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Lateral gradients significantly enhance static magnetic field-induced inhibition of pain responses in mice – a double blind experimental study

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ABSTRACT

Recent research demonstrated that exposure of mice to both inhomogeneous (3-477 mT) and homogeneous (145 mT) static magnetic fields (SMF) generated an analgesic effect towards visceral pain elicited by the intraperitoneal injection of 0.6% acetic acid. In the present work we investigated behavioral responses, such as writhing, entry avoidance, and site preference with the help of a specially designed cage that partially protruded into the either homogeneous (ho) or inhomogeneous (inh) SMF. Aversive effects, cognitive recognition of analgesia, and social behavior governed mice in their free locomotion between SMF and sham sides. The inhibition of pain response in percent (I) was calculated as the ratio of numbers of writhings observed in the SMF and sham sides for the 0-10, 11-20, and 21-30 min periods following the challenge. In accordance with previous measurements analgesic effect was induced in exposed mice (I_{ho}=64%, p<0.0002 and I_{inh}=62%, p<0.002). No significant difference was found in the site preference ($SMF_{ho,\ inh}$ *vs* sham) of mice indicating that SMF is neither aversive, nor favourable. Comparison of writhings observed in the sham *vs* SMF side of the cage revealed that SMF exposure resulted in significantly less writhings than sham (I_{ho}=64%, p<0.004 and I_{inh}=81%, p<0.03). Mice spent significantly longer times together on the same side of the cage (p<0.003). Deeper analysis revealed that the lateral SMF gradient between SMF and sham sides could be responsible for most of the analgesic effect (I_{ho}=91%, p<0.02 and I_{inh}=54%, p<0.02).

Keywords: static magnetic field (SMF), gradient, analgesia, pain inhibition, aversive behavior

INTRODUCTION

Temporal fluctuations of magnetic fields typically in the low frequency range have been extensively investigated during the last few decades partly because of the potential medical applications of such external fields, and partly due to safety considerations of magnetic fields induced by general-purpose alternating current electric power supply. Effects of external magnetic fields might be different when applying time-varying magnetic field (including pulsed, gradient, alternating, and rotating) or static magnetic field (SMF). Permanent magnets typically generate SMF, the field strength of which remains constant in time for a long period. In contrast to temporal magnetic gradients, the role of the spatial (lateral or vertical) magnetic gradients on complex living organisms can be considered unexplored with few exceptions [Heinrich et al., 2011; László and Gyires, 2009; Okano et al., 2012].

Since Galen's indication several scientific experiments have been performed in order to understand the biological effect of external magnetic field exposure and to develop magnetic devices optimized for therapy. Recent studies in humans suggest chronic pain as a potential application field for magnetic therapy as a non-contact, non-invasive and cheap physiotherapeutic method: in osteoarthritis [Hulme et al., 2002], spine disorders [Linovitz et al., 2002], abdominal and genital pain [Holcomb et al., 2000], chronic pelvic pain [Brown et al., 2002], knee pain [Hinman et al., 2002], fibromyalgia [Alfano et al., 2001], myofascial pain syndrome [Smania et al., 2003], and diabetic neuropathic pain [Weintraub et al., 2009]. Precise human evaluation of the efficacy of several differently applied magnetic therapies has led to conflicting conclusions in meta-analysis: SMF therapy showed no benefits compared to placebo control [Pittler et al., 2007]. These contradictory results are mainly due to inevitable human factors: many clinical studies are limited because of subject awareness of the control group (lack of magnetic properties of sham devices is easily revealable), high variability in biological response to pain [Richmond, 2008], and incorrect or incomplete characterization of the applied magnetic field (as discussed in [Harlow et al., 2004]). Animal experiments were performed in different species in order to circumvent subject awareness, where biological responses to painful stimuli were studied. Antinociceptive effect against thermal or chemical stimuli has been observed, e.g., in snails [Prato et al., 2000], rats [Martin et al., 2004], and mice [Choleris et al., 2000; Del Seppia et al., 2003; Gyires et al., 2008; László et al., 2009]. Moderate SMF has proven to be effective in treating inflammatory agonist-induced edema in rats [Morris and Skalak, 2008], yet seemed to reduce the cognitive performance [Ammari et al., 2008; Lopuch, 2009]. Similarly to SMF, low frequency electromagnetic fields exhibit possible biological effect; both thermal aversive response and morphine-induced analgesia

were attenuated in snails [Prato et al., 2000; Tysdale et al., 1991], and decreased antioxidant enzyme capacity in rat brains [Falone et al., 2008]. Pain response modulation is successfully realized by shielding the external geomagnetic induction; stress-induced analgesic effect in mice has been reduced upon pre-stress treatment in a shielded environment [Choleris et al., 2002]. Possible molecular mediators of analgesic modulation include series of endogenous signal transducing peptides, such as β-endorphin, substance-P, and serotonin [Bao et al., 2006]. Constant, but increased induction compared to the geomagnetic field also exhibits several molecular, cellular level effects including nitric oxide signalization, vasodepressive tendency in rat hypertension models [Okano and Ohkubo, 2006]. However, in contrast to rats, short-term SMF exposure of humans does not result in significant reduction in either blood pressure or heart rate [Hinman, 2002]. The magnetic induction in currently applied SMF therapies typically ranges from 1 to 400 mT [Colbert et al., 2009; Pittler et al., 2007]. Analgesic effect on visceral pain of such SMF on mice has been demonstrated [László and Gyires, 2009].

The analgesic effect of SMF in mice in chemically-induced pain essay has been extensively studied and proved by our workgroup previously. In these studies the most significant visceral pain response reduction has been observed in mice by applying an array of high grade NdFeB cylindrical magnets with alternating polarity above and below the cage [László et al., 2007]. Applying external inhomogeneous SMF does not tend to influence the anxiety and behavioral pattern (ambulation, rearing) of mice, furthermore, does not affect the morphine-induced hyperlocomotion and antinociception [László et al., 2009]. The antinociceptive effect was shown to remain detectable several minutes subsequent to the cessation of the SMF exposure [László et al., 2009]. In these experiments the animals were constrained to either SMF or sham exposure without the possibility of free locomotion between the sham or SMF side and without a chance to study their site preference, or the role of crossing over the sham-SMF boundary.

In the present work we investigated (i) the possible aversive effect of SMF on mice with and without induced abdominal pain, (ii) the cognitive recognition of beneficial antinociceptive effects of SMF, and (iii) the role of other external contributing factors which may further improve the antinociceptive effect of SMF, particularly the role of lateral magnetic field gradients in a half SMF-half sham exposure chamber. Using this specific exposure chamber the animals were allowed to move unhindered between the SMF- and sham-exposed areas thus making the comparative analysis of their site preference and long-term pain response possible.

MATERIALS AND METHODS

1.1 Animals

Sixty three, 6 weeks old, male CFLP mice (24-26 g) were housed in groups of 5 in a room under a 12:12 light/dark cycle at 20±2^{0}C. Standard rodent pellet and tap water was provided *ad libitum*. The animals were randomly selected and distributed into experimental groups detailed in Table 1. All experimental procedures were carried out according to the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments (243/1988), and the Helsinki Declaration (EC Directive 86/609/EEC). The studies were in harmony with the Ethical Codex of Animal Experiments and were approved by the Animal Care Committee of Semmelweis University, Budapest (permission number: 22.1/606/001/2010).

1.2 Pain essay

The writhing test as described by Wende and Margolin [Wende, 1956] and modified by Witkin *et al*. [Witkin et al., 1961] was applied. The visceral pain was induced by the intraperitoneal (i.p.) injection of 0.6% acetic acid in a volume of 0.2 ml/mouse. As a result of chemical irritation a characteristic stretching and writhing movement could be observed. Right after the administration of acetic acid mice were put in a transparent cage made of Plexiglas exposed to sham or SMF for 30 min in a custom-made exposure chamber. Keeping in mind that mice are socially sensitive [37] 2-3 mice were placed in the cage simultaneously, and the number of writhings was monitored during the 0-5, 6-20, and 21-30 min periods after the injection of acetic acid. In all experiments a daily positive control was used, where the mice were given acetic acid and were sham-exposed. The sham apparatus assured the same dimensions and conditions as the SMF-exposure chamber, but the animals inside were not exposed to either inhomogeneous or homogeneous SMF beyond that of Earth. Negative control animals received physiological salt solution i.p. and were considered unchallenged.

