

Propofol Increases Presynaptic Inhibition of Ia Afferents in the Intact Human Spinal Cord

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Background: *In vitro* studies indicate that the primary molecular targets of propofol in the spinal cord are γ -aminobutyric acid (GABA) type A receptors. Because of the complexity of the central nervous system, specific GABA-mediated effects have not yet been isolated in humans. Here, the authors used heteronymous Ia facilitation of the soleus H-reflex from the femoral nerve as a specific pathway involving GABA to demonstrate a presynaptic GABA-mediated effect of propofol in humans.

Methods: The study was performed in 10 volunteers aged 23–32 yr. The soleus H-reflex was evoked by stimulation of the tibial nerve in the popliteal fossa. The stimulation current was adjusted to yield an unconditioned H-reflex of 15% of the maximal muscle response to electric stimulation of the tibial nerve. The soleus H-reflex was conditioned by stimulating Ia afferents from the quadriceps femoris in the femoral triangle. The stimulus was applied 0.3–0.4 ms after the onset of facilitation, to assure a purely monosynaptic excitatory postsynaptic potential from quadriceps Ia afferents to the soleus motoneuron. At least 45 conditioned (femoral and tibial) and unconditioned (only tibial) stimuli were applied in random order. The authors compared the amount of heteronymous H-reflex facilitation under a concentration of 2 μ g/ml propofol with control values obtained before and after the propofol infusion.

Results: H-reflex facilitation due to the conditioning stimulus during propofol administration was significantly ($P < 0.05$, *t* test) decreased by an average of 43% in all patients in comparison with the control values.

Conclusions: Although alternative explanations such as supraspinal effects cannot be ruled out completely, the findings of this study are most likely explained by a specific presynaptic effect of propofol. Strong evidence from neurophysiologic studies indicates that this effect is mediated by the GABA type A receptors.

MOLECULAR studies have provided evidence that propofol enhances the γ -aminobutyric acid type A (GABA_A) receptor activity at concentrations relevant for anesthesia.^{1–3} *In vitro* studies of ventral horn interneurons in cultured spinal cord tissue slices indicate that the primary molecular targets of propofol in the ventral horn are also GABA_A receptors.⁴ Also shown in rats, the immobilizing action of propofol is antagonized by GABA_A receptor blockade.⁵ In humans, the importance of γ -aminobutyric acid-mediated (GABAergic) effects is difficult to demonstrate because the complexity of the human

central nervous system can hardly be disentangled *in vivo*. Pharmacologic proof of receptor-specific effects is difficult because it would not be ethically justifiable to administer specific receptor antagonists that are commonly used in *in vitro* studies. However, using methods developed to study the physiology of central nervous system functions, it may be possible to isolate also specific pharmacologic effects. One important mechanism by which γ -aminobutyric acid (GABA) reduces the activity of the central nervous system is the presynaptic inhibition. Since the early descriptions by Frank and Fuortes⁶ and subsequent studies of Eccles *et al.*,^{7,8} the spinal cord has served as a useful model for studying the presynaptic inhibition in mammals. It is generally accepted that GABA generates presynaptic inhibition at axo-axonic synapses in the spinal cord.^{9,10} The clearest ultrastructural demonstration of GABAergic axo-axonic synapses that mediate presynaptic inhibition can be done at the synapse between the Ia afferent and the motoneuron.¹¹ Very recently, even the specific interneurons that release GABA to the terminals of the Ia afferents have been identified.¹² Pharmacologically, it was recognized long ago that presynaptic inhibition is reduced by the GABA antagonists bicuculline and picrotoxin.^{13–16} Based on these results, it has been shown later that the GABA_A receptor, rather than the GABA_B receptor (which is also present at the Ia terminals), plays the predominant role in presynaptic inhibition of Ia terminals.¹⁷ The activation of GABA_A receptors either inhibits the release of neurotransmitter (glutamate) from Ia afferents by blocking action-potential invasion into their terminals or by reducing the amplitude of propagated action potentials and thereby blocking or reducing Ca²⁺ influx. The reduced release of excitatory transmitter causes a reduction of monosynaptic excitatory postsynaptic potential (EPSP) in the target motoneuron. The postreceptor mechanism is still debated, whereas the role of GABA as the transmitter of presynaptic inhibition in the spinal cord is now undisputed (for review, see Rudomin and Schmidt¹⁸). Presynaptic inhibition is one of the few specific GABAergic effects in the spinal cord that can be analyzed *in vivo*. Here, we used a method first described by Hultborn *et al.*¹⁹ that allows to examine changes of ongoing presynaptic inhibition on Ia fibers independent of its origin in humans. This method has already been used in several other studies assessing different effects such as movement,^{20–23} nociceptive stimulation,²⁴ or baclofen administration^{25,26} on presynaptic inhibition. The aim of the current study was to use the presynaptic

