over the time of study. Furthermore, after the first 2–3 postablation weeks rats appeared to walk normally, and they satisfied the background EMG requirement for H-reflex elicitation with the same daily frequency as before ablation, indicating that there was no reduction in daily activity level.

**Electrode implantation.** Each rat was implanted with chronic stimulating and recording electrodes in the right hindlimb under general anesthesia [ketamine HCl (80 mg/kg ip) and xylazine (10 mg/kg ip)] and in aseptic conditions. To record soleus EMG activity, a pair of fine-wire electrodes was placed in the right soleus muscle. To elicit the soleus H-reflex, a nerve-stimulating cuff was placed on the right posterior tibial nerve just above the triceps surae branches. The cuff was closed by a suture that encircled the cuff. The Teflon-coated wires from the nerve cuff and the muscle passed subcutaneously to a connector plug secured to the skull with stainless steel screws and dental cement. Immediately after surgery, the rat was placed under a heating lamp and given an analgesic (Demerol, 0.2 mg im). Once awake, it received a second dose of analgesic and was returned to its cage and allowed to eat and drink freely. Rats recovered quickly and awakened, it received a second dose of analgesic and was returned to its heating lamp and given an analgesic (Demerol, 0.2 mg im). Once awake, it received a second dose of analgesic and was returned to its cage and allowed to eat and drink freely. Rats recovered quickly and resumed their normal activity within 1–3 h.

**H-reflex conditioning protocol.** Data collection began at least 30 days after the implantation surgery and continued 24 h/day, 7 days/wk for 70–90 days. During this period, the rat lived in a standard rat cage with a 40-cm flexible cable attached to the skull plug. The cable, which allowed the animal to move freely about the cage, carried the wires from the electrodes to a commutator above the cage that connected to an EMG amplifier (gain 1,000, bandwidth 100–1,000 Hz) and a nerve-cuff stimulation unit. The rat had free access to water and food, except that during H-reflex conditioning it received food mainly by performing the task described below. Animal well-being was carefully checked several times each day, and body weight was measured weekly. Laboratory lights were dimmed from 2100 to 0600 daily.

Stimulus delivery and data collection were under the control of a computer, which monitored soleus EMG activity (sampled at 5,000 Hz) continuously for the entire period of data collection. The soleus H-reflex was elicited as follows. Whenever the absolute value (equivalent to the full-wave rectified value) of background (i.e., ongoing) EMG activity in the soleus muscle remained within a predefined range [based on the rat's typical soleus EMG level as it moved about the cage; usually 1–2% of maximum possible EMG activity as assessed by maximum M-response (i.e., direct muscle response)] for a randomly varying 2.3- to 2.7-s period, the computer initiated a trial. In each trial, the computer stored the most recent 50 ms of soleus EMG activity (i.e., the background EMG interval), delivered a monophasic stimulus pulse through the cuff on the posterior tibial nerve, and stored soleus EMG activity for another 100 ms. In the course of its normal activity, the animal usually satisfied the background EMG requirement, and thus received nerve-cuff stimulation, 2,500–6,800 times/day.

Stimulus pulse amplitude and duration were initially set to produce a maximum H-reflex (as well as an M-response that was typically just above threshold). Pulse duration remained fixed (usually 0.5 ms; 0.1 ms in the few rats in which the 0.5-ms pulse produced a stimulus artifact that impinged on the M-response). After each trial, pulse amplitude was adjusted by the computer so as to maintain the average absolute value of EMG activity in the M-response interval (typically 2.0–4.5 ms) unchanged throughout data collection. This ensured that the effective strength of the nerve stimulus was stable throughout the experiment despite any changes that occurred in nerve cuff electrode impedances or in other factors (Chen and Wolpaw 1995; Wolpaw 1987). Thus throughout the entire period of data collection, both the background EMG activity (reflecting soleus motoneuron tone at the time of H-reflex elicitation) and the M-response (reflecting the effective strength of the nerve cuff stimulus) remained stable.

