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Binding of [^{3}H]MK-801 in subcellular fractions of *Schistosoma mansoni*: Evidence for interaction with nicotinic receptors

Renata Fittipaldi Pessôa, Newton Gonçalves Castro, François Noël *

Departamento de Farmacologia Básica e Clínica, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Cidade Universitária, 21941-590 Rio de Janeiro, Brazil

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Abstract

Several studies have suggested that L-glutamate is a putative neurotransmitter in *Schistosoma mansoni*. Recently, we detected the presence of low-affinity binding sites for [^{3}H]kainic acid in the heterogeneous (P_1) subcellular fraction of *S. mansoni*. In an attempt to characterize *N*-methyl-D-aspartate (NMDA) receptors in this worm, we performed binding assays with [^{3}H]MK-801, a NMDA non-competitive antagonist, in the P_1 fraction of adult *S. mansoni*. In competition experiments, MK-801 (IC_{50} ~200 μM) and ketamine (IC_{50} ~500 μM) exhibited a low affinity for the sites labeled with [^{3}H]MK-801. Along with the lack of modulation of this binding by glutamatergic agonists and antagonists and the absence of stereoselectivity for MK-801 isomers, these results suggest that [^{3}H]MK-801 could label a site different from the classical NMDA receptor in *S. mansoni*. Based on the evidences that MK-801 interacts with mammalian muscle and central nervous system nicotinic receptors as a low-affinity noncompetitive antagonist, we have investigated the effects of MK-801 on the nicotine-induced flaccid paralysis of the worm, in vivo. The motility of *S. mansoni* was quantified by image analysis through a measure of displacement of the worm's extremities. In the presence of (−)-nicotine (10–100 μM), we observed an immediate paralysis of the worms, that was inhibited by 1 mM MK-801. Besides nicotine, choline (10–50 mM) was also able to inhibit the worm's motility. As a conclusion, we suggest that [^{3}H]MK-801 binds to nicotinic receptors, and not NMDA receptors, in subcellular fractions of *S. mansoni*.

Keywords: *Schistosoma mansoni*; Dizocilpine; Glutamate; Platyhelminth; MK-801; Nicotine

1. Introduction

Schistosomiasis is a public health problem in many developing countries, and *Schistosoma mansoni* is the most widespread causative parasite [1]. Control of the disease by chemotherapy has relied heavily on praziquantel, potentially allowing drug-resistant parasites to emerge [2]. The nervous system of this parasite has been viewed as a likely target of chemotherapeutic attack [3], since new chemotherapeutic agents acting specifically on the worm's neuronal signaling pathways could be selective, producing minimal side effects on the host [4]. In the last decade, the use of chemical, histochemical and immuno-histochemical techniques indicated the presence of some candidate transmitters or modulators in the nervous system of platyhelminths, including several peptides, acetylcholine, 5-HT, dopamine, GABA and glutamate [3].

In the cestode *Hymenolepis diminuta*, an intense glutamate-like immunoreactivity (Glu-IR) has been demonstrated in cell bodies and fibers of adult worms [5]. In trematodes, such immunoreactivity has been described in adult *Fasciola hepatica* [6] and in daughter sporocysts and cercariae of *Trichobilharzia ocellata* and *S. mansoni* [7]. In adult *S. mansoni*, the existence of a glutamate transporter in isolated muscle cell of adult worms [8] and the characterization of low-affinity binding sites for

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; AP5, 2-amino-5-phosphonopentanoic acid; DNQX, 6,7-dinitroquinoline-2,3-dione; EDTA, ethylenediaminetetraacetic acid; 5-HT, 5-hydroxytryptamine creatine sulfate; NMDA, *N*-methyl-D-aspartate; NMDG, *N*-methyl-D-glucamine; PMSF, phenylmethylsulfonyl fluoride; POPOP, 1,4-bis-[2-(5-phenyloxazolyl)]-benzene; PPO, 2,5-diphenyloxazole; Tris, tris(hydroxymethyl) aminomethane

* Corresponding author. Tel.: +55 21 2562 6732; fax: +55 21 2562 6732.
E-mail address: fnoel@pharma.ufrj.br (F. Noël).