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Received from the Department of Anesthesiology, Charité-Universitätsmedizin Berlin, Campus Mitte, Berlin, Germany. Submitted for publication August 22, 2005. Accepted for publication January 10, 2006. Supported by grant No. Re1534/2-3 from Deutsche Forschungsgemeinschaft, Bonn, Germany. Presented in part at the joint meeting of the Physiological Society and the Federation of European Physiological Societies, Bristol, United Kingdom, July 20–23, 2005.

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inhibition as a way to dissect the complicated spinal motor network as far as possible and possibly demonstrate a specific GABAergic effect of propofol in humans. We hypothesized that propofol, because of its GABAergic properties, would increase presynaptic inhibition. This study could therefore link results obtained from *in vitro* studies that have shown the importance of GABAergic interactions for the anesthetic effect of propofol with the clinical effects of propofol that can be observed in humans.

Materials and Methods

Subjects

After approval of the local ethics committee (Charité, Berlin, Germany) and written informed consent of the subjects were obtained, the study was performed in 10 (3 female) volunteers who had an American Society of Anesthesiologists physical status of I. During the entire study period, each subject was comfortably seated in a reclining arm chair, with the hip semiflexed at 120°, the knees slightly flexed at 160°, and the ankle at 110°.

Instrumentation

The H-reflex was elicited with a rectangular pulse of 1 ms in duration (Digitimer DS7A; Digitimer Ltd., Welwyn Garden City, United Kingdom) by stimulation of the posterior tibial nerve with the cathode (gold-plated half-ball electrode, diameter: 7.5 mm) in the popliteal fossa and the anode placed just above the patella. Reflex responses were recorded with adhesive Ag/AgCl electrodes (Medicotest "blue point"; Istrykke, Denmark) placed over the soleus muscle. The single stimulus in the poplitea fossa activates Ia fibers from soleus spindle sense organs in the tibial nerve that project monosynaptically onto the soleus motoneurons, which finally causes a contraction of the soleus muscle that can be recorded as the compound muscle action potential (fig. 1). The compound muscle action potential was amplified 500-fold with a band pass filter ranging from 20 Hz to 3 kHz (Neuropack 4 mini; Nihon Kohden, Tokyo, Japan), digitalized with a sampling rate of 5,000 Hz (Mikro 1401 mk II; CED Ltd., Cambridge, England) and stored on a mobile computer hard disk. The peak-to-peak amplitude of the H-reflex was measured on-line using Signal 3.01 (CED Ltd.). The sensitivity of the H-reflex to inhibitory and facilitatory effects depends on its size.²⁷ During propofol administration, the amplitude of the H-reflex is suppressed. Therefore, the unconditioned H-reflex amplitude had to be adjusted by increasing the stimulus current of the test volley. In all measurements, the amplitude of the unconditioned H-reflex was tuned to be 15% of the maximal muscle response to electric stimulation of the posterior tibial nerve (Mmax). Mmax typically decreases during the time course of any kind of