M-response size was defined as the average absolute value of EMG activity in the M-response interval minus the average absolute value of background EMG activity. H-reflex size was defined as the average absolute value of EMG activity in the H-reflex interval (typically 6–10 ms) minus the average absolute value of background EMG activity and was expressed in units of average background EMG activity (Chen and Wolpaw 1995).

Under the control mode, the computer simply digitized and stored the absolute value of EMG activity from each muscle for 100 ms after the stimulus. Under the down-conditioning mode, it gave a food pellet reward 200 ms after the nerve stimulation if the average absolute value of soleus EMG activity in the H-reflex interval was below a criterion value. The criterion value was set and adjusted as needed each day so that the rat received an adequate amount of food (e.g., ~800 reward pellets/day for a 450-g rat). Each rat’s number of trials/day, background EMG activity, and M-response size remained stable throughout data collection.

Seven IO-ablated rats [IO-AC (acquisition) rats] were studied under the control mode for 20 days to determine the control (i.e., initial) H-reflex size. Each was then exposed to the down-conditioning mode for 50 days. The last 10 control-mode days and the last 10 conditioning days i.e., days 41–50 of down-conditioning) provided the data used to assess the impact of IO ablation on the acquisition of soleus H-reflex down-conditioning. To assess the long-term impact of IO ablation itself on H-reflex size, five additional rats [IO-Con (control) rats] were studied under the control mode for 20 days and then subjected to IO ablation. Data collection then continued under the control mode for 50 days (2 rats) or 70 (3 rats) more days.

**Histology.** At the end of data collection, each rat received an overdose of pentobarbital sodium (ip) and was then perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). The EMG electrodes, nerve cuff, and tibial nerve were examined, and the right and left soleus muscles were removed and weighed. Five unimplanted naive control (NC) rats with comparable body weights were similarly perfused and processed to provide normal IO and cerebellar histological data with which to compare the data from the IO-ablated rats.

The brain was removed and postfixed in the same fixative overnight. The area encompassing the IO and the cerebellum was blocked and washed with 0.05 M phosphate-buffered saline (PBS, pH 7.4), infiltrated with 30% sucrose for 24 h, embedded in OCT compound (Tissue-Tek), and frozen with dry ice. Transverse serial sections of IO and cerebellum were cut at 20 μm and 12 μm, respectively, and mounted onto precoated glass slides (Superfrost; Fisher).

Every fourth IO section was stained with cresyl violet, and every fifth cresyl violet-stained section was photographed with an Olympus BH2-RFCA microscope equipped with an Olympus DF70 digital camera. Thus for each animal four sections from rostral to caudal, corresponding to Ruigrok’s level (L)20, L16, L12, and L9 (Ruigrok 2004; Ruigrok and Voogd 2000), were used for quantitative analysis of the IO ablation. In these sections, we counted, on both right and left sides, the number of IO cells with diameters of at least 10 μm and time of H-reflex elicitation and the M-response (reflecting the effective strength of the nerve cuff stimulus) remained stable.

To evaluate the impact of IO ablation on olivocerebellar projection fibers, cerebellum vesicular glutamate transporter 2 immunoreactivity (VGLUT2-IR) was assessed with a standard avidin-biotin complex-peroxidase system (ABC Elite; Vector Laboratories, Burlingame,
CA). Every tenth cerebellar section was selected for processing. Briefly, the sections were first washed with PBS containing 0.1% Triton X-100 (PBST, pH 7.4) three times (10 min each), blocked with 7% normal goat serum for 90 min, and incubated overnight with monoclonal anti-VGLUT2 antibody [Millipore, 1:1500 dilution in PBST containing 2% bovine serum albumin (BSA)] in a humid chamber at 4°C. The sections were then washed again and incubated with biotinylated goat anti-mouse secondary antibody (1:200 in PBST) for 1.5 h. After endogenous peroxidase activity was quenched by 0.3% H$_2$O$_2$, the sections were reacted with the avidin-biotin complex (1:100 in PBS) for 1.5 h. Finally, the sections were reacted with 0.05% diaminobenzidine (DAB) solution containing 0.006% H$_2$O$_2$ for 14 min for color development.