1.3 Exposure conditions

1.3.1 Inhomogeneous SMF (inhSMF) exposure

InhSMF was induced with an exposure system that was developed, validated, and optimized for small animal experiments (#11 in [László et al., 2007]). Shortly, the device consisted of an upper and lower iron plate covered with 10x10 mm (diameter x height) cylindrical neodymium-iron-boron (NdFeB) N50 grade magnets (B_r=1.47 T) on one side each. The lateral periodicity of the

inhSMF was 10 mm. The individual magnets on both plates were placed next to each other with alternating polarity. Magnets facing each other on the 2 plates were oriented with opposite polarity. The plates were fixed in a holder with 50 mm vertical separation between the upper and lower magnet arrays thus realizing an exposure chamber size 140x140x46 mm (length x width x height). Magnetic coupling applied between the matrices (the upper and lower magnet arrays were coupled through a vertical ferromagnetic plate). The front iron wall of the chamber was removed in order to allow better view for the observer. Figure 1 shows the schematic arrangements plus scanned magnetic induction maps in the isocenter of the magnetic arrangements at 3 mm from the magnets' surface. In the isocenter the asymmetric induction from the matrix edges do not distort the map. Magnetic field dosimetry was performed separately from the animal experiments by means of a 5 V calibrated ratiometric linear Hall-effect sensor of 12.3 mV/T sensitivity (model UGN3503, Allegro MicroSystems, Inc., Worcester, MA, USA). The typical peak-to-peak magnetic induction values along the axis of a NdFeB magnet in the isocenter were 476.7±0.1, 12.0±0.1, and 2.8±0.1 mT, whereas the average lateral gradient values between 2 neighbouring local extremes were 47.7, 1.2, and 0.3 T/m at 3, 15, and 25 mm from the surfaces of plates, respectively.

The custom-fabricated *inhSMF&sham* was similar to the inhSMF chamber with the difference that the ferrous plates were twice as wide and, consequently, the exposure chamber was 140x280x46 mm. Half of the exposure chamber in width did not contain magnets. The typical magnetic induction values were similar to that of Fig. 1A, but the induction was close to zero (only the geomagnetic field was needed to be taken into account) in the non-magnetic side of the chamber.

1.3.2 Homogeneous SMF (hoSMF) exposure

HoSMF generator was similar to #16 in [László et al., 2007], just the magnetization of the single-block ferrite magnets was different. The average homogeneous SMF value in the exposure chamber was 145±5 mT.

HoSMF&sham in this arrangement contained similar magnets than in hoSMF, but the size of the ferrous housing of the chamber was double, as in inhSMF&sham. The magnetic induction value in the non-magnetic side was close to zero (geomagnetic field).

Double hoSMF also contained ferrite block magnets and was double size in width as in hoSMF&sham, but the full length of the exposure chamber was equipped with ferrite block magnets on both sides. The magnetic induction was again 145±5 mT on both sides.

The above description of the inhSMF and hoSMF arrangements complies the requirements Colbert *et al.* [Colbert et al., 2009] proposed for standardization.

The exposure chambers allowed us to insert either a 140x140x46 mm (cage1) or a 140x280x46 mm (cage2) perforated Plexiglas animal cage with air holes into the exposure chambers (see Fig. 1). An air-permeable opaque material covered the cage on 4 sides to provide illumination conditions similar in the exposure chamber and in the sham experiment. Only the front side of the cages was transparent to visible light. The support under the cage was always flat and stable. In the *sham only* arrangement we used a chamber that looked exactly like the hoSMF generator but contained no magnets, i.e., there was no magnetic induction but the geomagnetic field. In double-length chamber arrangements (inhSMF&sham and hoSMF&sham) one side of the exposure chamber was considered inhSMF or hoSMF, the other sham, in case of the double hoSMF, both sides were hoSMF. Experiments were recorded by a high resolution video camera (PowerShot G9, Canon Inc., Tokyo, Japan). The numerical evaluation of the results was carried out on the basis of these video clips, where SMF- and sham-exposure systems were disguised thus providing double blinding manner.

The exposure was whole body, while animals were free to move around in the cage. InhSMF exposure was shown not to introduce significant changes in the locomotor activity or anxiety behavior of animals [László et al., 2009].

The lighting conditions inside the cage were basically independent of the location of the mouse within the cage. A light gradient only occurred between the (transparent) front and the (opaque) back side of the cage. The arrangement was illuminated with halogenous lights from above during the experiments. The shaded area in the back was always bigger than 94x140 or 94x280 mm during the experiments. The halogenous lamps generated a scattered light in the shaded area of the cage between 3.9-10.6 lx.

Background noise was also measured; it never exceeded 52 dBC in the animal laboratory throughout the experiments. No site difference was found between the left and right sides of different cage configurations used.

1.4 Monitoring of locomotion

Locomotion was monitored in case of inhSMF&sham by measuring durations of staying in one or in the other (sham or inhSMF) side of the cage. The total durations were then compared. Two animals were in the cage at the same time, one of which received i.p. acetic acid according to the writhing test. One of the animals was marked (in a randomized order in a series). The inhSMF and the sham side of the inhSMF&sham chamber were also swapped between experiments in a

randomized order, because of the potential, but not verified lateral differences in lighting and noise amplitudes.

1.5 Statistics

We defined the measure of effectivity of a treatment (inhibition or effect) in percent as $M = 100\left(1-\langle x\rangle / \langle y\rangle\right)$, where $\langle x\rangle$ is the average number of a quantity in the treated group and $\langle y\rangle$ is that in the compared (most often control) group. When calculating the inhibition of pain response (I) in a similar form, the examined quantities were the numbers of writhings of exposed $\langle x\rangle$ and control $\langle y\rangle$ animals. The measure of effectivity and the inhibition of pain response are positive numbers. Beside M and I, we also estimated the F value, the critical value of F (F_{critical}), the p value, and the η^2 value by one-way ANOVA. In an expression $F_a=b$, $a=F_{\text{critical}}$, and $b=F$. We accepted statistical significance at the 95% confidence level, if $p<0.05$.

RESULTS

1. Effect of magnetic field exposure and cage size on response I (%) upon acute peritoneal pain in mice

Writhing number comparison between inhSMF/hoSMF and sham exposed groups (Group 1s vs 1i; Group 2s vs 2h; Group 3s vs 3h)

To analyze the sham to inhSMF or to hoSMF side preference and possible pain-induced locomotion of animals, a daily control arrangement was provided also in order to testify and validate the previously described antinociceptive effect of inhSMF or hoSMF [László et al., 2007]. Applying inhSMF (Group 1s *vs* 1i): the difference in the writhing numbers between unexposed and inhSMF exposed groups was statistically significant. The average writhing numbers decreased from 6±1.53, 36±2.08, 21±1.73, and 63±5.13 to 1.67±0.88, 10.33±1.20, 11.67±1.45, and 23.67±1.20 in the 0-5, 6-20, 21-30, and 0-30 min periods, respectively. This represents I_{inh}=62% for the total 30 min period of time, see Fig. 2. Table 1 shows the detailed results for all situations in this point 1.

Furthermore, in the hoSMF exposure situation in the short cage (Group 2s *vs* 2h): I_{ho}=64%, and in double-sized cage (Group 3s *vs* 3h): I=60% (see Table 1 and Fig. 2). By combining fresh data regarding hoSMF exposure conditions with those from the literature [Bao et al., 2006; Okano and Ohkubo, 2006] we obtain the result shown in Fig. 3. The dominant component of the magnetic induction in the 20-450 mT range was dorsal-ventral, in the 3 T case (clinical MRI) it was cranial-caudal.

Writhing number comparison between inhSMF/hoSMF and sham sides of double-sized cage (cage2) (Group 4h; Group 5i)

The double-sized cage was suitable to look for differences in the writhing number between the sides: sham and SMF of inhSMF&sham and of hoSMF&sham. Group 5i (inhSMF) showed I_{inh}=75%. For hoSMF exposure Group 4h resulted I_{ho}=81% (see Fig. 4).

Effect of cage size on writhing numbers (Group 1s vs 6s; Group 2h vs 3h)

If we compared the writhing numbers of challenged mice (Group 1s *vs* 6s) in the sham cages (short cage *vs* sham side of double-sized cage), we found no significant difference (I=9%). The same holds for challenged mice exposed to hoSMF (Group 2h *vs* 3h, I=1%) (see Fig. 5).

Exclusive effect of magnetic field gradients (Group 1i vs 5i; Group 2h vs 4h; Group 3h vs 4h; pooled Group 4h and 5i vs pooled 1s and 2s)

Furthermore, animals in the magnetic side of inhSMF&sham (Group 5i) showed significantly fewer pain syndromes than those in short cage under inhSMF exposure (Group 1i). This effect was demonstrated by I_{inh}=68% (see Table 1).