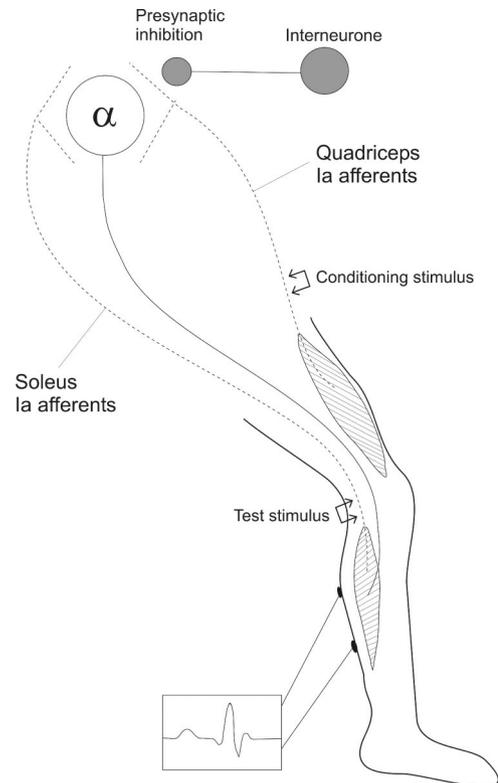


Fig. 1. Experimental paradigm. The soleus H-reflex (test stimulus) is elicited by an electrical stimulation of the tibial nerve. It is conditioned by a volley of Ia afferent fibers from the quadriceps muscle that run in femoral nerve (conditioning stimulus). These Ia afferents are subject to presynaptic inhibition *via* axo-axonic contacts of γ -aminobutyric acid-mediated interneurons.

experiment,²⁸ probably because of continuous dislocation of the stimulation electrode from the optimum position chosen at the beginning of the experiment. Therefore, Mmax was determined during each condition (control, propofol, second control) by increasing the stimulus current in steps of 10 mA until further increase in stimulus current would not result in a further increase of the M-wave amplitude. To quantify the suppressive effect of propofol on the unconditioned H-reflex, the maximal H-reflex amplitude (Hmax) was determined by increasing the stimulating current of the tibial nerve until a maximal H-reflex amplitude could be obtained. At least 10 Hmax values were averaged. The Hmax was expressed as a fraction of Mmax (Hmax/Mmax ratio) and was determined during each condition (control, propofol, second control). The conditioning stimulus was applied to the ipsilateral femoral nerve (fig. 1). The cathode (half-ball 12.5 mm in diameter) was placed in the femoral triangle just lateral of the femoral artery, and the anode was placed on the back of the thigh. A rectangular pulse of 1 ms in duration was delivered (Neuropack 4 mini). The stimulus intensity was adjusted to be 15% above the threshold for the motor response in the quadriceps muscle. It was kept constant throughout the entire experi-

ment. This conditioning pulse stimulates Ia fibers in the femoral nerve that derive from the M. quadriceps femoris muscle spindles. It has been shown in humans that collaterals of these Ia fibers project monosynaptically to the soleus motoneurons.²⁹ These projections are subject to presynaptic inhibition (fig. 1). Therefore, a constant conditioning stimulation, provided that there is no change in presynaptic inhibition, causes a constant EPSP in the corresponding soleus motoneurons. Because this EPSP cannot be measured directly, it is assessed by an appropriately timed soleus H-reflex (as described previously in this paragraph) conventionally called test reflex. A collision of the EPSPs from both soleus and quadriceps Ia fibers onto the soleus motoneuron leads to an increase of the H-reflex amplitude (some motoneurons that are not excited by the soleus Ia volley alone get excited by the additional EPSP from the conditioning stimulus, which drives the EPSP over the excitability threshold). If both the size of the test H-reflex and the stimulus strength of the conditioning stimulus are kept constant, the increase of presynaptic inhibition is revealed by a decrease of H-reflex facilitation. The smaller the reflex facilitation is, the larger the presynaptic inhibition is.

Experimental Protocol

At least 1 day before the experimental procedures, test measurements (without propofol administration) were performed to screen for subjects who would qualify for the electrophysiologic part of the study. Only subjects with a facilitation of the H-reflex by the conditioning stimulus of at least 15% were included. Because of the variability of H-reflex amplitude over time, a smaller facilitation would increase the number of averages necessary to demonstrate significant effects and thus make the measurement unfeasible. These test measurements also allowed the individuals to become familiar with the experiment. Subjects fasted at least 6 h before the beginning of the propofol administration. Standard monitoring (noninvasive blood pressure monitoring, electrocardiography, and pulse oximetry) and an intravenous access *via* a forearm vein were established before the study period. Propofol was infused intravenously *via* a computer-controlled infusion pump (Base primea; Fresenius, Bezins, France), programmed using the weight- and age-corrected pharmacokinetic parameter set of Schneider *et al.*³⁰ End-tidal carbon dioxide concentration was monitored with a tight-fitting facemask every 3 min when subjects were unconscious. The Bispectral Index was recorded with an Aspect XP[®] monitor (Aspect Medical Systems, Newton, MA). The Observer's Assessment of Alertness/Sedation score was determined immediately before and after the experimental run during propofol administration.