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[^{3}H]kainic acid in the heterogeneous (P_1) subcellular fraction [9] provide further evidence of a glutamatergic transmission. This pathway could be involved in the control of worm motility, since kainic acid, an agonist of non-NMDA receptors, induced a major and reversible alteration of the worm motility pattern (inhibition of peristaltic contraction waves and induction of cork-screw-like coiling of the body of the worm) that was blocked by 1 mM DNQX, a non-specific antagonist at AMPA/kainate receptors [9]. Thus, glutamate is to be considered a putative neurotransmitter in *S. mansoni*, although not all classical criteria that define a neurotransmitter have been met hitherto.

Due to the important role of glutamatergic NMDA receptors in the central nervous system of vertebrates and to the suggestion of NMDA receptor homologues in the schistosome EST database [10], we decided to look for evidence of NMDA receptors in adult *S. mansoni*. For this purpose, we used binding assays with [^{3}H]MK-801 as radioligand, because this drug acts as a non-competitive antagonist of the mammalian NMDA receptor due to its binding into the channel pore [11,12] and it has been successfully used to characterize high-affinity binding sites in *Caenorhabditis elegans*, a free-living nematode [13].

2. Materials and methods

2.1. Preparation of subcellular fractions of S. mansoni

Adult male *S. mansoni* (BH strain) were obtained by perfusion of Syrian hamsters infected 6 weeks previously with approximately 400 cercariae [14]. The male and female worms were physically separated using a Pasteur pipette.

About 1500 male worms were then homogenized in a Dounce homogenizer at 4 °C in 0.25 M sucrose solution (5 mM Tris–HCl pH 7.4) using three sequences of 10 passes of the pestle. The homogenate was centrifuged to obtain four pellets (P_1, P_2, P_3, P_4) sedimenting respectively at $300 \times g_{av}$ (5 min); $1000 \times g_{av}$ (10 min); $8000 \times g_{av}$ (10 min) and $100{,}000 \times g_{av}$ (1 h). These fractions have been previously characterized by electronic microscopy as heterogeneous (P_1), nuclear (P_2), mitochondrial (P_3) and microsomal (P_4) [15].

2.2. Preparation of rat brain cerebellar membranes

Rat brains were homogenized at 4 °C in six volumes of ice-cold 0.32 M buffered sucrose (pH 7.4) per gram of organ. After centrifuging at $800 \times g_{av}$ for 10 min, the supernatant was centrifuged at $12{,}000 \times g_{av}$ for 1 h. The pellet was re-suspended in water, re-homogenized and centrifuged at $100{,}000 \times g_{av}$ for 20 min. This procedure was repeated once to obtain the final pellet re-suspended in 50 mM Tris–acetate buffer and stored at −80 °C until use. Before use, the preparation was further washed by dilution at room temperature and centrifugation.

2.3. Binding assays

P_1–P_4 fractions of *S. mansoni* (100 μg protein) were incubated for 15 min at 25 °C in 0.5 ml of 5 mM Tris–HCl buffer, pH 7.2, and 10 nM [^{3}H]MK-801 (28.9 Ci/mmol, New England Nuclear Life Science Products, USA). Bound and free [^{3}H]MK-801 were separated by rapid filtration under vacuum on glass fiber filters (GMF 3 type from Filtrak, Germany) followed by 4 ml washes with ice-cold 5 mM Tris–HCl cold buffer (pH 7.2). After drying, filters were added to a scintillation mixture [POPOP (0.1 g/l) and PPO (4.0 g/l) in toluene], and the radioactivity was measured with a Packard Tri-Carb 1600 TR liquid scintillation analyzer. Specific binding was defined as total binding minus binding measured in the presence of 3 mM ketamine or 1 mM unlabelled MK-801. Experiments with membranes prepared from rat brain (without cerebellum and brainstem) were performed as described in Lima et al. [16].

2.4. In vivo studies

Male cercariae of *S. mansoni* (BH strain) were obtained by light stimulation of snails (*Biomphalaria glabatra*) previously infected with a single miracidium. Newborn mice were then infected percutaneously with approximately 150 male cercariae and were killed 45 days later by cervical dislocation [9].