The situation is similar under hoSMF exposure (Group 2h *vs* 4h). The effect is I_{ho}=91% (Group 4h), see Fig. 6. However, in this case we can also compare Group 3h to 4h: I_{ho}=91% (see Fig. 7).

When we compared the writhing numbers of sham exposed mice in the short cage (Group 1s and 2s pooled) with those in the sham side of the double-sized cage (Group 5i and 4h pooled), we found that mice in the double-sized cage showed fewer pain responses representing I=90% (see Fig. 7).

HoSMF vs inhSMF exposure (Group 4h vs 5i)

If we compare writhing numbers of Group 5i and 4h, we can see the differences in the homogeneity of SMF. The inhibition of the hoSMF exposure (Group 4h) was undefined (all 6 writhing numbers were zero), 83%, 78%, and 81% (SMF *vs* sham side), while it was 67%, 64%, 64%, and 64%, respectively in Group 5i in the 0-5, 6-20, 21-30, and 0-30 min periods. The inhSMF exposure condition was more beneficial in inhibiting pain: I_{inh}=78% (see Fig. 8).

2. Effect of magnetic field exposure (M) on locomotion and social behavior in mice with acute peritoneal pain (control group vs challenged animals exposed to inhSMF&sham)

Table 2 shows the results concerning locomotion of this experiment in inhSMF&sham with double-sized cage (*control vs challenged animals exposed to inhSMF&sham*). No significant differences between the groups could be identified.

In the control group, 5 out of 9 mice spent more time in the inhSMF side of the inhSMF&sham arrangement than in the sham side, in the acetic acid-challenged group just opposite; 5 out of 9 mice spent more time in the sham side. The difference was not significant.

Unchallenged mice crossed the boundary between the two sides 632 times, while challenged mice did this 551 times. The difference was not significant.

Then we looked for the differences in every 10 min interval. In the first 10 min, when mice make exploratory ambulation in general, the challenged mice spent significantly more time in the sham side, than in the other (M_{inh}=21%, p<0.01). In the second 10 min, when mice started to adapt to their new environment, the unchallenged mice spent significantly more

time in the inhSMF side, than in the other (M_{inh}=42%, p<0.005). Comparing the 10 min intervals to each other, we realized that the first 10 min made the difference. Excluding this exploratory 10 min period of the estimate, we found that the only significant difference was that unchallenged mice spent more time in the inhSMF side, than in the other (M=24%, p<0.05).

We can confirm the fact that mice are socially sensitive, since mice spent considerably longer times together, i.e., in the same side of cage2, than separately (*control vs challenged animals exposed to inhSMF&sham*). Almost 19 out of 30 min mice were together representing M_{inh}=38%, $F_{4.49}$=12.51, p<0.003, η^2=44%.

DISCUSSION

Establishing daily control in accordance with previous models

Both the experimentally applied hoSMF and inhSMF arrangements (inhSMF, hoSMF, and double hoSMF) resulted in a pain inhibition of above 60% in challenged mice. Although animal numbers were small in some groups (e.g., Group 1i), the results of the inhSMF measurements are in accordance with previous measurements performed with the optimal cylindrical high-grade NdFeB magnets with alternating polarity and magnetic coupling between the upper and lower array of magnets [Gyires et al., 2008; László et al., 2007]. However, the calculated inhibition in our current experiments was found to be lower compared to previous ones with similar magnets and treatment (62% *vs* 79%). This observed difference is most likely due to the small number of treated animals in the present experiments. The writhing numbers (average ± standard error of the mean) in the total 30 min period of sham treated animals subsequent to i.p. acetic acid challenge in the previous experiments were 71±1 [László et al., 2007] or 71±5 [Gyires et al., 2008], and in the current tests they were 63±5. These are comparable with each other indicating that acetic acid treatment resulted in a pain response with similar magnitude regardless the variability of individual animals. HoSMF was found to be effective at a similar rate: pain inhibitory effect resulted in 64% and 60% merit in the small and in the double-sized cages, respectively. Few literature data are accessible on the behavioral effects of hoSMF on awake integer animals: applying 3 T homogeneous SMF on mice resulted in 68±2% antinociceptive activity in a similar pain essay [László and Gyires, 2009]. This previous average value is in accordance with our results, but the average magnetic induction in case of the applied ferrite block magnets is one magnitude below the one used in the cited experiment, and the direction of the main component of SMF is also 90^{o} different. This difference suggests a threshold induction value of hoSMF for effective antinociceptive response over which the response is constant or saturated as shown in Fig. 3.

Locomotion

Mice were free to move in the double-sized cage2 allowing them to choose their resting place on their own. In contrast to rats exposed to 7 T magnetic induction [Houpt et al., 2003] mice in our experiments showed no behavioral signs of aversion against SMF. A possible physiological mechanism behind circling and the development of taste aversion in rats subsequent to SMF exposure could be the vestibular activation [Cason et al., 2009]. The

proper characterization of advantageous and eventually harmful biological effects of the applied SMF has become an important issue since the widespread utilization of magnetic resonance imaging (MRI) devices. Magnetic induction typically varies between 0.5 and 3 T for human diagnostic MRI, for research and experimental purposes it can be up to or even over 10 T. According to guidelines from e.g. the U.S. Food and Drug Administration 8 T is the highest magnetic induction without significant risk to human health [Atkinson et al., 2007]. Based on our findings it is highly probable that moderate inhSMF or hoSMF arrangements in our experiments do not result in significant vestibular excitation. Unchallenged animals spent significantly more time in the inhSMF/hoSMF side of cage2 (p<0.04) supporting that neither SMF was aversive. Subsequent to the verification that SMF was not aversive; our next aim was to test whether the exposure was preferentially chosen by the challenged mice. In our visceral pain essay challenged mice spent approximately the same time in the inhSMF/hoSMF and in the sham side of cage2, there was no significant site preference for the challenged animals. During the total 30 min duration of a single exposure to inhSMF/hoSMF it is unlikely that conditioned learning has arisen: several hours are required to develop conditional taste aversion in mice [Lockwood et al., 2003]. Mice in our measurements failed to recognize the beneficial effects of inhSMF/hoSMF within the 30 min time limit of the experiments; the challenged group did not prefer either SMF to sham. When introduced to a new environment animals started to explore their novel cage. This exploratory activity usually takes several minutes, and can be quantified based on the numbers of exploratory rearings [Strekalova et al., 2004]. This activity of both challenged and unchallenged mice was supported by our observations; the analysis of separated 10 min intervals (0-10, 11-20, and 21-30 min) revealed that the behavior of the animals during the first 10 min of the experiment is mainly governed by their random exploratory motions. No significant difference was found when cumulative 0-20 min data were compared with 0-30 min cumulative data on time spent in the inhSMF/hoSMF and in the sham side. A possible explanation of the lack of inhSMF/hoSMF preference of challenged mice was that crossing the boundary (with a strong lateral gradient of inhSMF/hoSMF) caused enough pain inhibition, staying or moving in the inhSMF/hoSMF side did not provide excess analgesia.

Effect of cage size

According to our measurement data behavioral pattern and pain response (number of writhings) of challenged animals in the small cage and in the double-sized cage did not differ significantly when hoSMF or inhSMF was applied. Stress and aggression is not only

dependent on the area per animal, but also on the number of animals in the same cage. Other authors reported an increased number of agonistic encounters below 125 cm^2 area per mouse, when 3-8 mice were accommodated in the same cage [Van Loo et al., 2001]. In our experiments the area per mouse in case of the double-sized cage varied between 130-196 cm^2 and 2 or 3 animals were simultaneously in the cage. Agonistic encounters were not observed during the 30 min of experiments. This observation together with the similar numbers of writhing excludes the stressor role of the small cage.

Effect of social behavior

Regarding the previous paragraph, no agonistic encounters, but rather social cooperation was recordable between unchallenged and challenged mice in the double-sized cage. Mice spent almost 2/3 of the total experimental period together (either in the sham or inhSMF/hoSMF side of double-sized cage), which is supported by other observations applying the same, with 0.9% acetic acid induced visceral pain essay [Langford et al., 2006]. As a proof of empathy based on visual perception, cage mates recognize the pain behavior of each other mutually. If both mates were challenged with similar noxious stimuli, then the behavioral response of one animal tended to imitiate that of the other. Because in our experiments only one mouse was challenged inside the cage at a time, the only applicable measure of cooperative behavior was the time spent together. Unchallenged mice did not perform writhing behavior and they most likely did not influence the response of the challenged ones.