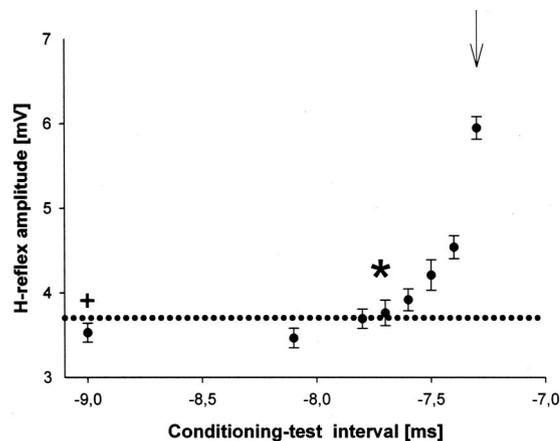


Fig. 2. Time course of the heteronymous Ia facilitation of the soleus H-reflex. By convention, the timing of the test pulse (soleus H-reflex) is given with reference to the conditioning pulse (Ia afferents in the femoral nerve). Because both pulses are to reach the spinal cord simultaneously, the test reflex must be delivered before the conditioning pulse because the distance from the site of stimulation of the H-reflex (popliteal fossa) to the spinal cord is longer than the distance from the femoral triangle to the spinal cord. In such cases, the conditioning test interval is said to be negative. Cross = unconditioned H-reflex; asterisk = beginning of facilitation; arrow = interval chosen for measurements cycles. The dotted line represents the cutoff line of 5% above the unconditioned H-reflex amplitude. Error bars represent SEMs.

Measurements, Data Processing, and Data Reduction

Only the first 0.5 ms of facilitation from the femoral nerve onto the soleus motoneurons has been demonstrated to be purely monosynaptic.³¹ Beyond that time window, the heteronymous facilitation can be contaminated by any other effects, such as Ib inhibition or oligosynaptic pathways. Therefore, we first established the earliest interval between the soleus H-reflex stimulation (test volley) and the femoral nerve stimulation (conditioning volley) at which it was possible to elicit a facilitation of at least 5% using steps of 0.1 ms, after roughly estimating the onset in steps of 0.4 ms. To determine the onset of heteronymous facilitation, we compared equal sample sizes of 25–35 stimuli for the conditioned and unconditioned H-reflex with different interstimulus intervals. Statistical significance was examined with analysis of variance (Graphpad Prism Version 3.0; San Diego, CA) including a Dunnett post test ($P < 0.05$). To obtain a more sizeable facilitation within the time window of monosynaptic facilitation, the interval chosen for all measurements was 0.3–0.4 ms longer than this and remained unchanged throughout measurements. Figure 2 shows an exemplary time course of the effect of the conditioning (1.15 * motor threshold, *i.e.*, the stimulus current necessary to evoke a muscle potential significantly different from background noise) stimulation from the femoral nerve onto the soleus H-reflex under control conditions.

We compared the amount of heteronymous H-reflex

facilitation under pseudo-steady state concentrations of 2 $\mu\text{g/ml}$ propofol with the averaged control values obtained before and 35 min after the end of propofol infusion. At each experimental condition (control, propofol, second control), a series of at least 45 unconditioned and 45 conditioned H-reflexes, delivered every 6 s, was recorded. Conditioned and unconditioned reflexes were randomly alternated. To compare the data of the different experimental conditions (control before propofol, propofol, control after propofol), the H-reflex amplitude is always expressed as a fraction of M_{max} . Mean and SEM were calculated for all measurements off-line. The difference in the magnitude of heteronymous facilitation between control and propofol was tested using analysis of variance (Graphpad Prism Version 3.0) including a Tukey posttest ($P < 0.05$). The amount of facilitation was expressed relative to the average unconditioned H-reflex in the corresponding measurement.