VGLUT2-IR is a selective marker for climbing fibers and their terminals in the molecular layer of cerebellar cortex (Fremeau et al. 2001; Kaneko et al. 2002). Thus, for each rat, we quantified VGLUT2-IR in the molecular layer. Every fourth VGLUT2-labeled section was analyzed. For each of these sections, 10 photomicrographs were taken with an Olympus BX61 microscope equipped with a Hamamatsu CCD digital camera ($\times100$ magnification, fixed illumination). These photomicrographs were distributed randomly across the entire lateral to medial dimension of the cerebellar cortex; they thereby sampled the entire cerebellar cortex of the section. In each photomicrograph, the density of VGLUT2-IR terminals was traced with the ImageJ program (version 1.48v). For each IO rat, cerebellar VGLUT2-IR was calculated in percentage of normal as [(average VGLUT2-IR density of the IO rat)/(average VGLUT2-IR density for the 5 NC rats)] $\times$ 100.

It is important to note the steps taken to ensure that tissue processing and analysis were reliable and unbiased. Sections from NC rats and IO-ablated rats were routinely processed together. Furthermore, all quantitative analyses (i.e., of IO neurons and cerebellar VGLUT2-IR labeling) were performed in a blinded manner (i.e., the evaluator did not know whether a given slide came from an NC rat or an IO-ablated rat).

**Data analysis.** To assess the effects of IO ablation on acquisition of a down-conditioned H-reflex, a paired t-test was used to compare the average H-reflex sizes of IO-AC rats for the last 10 days of down-conditioning (i.e., days 41–50 of the 50-day down-conditioning period) to their average H-reflex sizes for the last 10 control-mode days prior to the beginning of the down-conditioning period. To compare the effects of IO ablation on down-conditioning with the effects of other lesions assessed in earlier studies, an ANOVA followed by the Bonferroni test was used to compare the average H-reflex size for each 10-day period after IO ablation to the average H-reflex size for the last 10 preablation days. If an effect was found, Dunnett’s multiple comparisons method was used to identify those 10-day periods that differed significantly from the average of the last 10 control-mode days.

**RESULTS**

Animals remained healthy and active throughout data collection. Body weight increased from 269–537 [375(±68 SD)] g at the beginning of the study to 528–716 [590(±57)] g at the time of perfusion. Right and left soleus muscle weights (measured as % body wt) averaged 0.044(±0.004 SE)% for the right and 0.042(±0.003)% for the left. They did not differ significantly from each other (P = 0.16 by paired t-test) and did not differ from soleus muscle weights of normal rats (Chen et al. 2001a, 2001b, 2002, 2005, 2006a, 2011; Chen and Wolpaw 1995, 1997, 2002). Examination of the nerve cuffs revealed the expected connective tissue investment of the wires and apparent good preservation of the nerve inside the cuff.

**IO ablation and its effects on climbing fibers.** IO ablation was largely effective. In the IO-ablated rats (IO rats), IO cell counts (i.e., % of IO remaining) averaged 35.6(±7.1)% for the left and 0.042(±0.004 SE)% for the right (P = 0.16 by paired t-test) and did not differ from soleus muscle weights of normal rats (Chen et al. 2001a, 2001b, 2002, 2005, 2006a, 2011; Chen and Wolpaw 1995, 1997, 2002). Examination of the nerve cuffs revealed the expected connective tissue investment of the wires and apparent good preservation of the nerve inside the cuff.

As Fig. 1, H–K, show, the marked loss of IO neurons was accompanied by a marked loss in climbing fiber inputs to the cerebellum. IO ablation produced widespread degeneration of VGLUT2-IR terminals in the molecular layer of cerebellar cortex. [VGLUT2-IR is a selective marker for climbing fibers and their terminals in this layer of cerebellar cortex (Fremeau et al. 2001; Kaneko et al. 2002).] VGLUT2-IR in the molecular layer of IO-AC and IO-Con rats (e.g., Fig. 1K) averaged 30.1(±2.4 SE)% and 27.4(±4 SE)%, respectively, of that in NC rats (e.g., Fig. 1L) [P < 0.0001 for IO-AC or IO-Con vs. NC; P > 0.05 for IO-AC vs. IO-Con (ANOVA followed by Tukey HSD test)] (Fig. 1J).