Cross interactions

As previously concluded, mice did not recognize the antinociceptive effect of inhSMF/hoSMF; they did not prefer to spend more time exposed to inhSMF/hoSMF. For this reason the effectivity of inhSMF/hoSMF was analyzed comparing the numbers of writhing as being more objective signs of pain response. Significantly less pain syndromes were observed in mice being on either the hoSMF or the inhSMF side of double-sized cage compared to the sham side. This is in agreement with previous data, where pain responses were monitored only in cage1 either entirely exposed to inhSMF/hoSMF or keeping it as sham [Gyires et al., 2008].

Exclusive effect of SMF gradients

In order to understand whether inhSMF/hoSMF themselves or the spatial gradients of the inhSMF/hoSMF play a more important role in the antinociceptive action in visceral pain, we

performed a deeper analysis on the raw writhing data. Keeping in mind that cage size had no effect on visceral pain responses, animals in double-sized cage were free to cross the sham-inhSMF boundary in the inhSMF&sham arrangement and the sham-hoSMF boundary in the hoSMF&sham setup. Comparing the numbers of writhing in the sham side of double-sized cage to those in the small cage under sham conditions and the writhing in the inhSMF side of double-sized cage to those observed in inhSMF with small cage, significantly less pain responses were found in the double-sized cage. A plausible explanation of this finding is that it was not the magnetic field itself, but its gradient that was more important in analgesia. The same stands for the experiments in hoSMF, if we compare the analgesic action of mice in small cage exposed to hoSMF to mice in double-sized cage exposed to hoSMF&sham. Furthermore, cross-checking pain reactions in double-sized cage with hoSMF&sham with double hoSMF (where the hoSMF covers the entire cage area) the results were identical: analgesic effect was more prominent, where only half of the cage was exposed to hoSMF and mice were not restricted in motion. Time varying magnetic field gradients were shown to perform analgesic effects which could be enhanced by chemicals in rats [Martin and Persinger, 2004]. Crossing over the inhSMF/hoSMF boundary elicits similar physical effects to an Extremely Low Frequency (ELF) magnetic field on a static object [László, 2011]. ELF fields, where the frequency of magnetic induction variation is typically several Hz possess contradictory physiological effects on animals: they can induce opioid-peptide mediated analgesia in land snails [Bao et al., 2006; Prato et al., 2000] and mice [Del Seppia et al., 2007]. However, these conclusions need extra caution due to the small amount of animals taken into analysis.

HoSMF vs inhSMF exposure

Former research on optimizing SMF for analgesic therapy suggests that application of an inhSMF is preferable to a hoSMF [László et al., 2007]. Our data analysis supports this hypothesis; the inhSMF exposure condition resulted in stronger pain inhibition.

Aversiveness to moderate SMF exposure

Short term (5-30 min) exposure to strong SMF instantaneously triggers taste aversion in rats [Houpt et al., 2003]. The underlying behavioral and molecular processes are in focus of scientific interest, but many parameters are currently unclear. In several brainstem regions of rats exposed to such SMF, c-Fos induction occurred parallel to the taste aversion [Snyder et al., 2000] possibly underlining its role as a mediator. Experiments in deer mice revealed that

the conditional taste aversion is not instantaneous [Choleris et al., 2000]. Sexual steroids are also thought to play a role in the long term persistence of taste aversion: in ovariectomized rats taste aversion was found to be more prominent; however, estrogen replacement eliminated the aversive behavior. Male rats acquired a stronger initial aversion but extinguished faster than females [Cason et al., 2006]. Repeated exposure to SMF attenuates this biological response possibly due to habituation to vestibular perturbation subsequent to the exposure [Houpt et al., 2010]. The aversive response is abolished by labyrinthectomy, which further supports the possibility of vestibular stimulus upon SMF exposure [Cason et al., 2009]. Mice were similarly treated with high SMF resulting in similar aversive responses to those of rats [Lockwood et al., 2003]. One main aim of the present study was to analyze the aversiveness of the applied moderate inhSMF/hoSMF, whereas previous measurements [László et al., 2007] were performed under steady inhSMF/hoSMF or sham exposure condition without the possibility of free choice of resting position for the animals. In case of a prompt vestibular activation, aversive behavior and sham site preference would have been expected if the animals had been able to move freely across inhSMF/hoSMF and sham exposed sides of cages as used in the present experiment. This aversiveness might compete with the antinociceptive effect of the inhSMF/hoSMF, but the cognitive performance of mice can prevent the rapid realization of this beneficial effect of inhSMF/hoSMF. Another possibility that explains the absence of aversiveness might be due to the complex stimulation of the brain resulting in either sedative or anxiogenic activity. Anxiogenic effect of chronic exposition to ELF was observed in rats [Liu et al., 2008]. To exclude the possible anxiogenic or sedative effect of the moderate inhSMF/hoSMF exposure applied in the present study further experiments are necessary.

CONCLUSIONS

In the present series of experiments we proved that neither inhomogeneous, nor homogeneous static magnetic field (SMF) exposure in the applied range was aversive to mice. We realized that it was the exposure to the spatial gradient of the SMF that caused the pain inhibition in the writhing test rather than the exposure to the magnetic field itself. We confirmed that mice were socially sensitive, mice preferred to spend more time in the same side of the cage. We conclude that conditioning of mice with the applied short term SMF exposure is not possible; the cognitive perception of analgesia and inhomogeneous or homogeneous SMF site preference was not developed.

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CAPTIONS

Fig. 1 Panel A and C demonstrate line-scanned magnetic induction maps from an area of 40x40 mm in the isocenter of the magnetic arrangements in a distance of 3 mm from the magnets' surface. Panel A shows the inhomogeneous static magnetic field (SMF), Panel C shows the homogeneous SMF. Panel B and D show the schematics of SMF generators and animal cages. Panel B shows inhomogeneous SMF arrangements, Panel D shows homogeneous SMF ones. These panels are not in scale. The generators contain parallel ferrous plates for the magnets below and above the cage. While the ferrous house is shown in grey, magnets are white with a white or black top distinguishing between magnetic North and South poles.

Fig. 2 Number of writhings in the 30 min long writhing test in mice. SMF stands for static magnetic field (exposure), *n* is animal number in the group. Sham and inhomogeneous SMF-exposed single-sized cage1 (Group 1s, white and 1i, black), sham and homogeneous SMF-exposed single-sized cage1 (Group 2s, light grey and 2h, grey) and sham and homogeneous SMF-exposed sides of double-sized cage2 (Group 3s, squared and 3h, dense squared) are compared. Statistical significance relates to the corresponding sham-exposed control group, calculation was made by one-way ANOVA at 95% confidence level. Positive error bars represent standard error of the mean values.

Fig. 3 Inhibition of pain (*I* in %) induced by homogeneous static magnetic field (hoSMF) exposure in the writhing test in mice. The dominant component of the magnetic induction in the 20-450 mT range was dorso-ventral, in the 3 T case (clinical MRI) it was cranial-caudal or dextro-sinistral. Linear solid and dotted sections between marks and error bars guide the eye only. Error bars represent standard error of the mean values.

Fig. 4 Number of writhings of challenged mice in the sham and SMF-exposed sides of double-sized cage2 are compared in Group 5i (inhomogeneous SMF, white *vs.* black) and Group 4h (homogeneous SMF, light grey *vs.* grey).

Fig. 5 Effect of cage size: number of writhings of challenged mice in sham and homogeneous SMF-exposed single-sized cage1 (Group 1s, white and 6s, light grey) compared to that of sham and hoSMF sides of double-sized cage2 (Group 2h, grey and 3h, black).

Fig. 6 Exclusive effect of magnetic field gradients: number of writhings of challenged mice in the inhomogeneous (Group 1i, black) and homogeneous (Group 2h, light grey) SMF-exposed single-sized cage1 *vs.* number of writhings in the inhomogeneous (Group 5i, white) and homogeneous (Group 4h, grey) SMF-exposed sides of double-sized cage2 (Group 2h and 3h).

Fig. 7 Exclusive effect of magnetic field gradients (cont.): number of writhings of challenged mice in double homogeneous SMF-exposed double-sized cage2 (Group 3h, white) was compared to that of in SMF-exposed side of double-sized cage2 (Group 4h, black). Comparison of pooled writhing numbers of sham-exposed challenged mice in single-sized cage1 (Group 1s and 2s) and that of in the sham side of double-sized cage2 (Group 4h and 5i) is represented by the dotted grey bars.