Results

Test measurements (without propofol administration) were performed in 12 subjects. One subject felt discomfort and refused further examination. In one other subject, the conditioning stimulation of the femoral nerve did not cause any significant facilitation of the soleus H-reflex. Those two individuals were excluded from further measurement, and all of the other 10 subjects were included in the study. The administration of propofol caused a decrease in the Observer's Assessment of Alertness/Sedation score and in the Bispectral Index value in all but one subject. The median values under 2 $\mu\text{g/ml}$ propofol plasma concentration yielded 3 (range, 1-4) and 66 (range, 31-95) for the Observer's Assessment of Alertness/Sedation score and the Bispectral Index value, respectively. During propofol administration, all subjects breathed spontaneously, and end-tidal carbon dioxide concentrations remained less than 42 mmHg. The unconditioned H_{max} was reduced only moderately by this concentration of propofol (average reduction of $24 \pm 18\%$ [mean \pm SD]).

Propofol Effects on Heteronymous H-reflex Facilitation

Propofol reduced the heteronymous H-reflex facilitation of the soleus H-reflex. Exemplary tracings from one subject are shown in figure 3 for unconditioned and conditioned H-reflex before and during administration of propofol. The amplitude of the H-reflex displays considerable variability from stimulus to stimulus. However, with at least 45 stimulations averaged, stable mean values were achieved (fig. 4). At the time of the second control run (at least 35 min after the end of the propofol infusion), the amplitude of the facilitation returned to

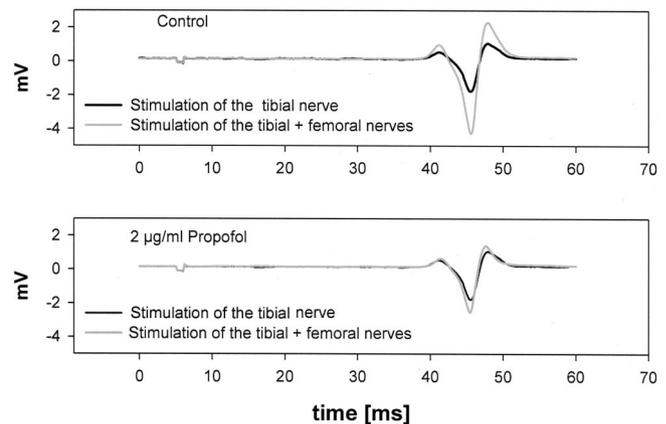


Fig. 3. Original tracings of the conditioned and unconditioned H-reflex under control conditions and 2 $\mu\text{g/ml}$ propofol. The conditioning stimulus leads to an increase of the H-reflex amplitude (gray line) in comparison with the unconditioned H-reflex amplitude (black line). During propofol administration, the H-reflex amplitude decreases. Because the sensitivity of the H-reflex to facilitation depends on the size of the unconditioned H-reflex, the stimulus strength of the H-reflex was adjusted so that the amplitude of the unconditioned H-reflex was of the same size as during control conditions (amplitudes of the black lines in both figures are about the same). The conditioning volley of the femoral nerve was kept constant under both conditions. Under control conditions, the conditioning volley leads to a greater increase (facilitation) of the H-reflex than under 2 $\mu\text{g/ml}$ propofol (gray line), i.e., the heteronymous Ia facilitation of the soleus H-reflex is suppressed by propofol.

control values (fig. 4). Because of the large intraindividual variability of the unconditioned H-reflex over time (fig. 4), the reduction was significant in only six subjects in comparison with controls before and in eight subjects after administration of propofol (analysis of variance with Tukey posttest, $P < 0.05$; fig. 5). However, pooling the data of the first (before propofol) and second (after propofol) control measurement for each subject leads to