About 12–15 adult male worms were recovered from the portal veins of mice 50 days after infection. The worms were collected using a stiletto, washed rapidly and then placed in a glass chamber with two wells containing 250 μl of a buffered saline solution, consisting of 82.5 mM Na^+, 4.1 mM K^+, 3.6 mM Ca^{2+}, 3.3 mM Mg^{2+}, 100.4 mM Cl^-, 79.9 mM glucose, 10 μM 5-HT and 15 mM HEPES, pH 7.4.

In each well, three worms were preincubated for 10 min at 37 °C. After that, the bath medium was exchanged with saline buffer containing the drugs. The antagonists were added just prior to the 10 min preincubation and remained present throughout the experiment.

Body area measurements were made by analysis of the images captured with a CCD camera using the Image-Pro® Plus program (Media Cybernetics, Silver Spring, MD, USA) according to previously published procedures [9]. A new methodology was created to quantify the worm's motility, based on the displacement of its extremities in a Cartesian plan. The *x*–*y* coordinates of the worm's extremities (A and B) were determined on the images, which were taken every minute ($x_{A,i}$, $y_{A,i}$, $x_{B,i}$, and $y_{B,i}$, at times $i = 1, 2, \ldots$). The relative displacement of each extremity (d_A and d_B) was calculated by the formula: $d_{A,i} = [(x_{A,i} - x_{A,i-1})^2 + (y_{A,i} - y_{A,i-1})^2]^{0.5}$, similarly applied for $d_{B,i}$. The "*displa-*

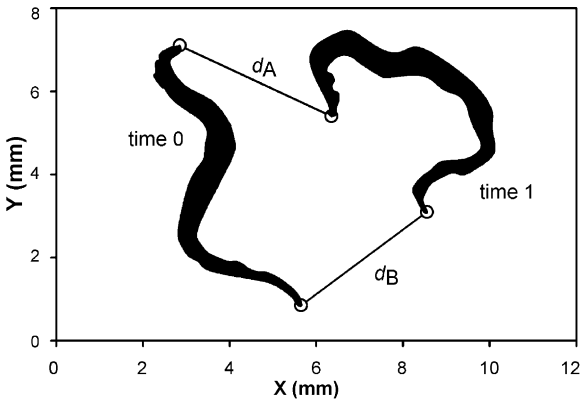

Fig. 1. Method of measurement of worm motility based on changes of the *x–y* coordinates of the body extremities. The figure illustrates two profiles of the same worm imaged 1 min apart. The extremities were marked on the image and the calibrated coordinates were obtained with the image analysis software. The displacement of each extremity was then calculated (d_A, d_B) and their average yielded the displacement index (d) for that 1 min interval.

cement index'' (d_i) was finally calculated by the formula: $d_i = 0.5(d_{A,i} + d_{B,i})$ (Fig. 1).

2.5. Statistics

Statistical comparisons of recoveries of MK-801 binding in the different subcellular fractions were determined by using ANOVA. Comparison of IC_{50} for competition curves of MK-801 isomers was performed using a parallel-line regression analysis with a test for differences in elevation [17]. Statistical comparisons of displacement values measured before and after addition of the drugs were determined by using one-way repeated measures ANOVA with the post hoc Student–Newman–Keuls test for multiple comparisons, and significance was accepted when $P < 0.05$.

2.6. Drugs

The drugs were purchased from Sigma Chemical Co. (5-HT, glycine, AP5, DNQX, kainic acid, (−)-MK-801, ketamine, (−)-nicotine), RBI Co. (L-glutamate) and Tocris Cookson Ltd. (NMDA, quisqualate, (+)-MK-801, methyllycaconitine, (*RS*)-AMPA).

3. Results

3.1. Binding assays

Specific binding sites for 10 nM [^{3}H]MK-801 were detected in all subcellular fractions (P_1–P_4), but the maximal recovery was obtained in the heterogeneous (P_1) fraction (Table 1). Therefore, this fraction was used to further characterize the binding of [^{3}H]MK-801 to *S. mansoni*.