Fig. 8 Homogeneous *vs.* inhomogeneous exposure: number of writhings of challenged mice in homogeneous (Group 4h, white) and inhomogeneous (Group 5i, black) SMF-exposed sides of double-sized cage2.

Table 1 Overview of results concerning the full, 30 min long writhing test in mice in different cages and under different exposure conditions (Animal groups 1-5, the letters indicate the exposure conditions as follows; s: sham, i: inhomogeneus static magnetic field, h: homogeneous static magnetic field). Intraperitoneal injection of 0.2 ml/mouse acetic acid was used to exert the acute visceral pain. *I* (%) stands for inhibition of pain.

Table 2 The statistical characteristics of time periods (s) mice spent in the static magnetic field (SMF side) or in the other side (sham side) of the animal cage protruding to SMF. Challenged mice received 0.2 ml/mouse acetic acid intraperitoneally at time point 0 unchallenged mice received physiological salt solution. *M* (%) stands for the measure of effectivity in percent. No significant differences between sides could be identified.

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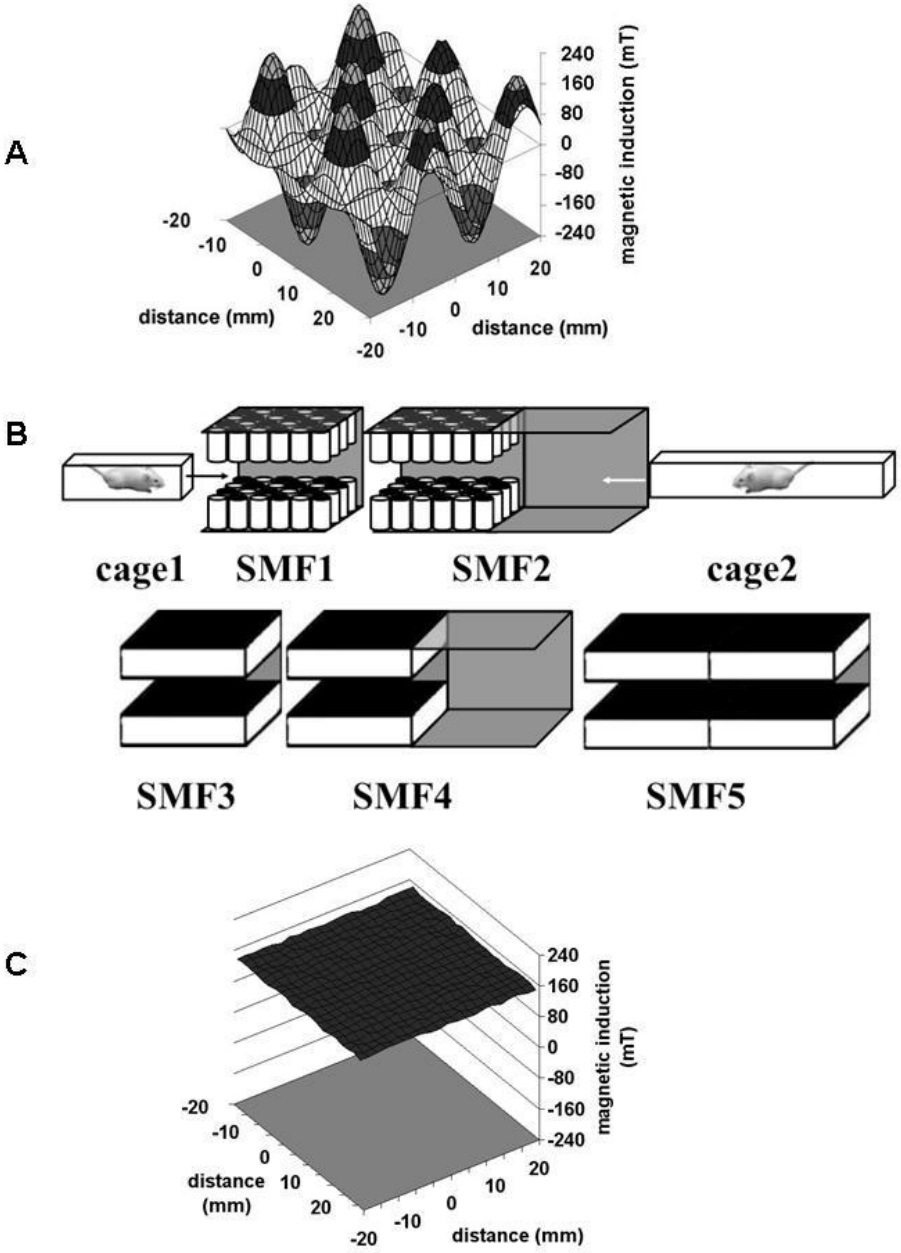
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0
-80
-160
-240
magnetic induction (mT)
-20
-10
0
10
20
distance (mm)
B
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SMF1
SMF2
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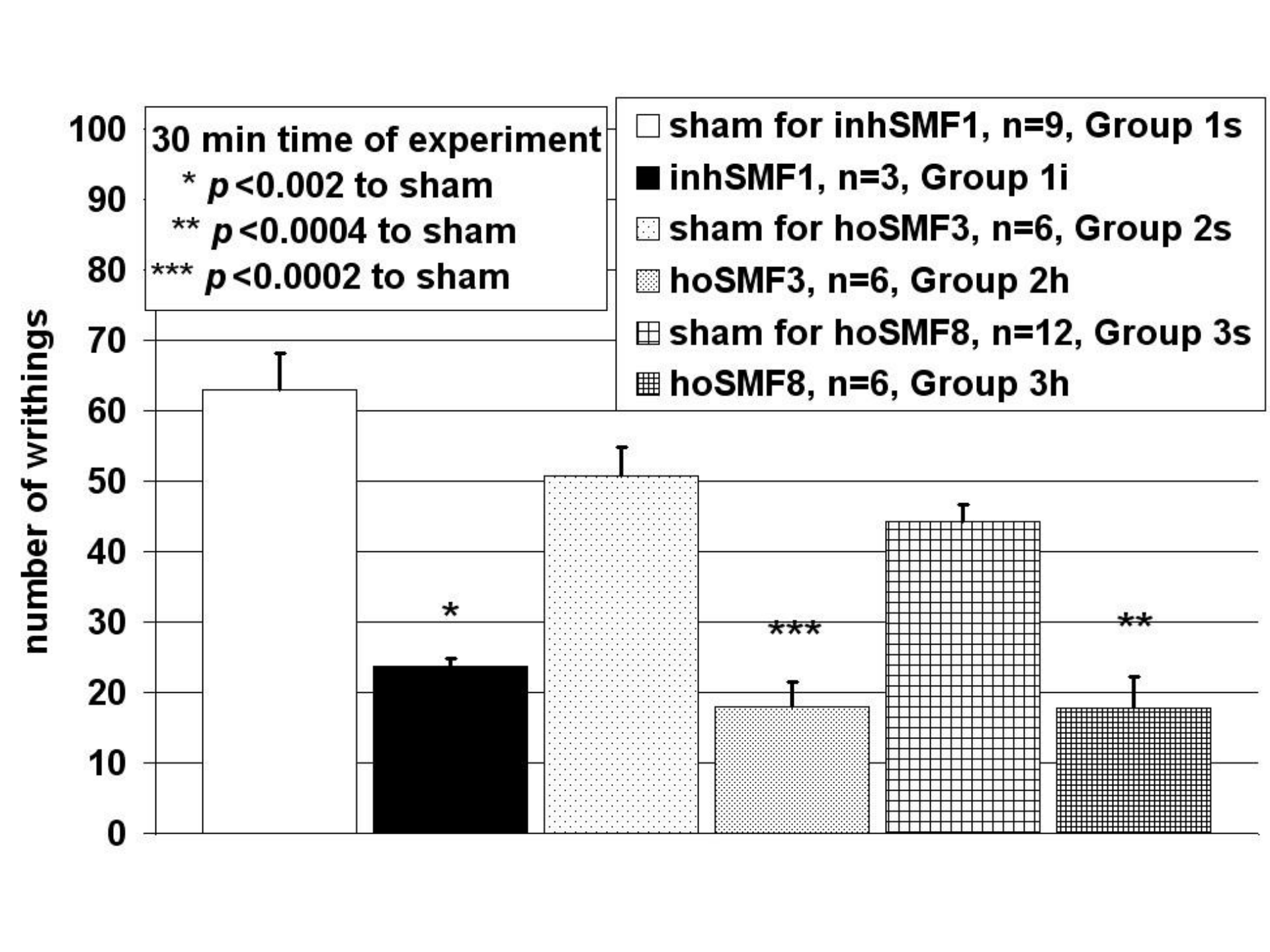
SMF4
SMF5
C
magnetic induction (mT)
distance (mm)