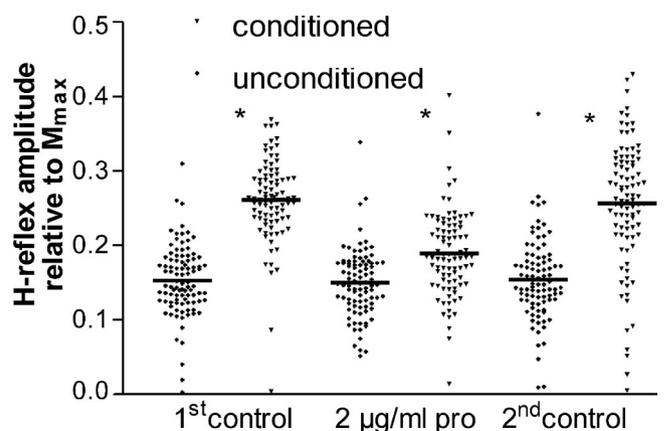


Fig. 4. Variability of the heteronymous Ia facilitation of soleus H-reflex in an exemplary subject. Individual measurements of H-reflex amplitude with (triangles) and without (crosses) conditioning stimulation in an exemplary subject. Data from 50 stimulations before (first control), during (2 mg/ml propofol), and after (second control) propofol administration. Lines indicate the mean value. Asterisks show significant difference in comparison with the propofol measurement (Tukey posttest, $P \leq 0.05$).