The pharmacological modulation of a binding reaction is best studied by comparing the IC_{50} values calculated from whole competition curves performed with different putative ligands, as was done with the rat brain membranes (see below). Unfortunately, such a protocol was not possible with *S. mansoni* due to the limitations of material. The protein yield was about 19.6 mg per 1500 worms. As a surrogate, we tested a single concentration of each putative ligand, generally equal to or near the IC_{50} value that we previously measured in rat brain (Fig. 2). [^{3}H]MK-801 binding to P_1 and to rat brain was not stimulated by either glutamate or glycine, which could suggest the presence of these endogenous aminoacids in our preparations. To test if the endogenous agonists were present and possibly activating NMDA receptors in the preparation, we used the classical competitive antagonists of glutamate and glycine, AP5 and kynurenic acid, respectively. Neither AP5 (100 μM) nor kynurenic acid (300 μM) decreased the binding of [^{3}H]MK-801 in *S. mansoni* P_1, but they were effective in the rat brain preparation (Fig. 2 and [11]). The ligands of the non-NMDA receptors, quisqualate, kainic acid and AMPA, each at 300 μM, did not alter the binding

Table 1
Subcellular distribution of 10 nM [^{3}H]MK-801 binding in subcellular fractions of *S. mansoni*

Fractions	[^{3}H]MK-801 bound recovery (%)[a]	[^{3}H]MK-801 bound (fmol mg protein^{-1})
P_1	72 ± 6[b]	918 ± 357
P_2	5 ± 1	122 ± 20
P_3	7 ± 1	141 ± 12
P_4	17 ± 5	156 ± 32

[a] % Recovery = 100 × binding (specific binding × protein content) in each individual fraction divided by the sum of the binding of the four fractions. Values represent mean ± S.E.M. from four different preparations, each experiment being performed in quadruplicate.
[b] $P < 0.05$, one-way ANOVA (P_1 vs. P_2, P_3 and P_4 fractions).

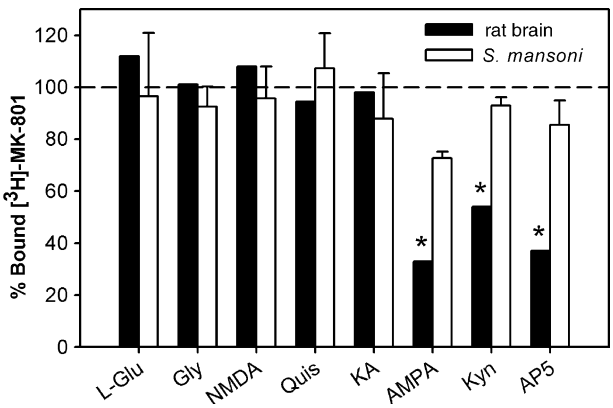

Fig. 2. Modulation of 10 nM [^{3}H]MK-801 binding to P_1 fraction of *S. mansoni* and rat brain preparation by classical glutamatergic ligands. The values of specific binding expressed as percent of control (mean ± S.E.M.) were obtained from three experiments performed in quadruplicate. The ligand concentrations were: L-glutamate (L-Glu), 1 μM (rat brain) or 100 μM (P_1); glycine (Gly), 30 μM; NMDA, 100 μM; quisqualate (Quis), 300 μM; kainic acid (KA), 300 μM; AMPA, 300 μM; kynurenic acid (Kyn), 300 μM; AP5, 100 μM. (*) Significantly different from control ([^{3}H]MK-801 binding in the absence of inhibitor), $P < 0.05$ (one-way ANOVA).