30 min time of experiment
* p <0.002 to sham
** p <0.0004 to sham
*** p <0.0002 to sham
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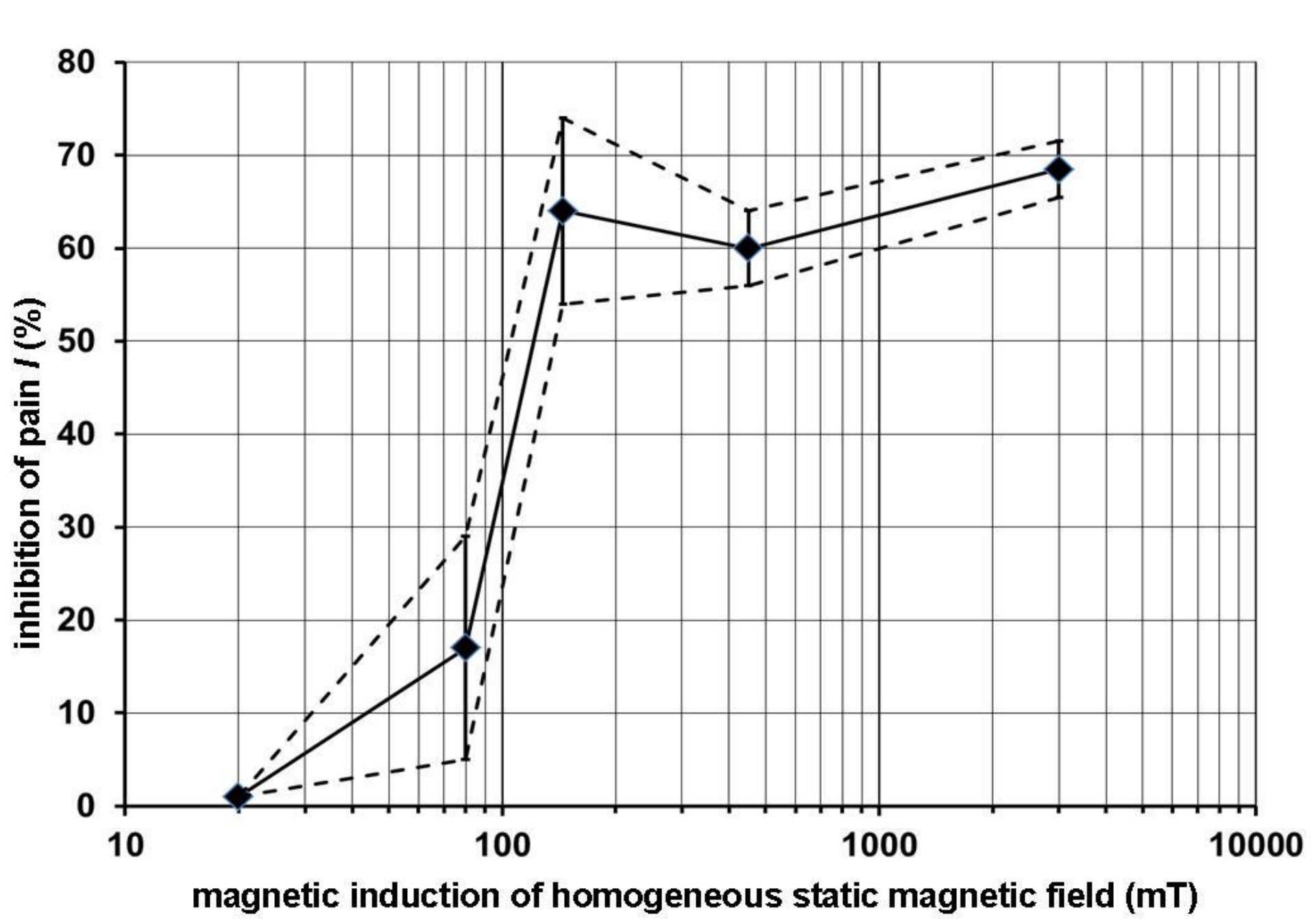
inhSMF1, n=3, Group 1i
sham for hoSMF3, n=6, Group 2s
hoSMF3, n=6, Group 2h
sham for hoSMF8, n=12, Group 3s
hoSMF8, n=6, Group 3h
number of writhings
100
90
80
70
60
50
40
30
20
10
0
*
***
**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70
60
50
40
30
20
10
0
inhibition of pain I (%)
10
100
1000
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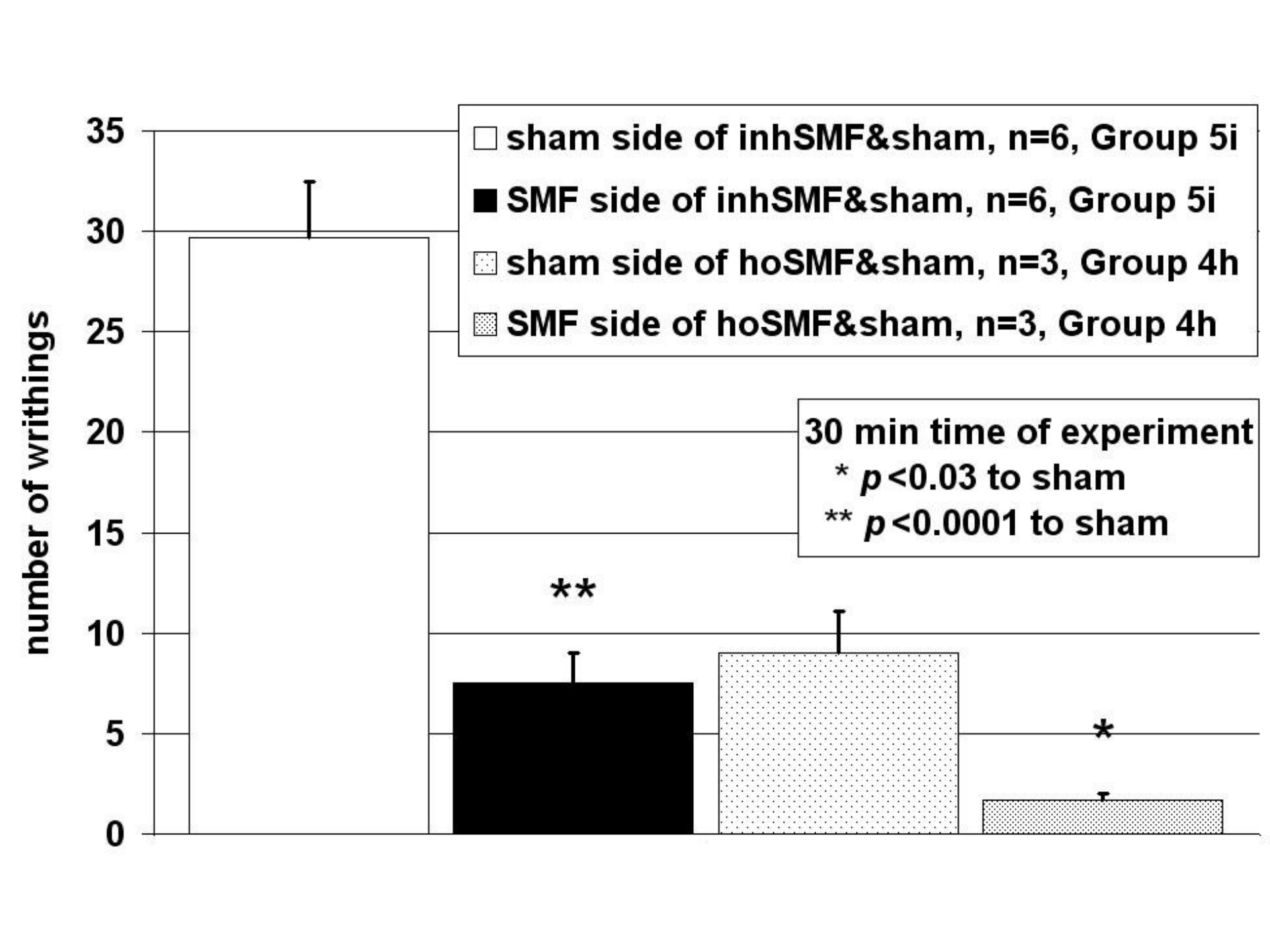
magnetic induction of homogeneous static magnetic field (mT)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SMF side of inhSMF&sham, n=6, Group 5i
sham side of hoSMF&sham, n=3, Group 4h
SMF side of hoSMF&sham, n=3, Group 4h
30 min time of experiment
* p<0.03 to sham