**Effects of IO ablation on H-reflex size in conditioned rats.**

In the five rats in which H-reflex data were collected under the control mode before and for 50–70 days after IO ablation (i.e., IO-Con rats), the H-reflex did not change. For none of the 10-day periods after IO ablation did H-reflex size differ significantly from that of the last 10 preablation days. These data are shown in Fig. 1L. They indicate that IO ablation does not affect H-reflex size in the 2 mo after ablation.

**Effects of IO ablation on acquisition of a down-conditioned H-reflex.** Figure 2A shows the data for a control-mode (i.e., preconditioning) day and a day at the end of down-conditioning from a normal rat (Fig. 2A, left) and an IO-AC rat (Fig. 2A, right). In both rats, background EMG level (i.e., level at time 0) and M-response (i.e., direct muscle response) size are stable. In the normal rat the H-reflex is much smaller after down-conditioning, while in the IO-AC rat it has not changed.

Figure 2B summarizes the impact of the 50-day exposure to the down-conditioning mode on H-reflex size in IO-ablated rats (i.e., IO-AC rats). Figure 2C shows the final values for the
individual IO-AC rats and contrasts them with the final values for normal rats (Chen and Wolpaw 1995, 1996, 1997, 2002; Chen et al. 2001a, 2001b, 2005, 2006a, 2006b; and unpublished data). H-reflex size for days 41–50 averaged 63(±2 SE)% (P < 0.001 vs. initial value, paired t-test) in the normal rats and 107(±7)% (P = 0.36 vs. initial) in the IO-AC rats. Down-conditioning was successful [i.e., the H-reflex decreased to ≤80% of its initial value (Chen and Wolpaw 1995; Wolpaw et al. 1993)] in 80% of the normal rats and in none of the IO-AC rats (P < 0.0001 by χ²-test).

Figure 2C also compares the final H-reflex sizes of IO-AC rats and normal rats to results from previous studies in rats with midthoracic transection of the CST and rats with ablation of the principal cerebellar output (DIN) (Chen and Wolpaw 1997, 2002, 2005). The final H-reflex sizes in the four groups of rats differ significantly by ANOVA (P < 0.001), and pairwise multiple comparison with the Tukey test indicates that the IO-AC, DIN, and CST rats differ significantly from normal rats (P < 0.001 for IO-AC and DIN vs. normal and P < 0.01 for CST vs. normal). No difference was found among the three different lesions (P > 0.64 for all). In sum, down-conditioning was successful in none of the IO-AC, DIN, or CST rats: IO ablation, like CST transection or DIN ablation, entirely prevents down-conditioning.

**DISCUSSION**

**Severity, specificity, and rapidity of IO ablation.** The quantitative IO lesion analysis described in RESULTS and illustrated in Fig. 1, A–G, indicates that the regimen of 3-AP injection followed 3.5 h later by nicotinamide destroyed most of the IO. Furthermore, this loss correlated with loss of climbing fiber inputs to cerebellum (Fig. 1, H–K). The IO specificity of this ablation regimen has been established by studies showing that it has minimal or no impact on other brain areas (e.g., Balaban 1985; Gasbarri et al. 2003; Llinas et al. 1975; O’Hearn and White 2006; Seoane et al. 2005; Watanabe et al. 1997). These studies also indicate that the IO lesion develops quickly, in the first 24 h after AP injection. Thus the acceptance and interpretation of the present data should not be compromised by concerns about the severity, specificity, or rapidity of the IO ablation.

**Possible role of inferior olive in H-reflex down-conditioning.** IO lesions are followed by histological and functional effects that evolve gradually and appear to reflect recovery processes or other long-term changes (Aoki and Sugihara 2012; Bardin et al. 1983; Benedetti et al. 1984; Lutes et al. 1992; Rossi et al. 1991a, 1991b). Nevertheless, IO ablation alone [like DIN ablation alone (Chen and Wolpaw 2005)] had no detectable...
long-term effect on H-reflex size (Fig. 1L). However, IO ablation [like DIN ablation (Chen and Wolpaw 2005)] entirely prevented down-conditioning of the H-reflex (Fig. 2).