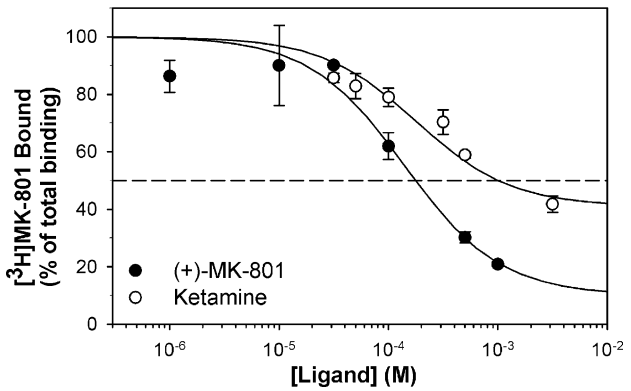

Fig. 3. Ketamine and MK-801 competition curves for 10 nM [^{3}H]MK-801 binding to P_1 fraction of *S. mansoni*. The curves were drawn using the parameters fitted by non-linear regression analysis using the model of a single class of independent binding sites. Each point represents the mean ± S.E.M. obtained from two experiments performed in quadruplicate. Note that the values of total binding were used in the ordinate and that the IC_{50} reported in Table 2 represent the concentrations of the ligands necessary to inhibit half of the specific binding (measured as the difference between the upper and lower plateaus) and not half of the total binding of [^{3}H]MK-801.

of [^{3}H]MK-801 to *S. mansoni*. Note that AMPA inhibited [^{3}H]MK-801 binding in the rat brain preparation only at the very high concentration used here.

Ketamine is another well-characterized ligand of the pore site of mammalian NMDA receptors, often used in competition assays with MK-801 [18]. For ketamine and MK-801, full competition curves were obtained, allowing the estimation of their (low) affinities through calculation of the IC_{50} (Fig. 3). Comparing these IC_{50} values with those obtained from rat brain, we conclude that ketamine and MK-801 have affinities about 500 and 20,000 times lower for the sites labeled with [^{3}H]MK-801 in *S. mansoni* than in rat brain, respectively (Table 2).

These atypical characteristics of MK-801 binding in *S. mansoni* along with reports that MK-801 blocks both peripheral and central nicotinic acetylcholine receptors (nAChRs) in vertebrates [19] led us to investigate if the [^{3}H]MK-801 binding sites detected in *S. mansoni* could correspond to nAChRs, instead of NMDA receptors. For this purpose, we explored the reported differences in stereoselectivity of the MK-801 sites in vertebrate NMDA receptors and nAChRs, by performing competition assays with the MK-801 stereoisomers in *S. mansoni*. Fig. 4A shows that (+) and (−)-MK-801 had essentially identical IC_{50} in *S. mansoni*, as observed for binding of MK-801 to

Table 2
Inhibition of [^{3}H]MK-801 binding in rat cerebral membranes and in P_1 fraction of *S. mansoni* by ketamine and MK-801

Compound	IC_{50} values (μM)[a]	
	Rat brain	*S. mansoni*
Ketamine	~1	500
MK-801	0.01	200

[a] Values represent the means obtained from two experiments performed in quadruplicate.