** p<0.0001 to sham
number of writhings
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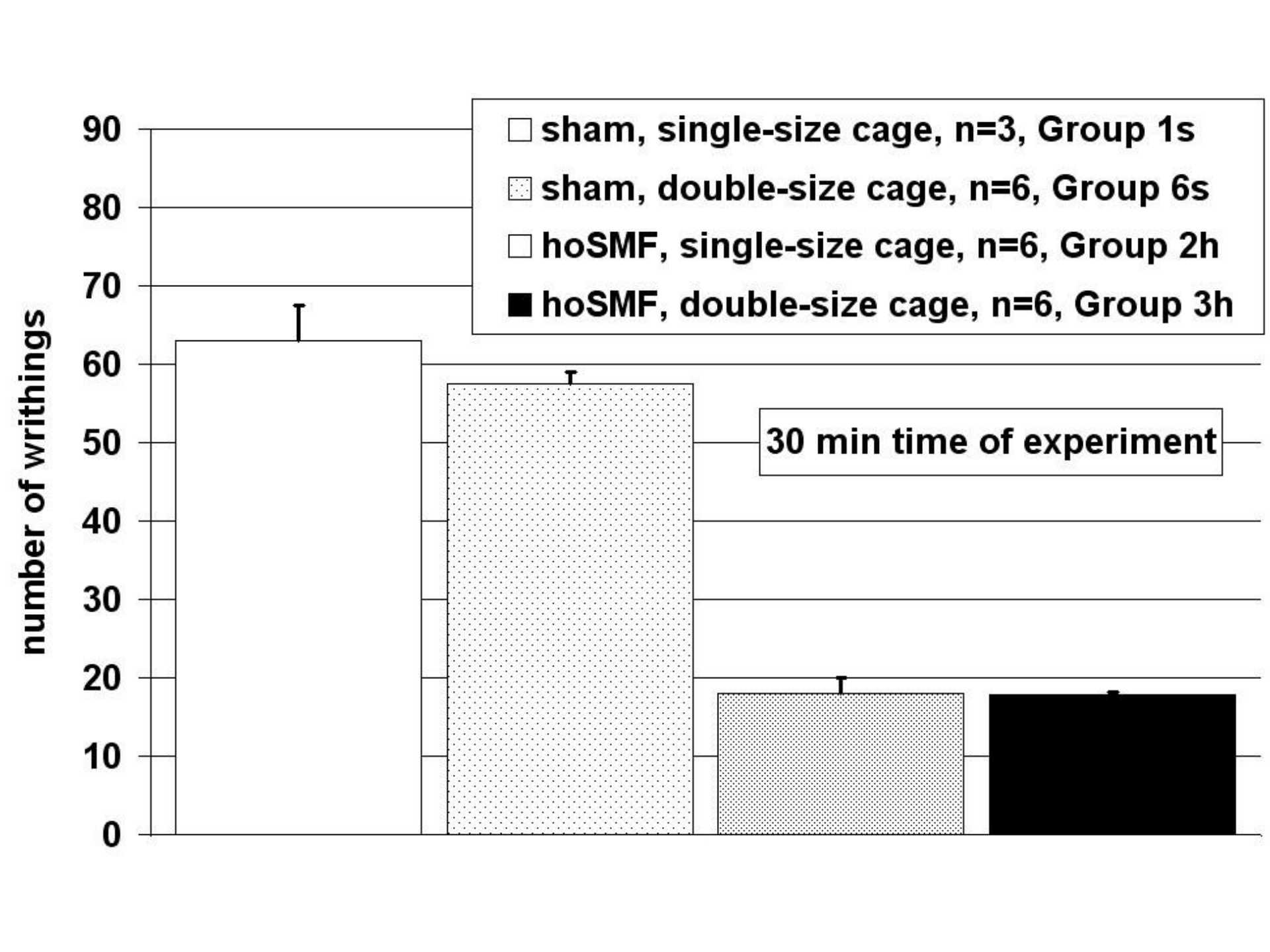
30
25
20
15
10
5
0
**
*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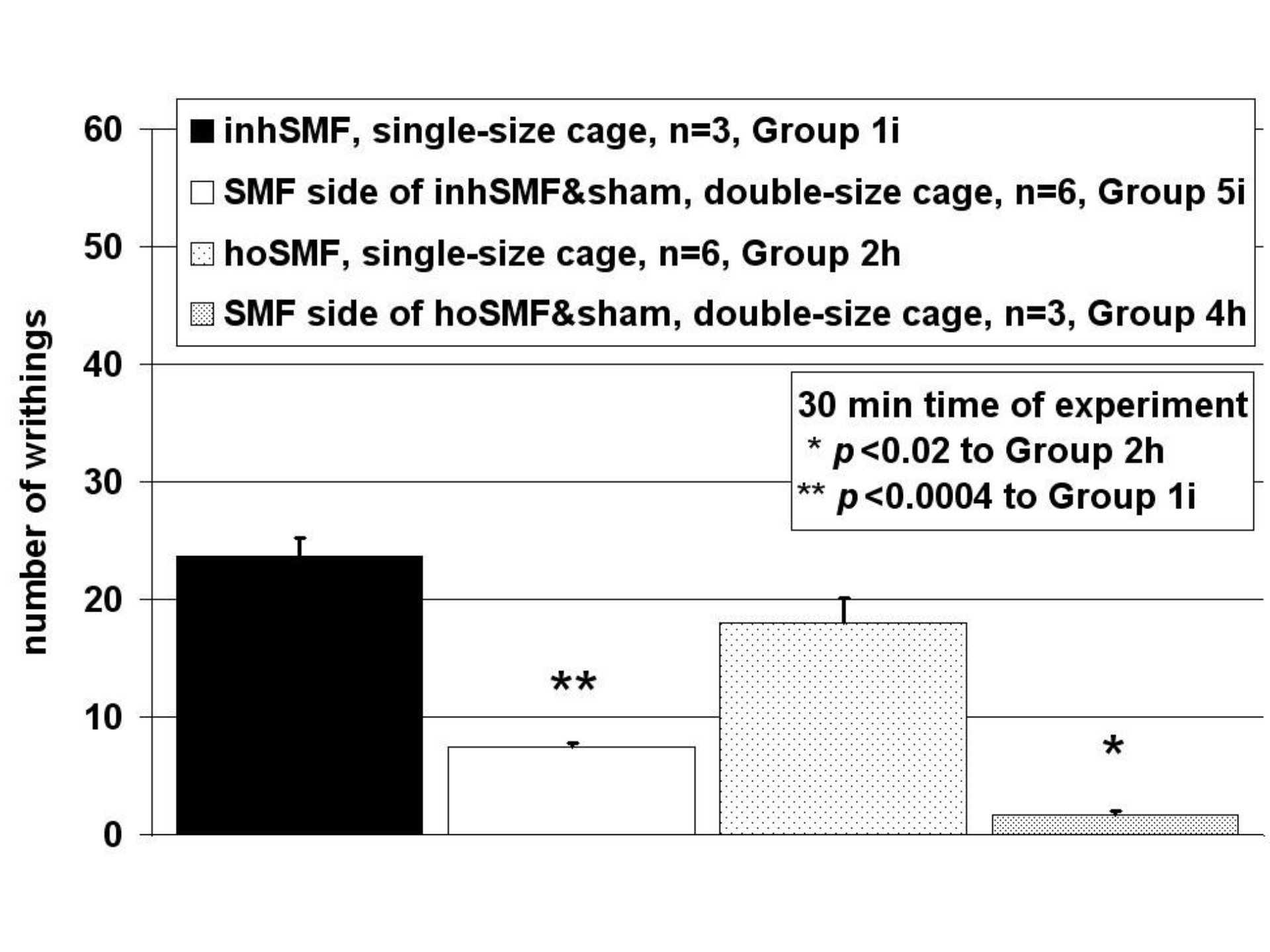
sham, double-size cage, n=6, Group 6s
hoSMF, single-size cage, n=6, Group 2h
hoSMF, double-size cage, n=6, Group 3h
30 min time of experiment
number of writhings
90
80
70
60
50
40
30
20
10
0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SMF side of inhSMF&sham, double-size cage, n=6, Group 5i
hoSMF, single-size cage, n=6, Group 2h
SMF side of hoSMF&sham, double-size cage, n=3, Group 4h
30 min time of experiment
* p<0.02 to Group 2h
** p<0.0004 to Group 1i
number of writhings
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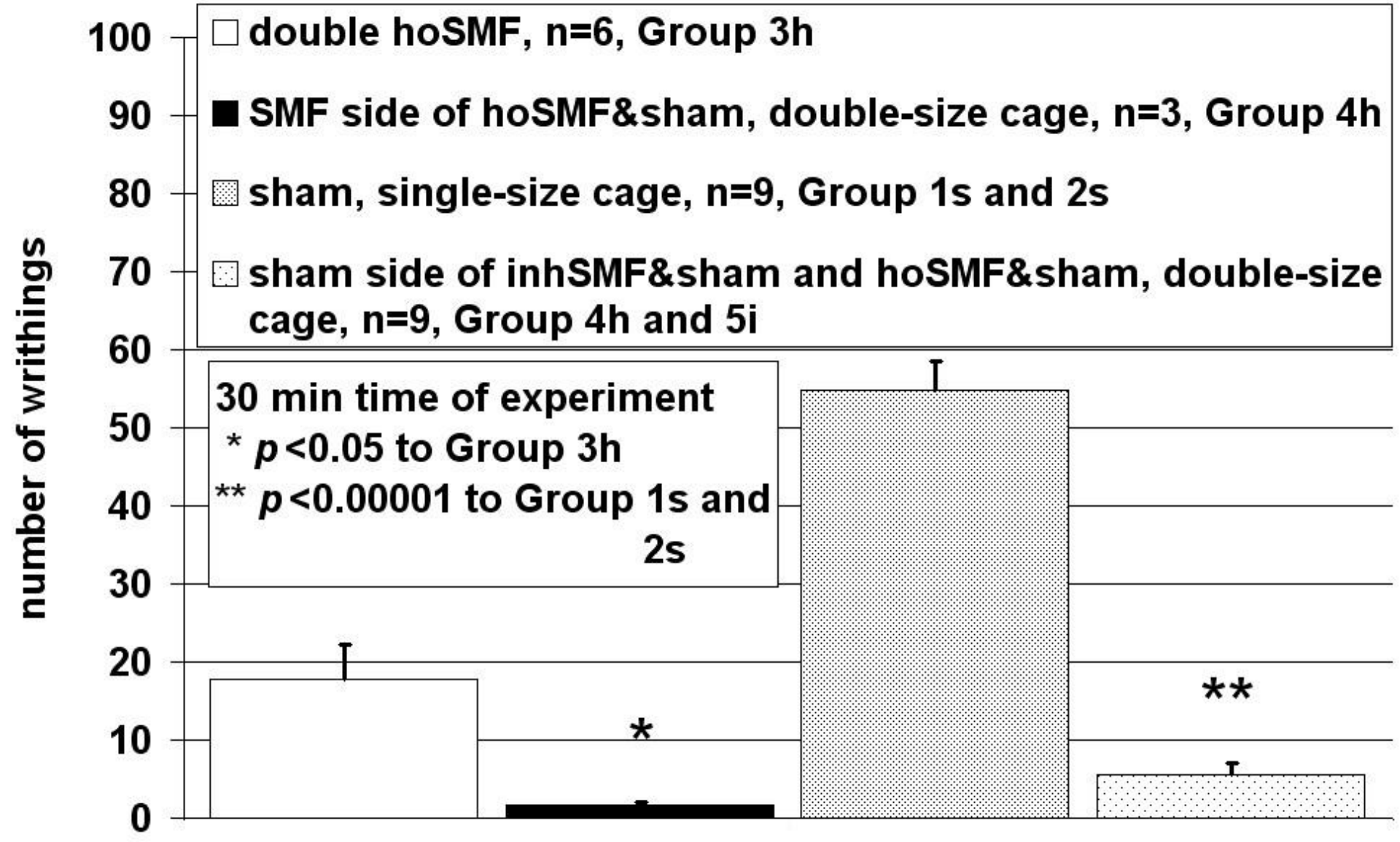
50
40
30
20
10
0
**
*