While H-reflex down-conditioning is prevented by DIN ablation or CST transection, it is not affected by transection of other major descending pathways, including the rubrospinal, reticulospinal, and vestibulospinal tracts (Chen and Wolpaw 1997, 2002, 2005; Wolpaw and Chen 2006). Thus it appears that the essential cerebellar contribution is output that goes to SMC (the principal origin of the CST) rather than to the spinal cord. This cerebello-cortical input might guide the CST activity that produces the spinal cord plasticity that is directly responsible for the smaller H-reflex. Alternatively, this input might simply be needed for the normal functioning of SMC that enables it to maintain the crucial CST activity. The present finding that IO ablation abolishes acquisition of down-conditioning as effectively as DIN ablation supports the hypothesis that the IO and the cerebellum guide the CST activity that changes the H-reflex. A nonspecific global effect of IO ablation on SMC function is less likely because the IO does not provide direct input to SMC; furthermore, IO ablation (like DIN ablation) had no lasting effect on animal well-being, gross motor behavior, or activity level (see METHODS).

Two other simple learning phenomena, vestibulo-ocular reflex (VOR) conditioning and eyelblink conditioning, are believed to depend on cerebellar plasticity produced by the conjunction of activity in particular mossy and climbing fibers (Boyd et al. 2004; Cheron et al. 2011; Freeman and Steinmetz 2011; Longley and Yeo 2014; Mauk et al. 2014; Schoneville et al. 2011; Thompson 2005; Welsh et al. 2005). The climbing fibers, which originate in the IO, are hypothesized to provide a teaching signal. A similar conjunction might underlie H-reflex conditioning. The mossy fibers could convey efference-copy activity that reflects current CST influence over the H-reflex arc (Leergaard et al. 2006; Ruigrok et al. 2015; Suzuki et al. 2012). The climbing fibers could indicate whether a reward occurs (e.g., whether the IO receives cortical input that reflects the click of the pellet dispenser or the taste of the food pellet) (see Ruigrok et al. 2015 for review of IO inputs). The cerebellar output to SMC resulting from this conjunction might increase the probability of CST activity that decreases the H-reflex and thereby increases the probability of reward. Confirmation of this possibility will entail demonstration that H-reflex conditioning is associated with activity in mossy and climbing fibers that differs for up- and down-conditioning, and that this activity is necessary and sufficient for changing the H-reflex.

Plasticity underlying H-reflex conditioning. H-reflex down-conditioning appears to depend on plasticity in both brain and spinal cord that functions as a hierarchy; plasticity in the brain (probably in SMC) induces and maintains the plasticity in the spinal cord that is directly responsible for the smaller H-reflex (Wolpaw and Chen 2006; see Wolpaw 2010 and Thompson and Wolpaw 2014 for review). The present results indicate that the IO has a key role in this hierarchy. Study of the impact of IO ablation on maintenance of H-reflex down-conditioning (Chen et al. 2014a), and comparison of this impact with that of DIN ablation (Wolpaw and Chen 2006), could further clarify the supraspinal plasticity that underlies acquisition and preservation of this simple motor skill.

H-reflex conditioning and motor learning. For several reasons, operant conditioning of the H-reflex is a uniquely valuable motor learning model. First and most simply, H-reflex changes similar to those produced by the conditioning protocol contribute to the acquisition of motor skills in normal life (e.g., Nielsen et al. 1993; see Pierrot-Deseilligny and Burke 2012 and Wolpaw and Chen 2010 for review). Second, H-reflex conditioning enables detailed analyses of how the many different motor skills in an individual’s repertoire modify and share the same spinal neurons and synapses (Chen et al. 2005, 2011, 2014b, 2014c; see Thompson and Wolpaw 2014 and Wolpaw 2010 for review). Third, H-reflex conditioning provides an effective new approach to improving functional recovery after partial spinal cord injuries or in other disorders (Chen et al. 2006b,
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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