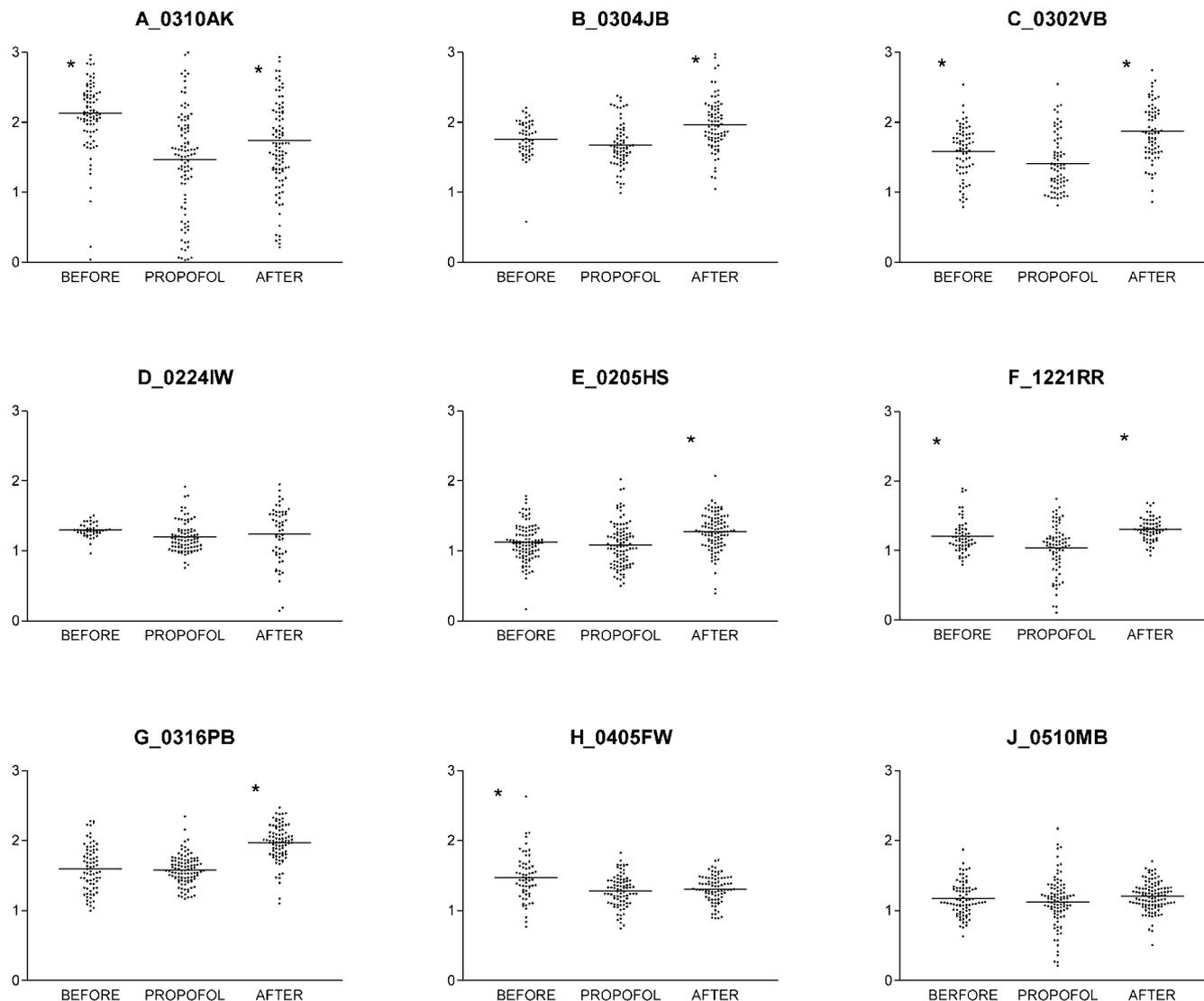


Fig. 5. Heteronymous Ia facilitation of soleus H-reflex in all other subjects (subject 1 shown in fig. 4). Amplitude of the conditioned H-reflex amplitude before, during, and after propofol administration. The amplitude is expressed as fraction of the mean unconditioned H-reflex in the corresponding measurement. Note the high variability of the H-reflex amplitude. Asterisks show significant difference in comparison to the propofol measurement (Tukey posttest, $P \leq 0.05$).

significant differences between propofol and control values in all individuals (Student t test, $P < 0.05$). The average reduction of H-reflex facilitation due to the conditioning stimulus during propofol administration was 37% in comparison with control values before and 44% after propofol administration. Suppression of facilitation correlated with neither the decrease of the Bispectral Index value nor the suppression of Hmax during propofol administration.

Discussion

In the current investigation, we studied the effect of propofol on the ongoing presynaptic inhibition of Ia afferents from the quadriceps femoris to the soleus motoneuron by using the method first described by Hult-

born *et al.*¹⁹ The principle of this method has been validated in animal experiments in which presynaptic inhibition of Ia afferents and postsynaptic events could be observed in intracellular recordings. These experiments have shown that the amount of heteronymous facilitation faithfully reflects the level of presynaptic inhibition of the Ia afferents projecting on the tested motor nucleus and is not affected by postsynaptic inhibition of the motoneurons.¹⁹ Objective evidence that activation of presynaptic pathways reduces EPSPs in motoneurons that originate from monosynaptic Ia afferents but not from descending pathways, which are free of presynaptic inhibition, further confirms the sensitivity of the method in measuring presynaptic inhibition.^{31,32} We have *a priori* selected only subjects with a facilitation of the H-reflex of greater than 15%, reaching significance in

Table 1. Amount of Facilitation in All Subjects

Subject	Before	Propofol	After
A	212 ± 9	147 ± 11	174 ± 12
B	176 ± 6	167 ± 6	196 ± 6
C	158 ± 6	141 ± 8	187 ± 8
D	129 ± 27	120 ± 4	124 ± 7
E	112 ± 4	108 ± 4	127 ± 4
F	121 ± 4	104 ± 5	131 ± 4
G	160 ± 5	158 ± 4	197 ± 4
H	147 ± 7	128 ± 4	131 ± 4
I	171 ± 7	126 ± 6	166 ± 8
J	117 ± 4	112 ± 5	121 ± 3

Facilitation of the H-reflex by femoral nerve stimulation before, during, and after propofol administration in all subjects. Presented as percent of the unconditioned H-reflex. Data represent mean ± SE.

a feasible number of stimulus repetitions. This potentially introduces a bias in the results. However, we have found no significant correlation between the magnitude of the facilitation and the magnitude of the effect of propofol (table 1). Regarding the raw data (fig. 5), the suppressive effect of propofol on the amount of H-reflex facilitation seems to be barely traceable in some subjects because of the large measurement variability. This results predominantly from the intraindividual variability of the H-reflex size itself but also from other disturbances, such as minimal alterations of the electrode positions due to movement of the leg or conductance variability of the skin due to transpiration during the course of the experiment. It is noteworthy that the measurements span a period of 4–6 h. To minimize the systematic errors that might have increased during the experimental time course, we also compared the propofol measurement with the pooled (before and after propofol administration) control measurements, which reveal significant differences in all subjects.