double hoSMF, n=6, Group 3h
SMF side of hoSMF&sham, double-size cage, n=3, Group 4h
sham, single-size cage, n=9, Group 1s and 2s
sham side of inhSMF&sham and hoSMF&sham, double-size cage, n=9, Group 4h and 5i
30 min time of experiment
* p<0.05 to Group 3h
** p<0.00001 to Group 1s and 2s
number of writhings
100
90
80
70
60
50
40
30
20
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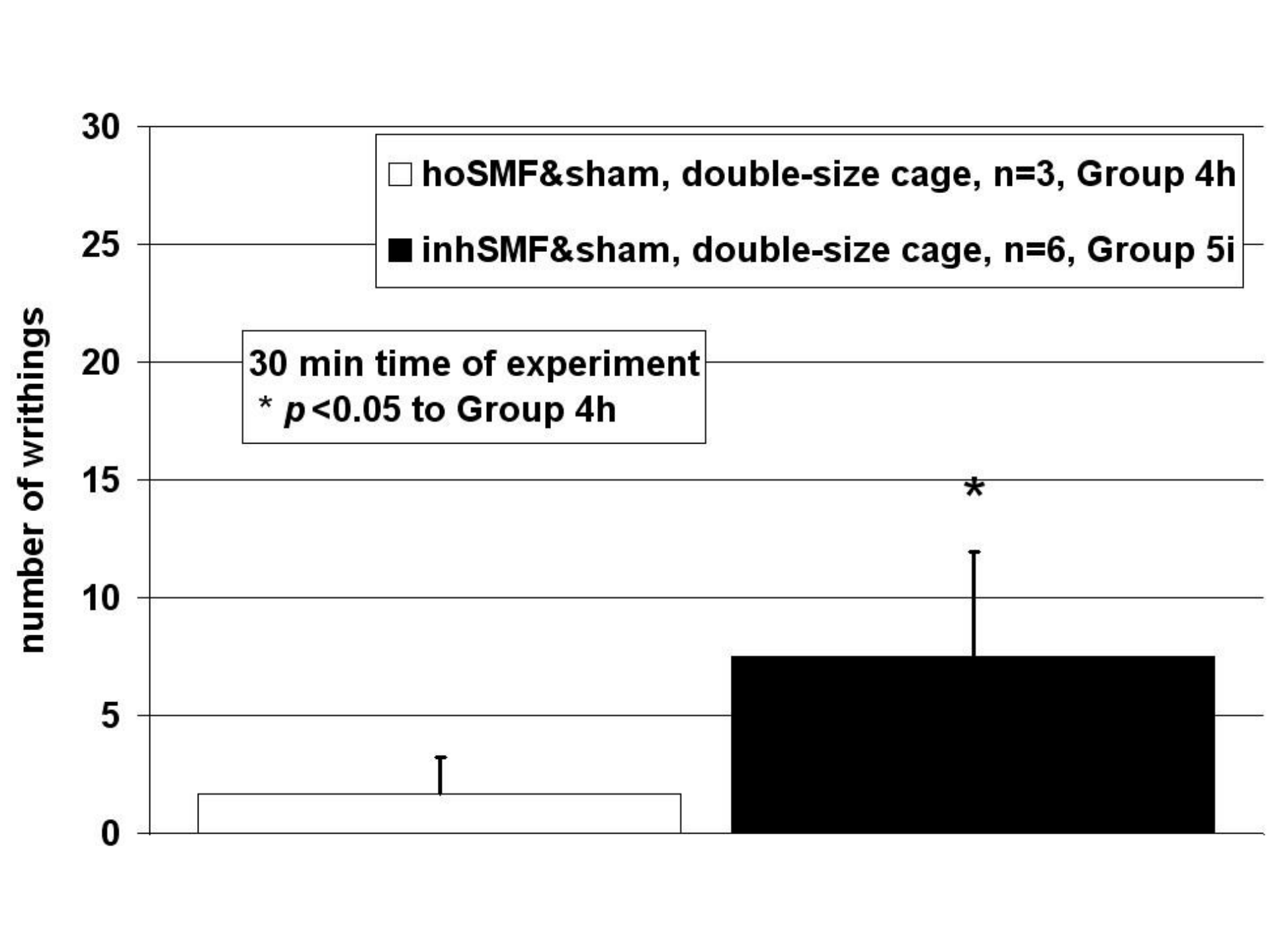
30
25
20
15
10
5
0
number of writhings
□ hoSMF&sham, double-size cage, n=3, Group 4h
■ inhSMF&sham, double-size cage, n=6, Group 5i
30 min time of experiment
* p <0.05 to Group 4h
*