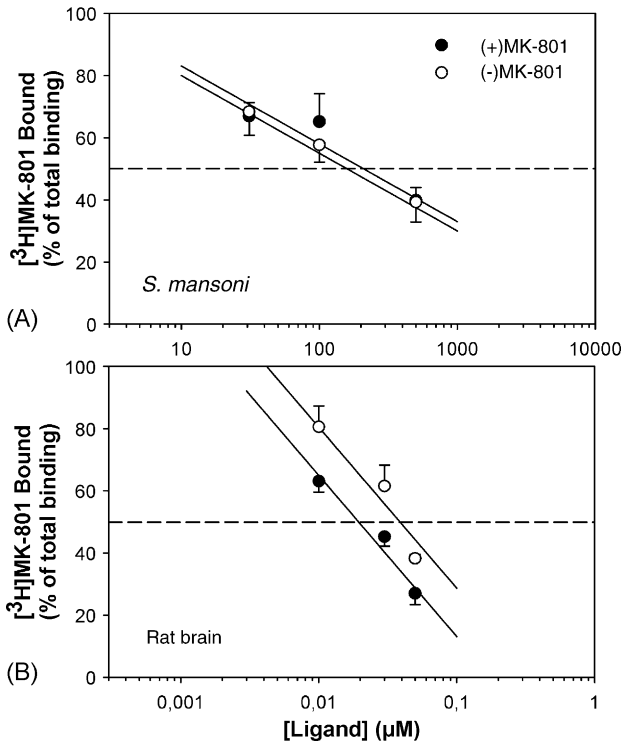

Fig. 4. Stereoselectivity of MK-801 binding to *S. mansoni* and rat brain. Each point represents the mean ± S.E.M. obtained from seven to eight samples for *S. mansoni* and three to eight samples for rat brain. The data were analyzed by ANOVA of the linear regression on the $\log_{10}$ concentrations. After establishing that the slopes were not significantly different ($P > 0.5$), a common slope was calculated for the two regressions, and the difference between elevations (i.e., affinities) was tested for significance [17]. (A) Effect of MK-801 stereoisomers on [^{3}H]MK-801 binding to *S. mansoni*. The elevations were not significantly different ($P = 0.80$, 44 d.f.). (B) Effect of MK-801 stereoisomers on [^{3}H]MK-801 binding to rat brain. The elevations were significantly different ($P = 0.006$, 28 d.f.), and the affinity ratio [(+)-MK-801/(−)-MK-801] was 1.99.

human nAChRs [20]. In contrast, (+)-MK-801 exhibited a two-fold higher affinity than its stereoisomer in our rat brain preparation (Fig. 4B), as expected for the binding of MK-801 to the mammalian NMDA receptor [21].

3.2. In vivo studies

To look for functional evidence of an interaction between MK-801 and nAChRs, we performed in vivo experiments. As reported earlier [9], the adult male worms exhibited a variety of spontaneous body movements in medium containing 5-HT (10 μM), including small and fast generalized shortening and lengthening of the body and propagating body waves along the anterior–posterior axis, similar to peristaltic waves (Fig. 5A–D). Application of (−)-nicotine 100 μM for 10 min induced an obvious flaccid paralysis of the worms, characterized by an intense decrease in motility (Fig. 5F and G). This effect appeared within 5 min of solution exchange, and was completely reversed upon washout of (−)-nicotine (Fig. 5H).

Initially, we analyzed the effects of (−)-nicotine looking for changes in the body area of the worms, a parameter that

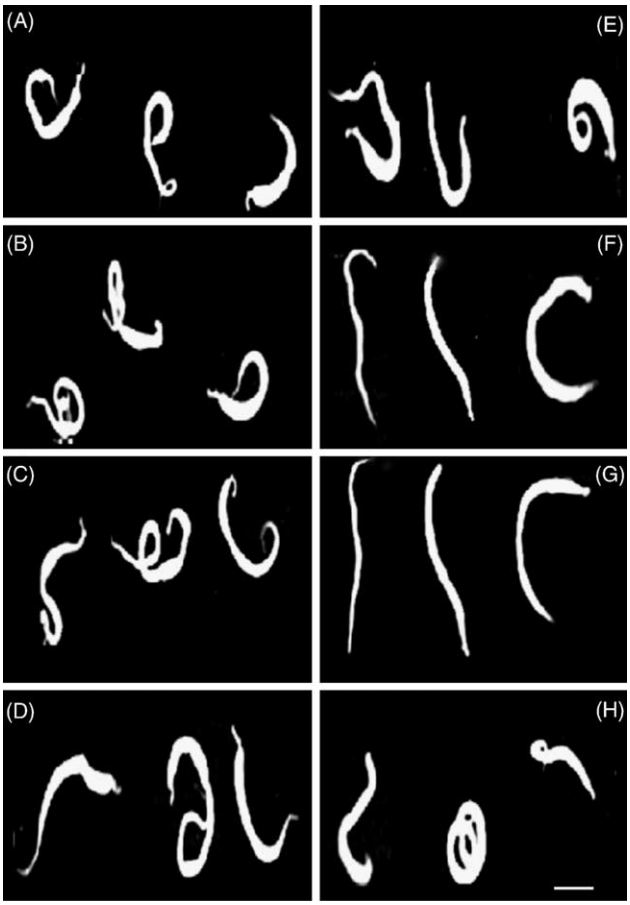

Fig. 5. Digitized images showing the changing body postures of adult male *S. mansoni* in a control experiment (A–D) or in a test of 100 μM (−)-nicotine (E–H). Control worms presented a variety of postures. After 2 min of exposure to 100 μM (−)-nicotine, added at the end of the 10 min-preincubation period, a marked flaccid paralysis of the worms was observed (F), that persisted until the drug was removed (G). The (−)-nicotine-induced response was reversed completely after washing (H). Time points of each image: (A and E) 5 min; (B and F) 12 min; (C and G) 20 min, (D and H) 25 min. Calibration bar: 4 mm.