The reduced amount of heteronymous Ia facilitation during a plasma propofol concentration of 2 µg/ml can be ascribed to an increase of presynaptic inhibition. As stated in the introduction, this presynaptic inhibition is a specific GABAergic effect mediated primarily by GABA_A receptors. To our knowledge, only one other study exists that addresses the influence of propofol on presynaptic inhibition in humans. Shimizu *et al.*³³ examined in seven patients the action of propofol (1 mg/kg, intravenous) on the second positive wave of segmental spinal cord evoked potentials, which may also reflect GABA_A receptor-mediated primary afferent depolarization associated with presynaptic inhibition. However, this method is less established than the one used in our study and cannot rule out multisynaptic effects. Both methods examining presynaptic inhibition in the spinal cord cannot exclude the effect of propofol on supraspinal influences on presynaptic inhibition. Specifically, reticulospinal pathways might modulate presynaptic inhibition.³⁴ However, reversible spinalization by cold block in anesthetized cats^{35,36} decreases the tonic level of presynaptic

inhibition. Therefore, one can conclude that tonic supraspinal effects rather enhance than inhibit tonic presynaptic inhibition. An activation of these enhancing pathways during propofol may not be completely ruled out. Further experiments with drugs such as nitrous oxide, which cause a similar degree of sedation without major effects on GABA_A receptors, might prove this assumption. The method we used in this study assures a purely monosynaptic connection between the Ia afferents in the femoral nerve to the soleus motoneurons. However, it cannot be excluded that other presynaptic effects of propofol, such as interactions with presynaptic Na⁺ or Ca²⁺ channels, contribute to the reduced EPSP on the soleus motoneuron, although these are not assumed to be major molecular targets of propofol.³⁷ An alternative explanation to an effect of propofol on GABA_A receptors might also be a propofol-induced activation of the inhibitory interneuron, leading to an enhanced release of GABA. This seems unlikely, however, because propofol has been shown to decrease, not increase, the firing rate of spinal neurons.⁴

An ideal but, for ethical reasons, impossible way to prove a receptor specific effect would have been to antagonize the propofol effect by using GABA_A receptor-specific antagonists. That could possibly be examined in animal experiments with spinal and/or systemic administration of the antagonist. Animal experiments similar to those performed by Hultborn *et al.*¹⁹ to show the validity of the method would also permit simultaneous recording in the Ia afferent and the corresponding motoneuron to give direct evidence for presynaptic effects of propofol. Animal experiments, however, can only give complementary evidence, because (1) data in such experiments often cannot be acquired under control conditions without any anesthetic and (2) there are differences in the connectivity of the motor system between primates and other mammals. To further evaluate the supraspinal influences, this study protocol could be performed in volunteers with a complete hemiplegia or tetraplegia after the phase of spinal shock. The results obtained in this study lead to the question: Does the enhanced presynaptic inhibition contribute to propofol-mediated immobility? Considering the very low concentrations at which this effect occurs, this seems unlikely. The C₅₀ for the suppression of motor responses to painful stimuli is higher than 10 µg/mg,³⁸ and all subjects moved spontaneously during the experiments. GABA_A receptor blockade has been shown to be relevant for the immobilizing action of propofol,⁵ and GABA_A receptors are the most important target of propofol in the spinal cord,⁴ but GABA_A receptors are not exclusively localized presynaptically, and propofol effects do not specifically occur in the spinal cord. The mechanism shown here, presynaptic inhibition, strongly suppresses peripheral input to motoneurons but does not alter spinal motoneuron excitability itself. Corticospinal input to motoneu-

rons is not subject to presynaptic inhibition, and thus, voluntary movements are not affected by this mechanism. However, the increased presynaptic inhibition may be the reason for impaired motor coordination after general anesthesia with propofol. In addition, this property of enhancing presynaptic inhibition makes propofol an ideal antispastic agent especially benefiting those patients with some remaining voluntary motor functions.

During propofol anesthesia, the spinal cord may not be the only site where propofol enhances presynaptic GABA_A receptor-mediated presynaptic inhibition. Although these effects cannot be examined in isolation in humans, they may contribute to the general anesthetic effects of propofol, *i.e.*, among others, hypnosis, amnesia, and immobility.

With our study, we have attempted to dissect the complicated network of spinal motor control as far as possible *in vivo* in humans. The results show that propofol reduces Ia facilitation from the femoral nerve to the soleus H-reflex. Because the method assures a purely monosynaptic connection, this effect should be mediated by increased GABAergic presynaptic inhibition on Ia afferent fibers. Although we cannot completely rule out alternative explanations, we most likely have demonstrated *in vivo* in humans an effect of propofol specifically mediated by GABA_A receptors. Because of the complexity of the network *in vivo*, studies like this cannot yield definite answers but will need to be complemented by animal and *in vitro* research.

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