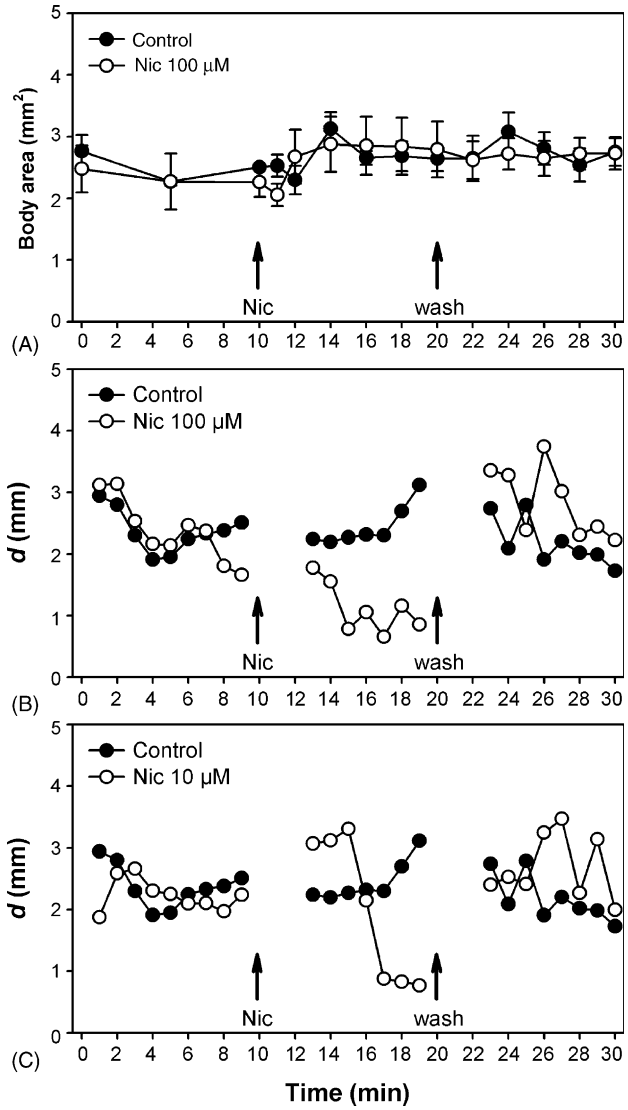

Fig. 6. Time course of the effect of (−)-nicotine on the body area and motility of *S. mansoni* adult males. After 10 min in saline (control) solution (pre-incubation), the medium was changed for (−)-nicotine (○) or control saline (●). After 10 min of incubation, the worms were washed twice with saline solution. (A) Changes in body area upon exposure to 100 μM (−)-nicotine. Each point represents the mean ± S.E.M. obtained from four experiments with three worms each ($n = 12$). The differences were not significant for this parameter. (B) Changes in motility upon exposure to 100 μM (−)-nicotine, same experiments as in (A). The average of the values of d (see Fig. 1) at times 6–8 min (d_6–d_8), was significantly different from the average of d_{16}–d_{18}, which was also different from the average of d_{26}–d_{28}, as determined by one-way repeated measurements ANOVA ($P < 0.05$). (C) Changes in motility upon exposure to 10 μM (−)-nicotine, in another set of experiments. The 3-min average of d during the exposure (d_{16}–d_{18}) was also significantly different from the control (d_6–d_8) and washout averages (d_{26}–d_{28}). The gaps in the plots in B and C correspond to the solution exchange periods, when the worms are dragged by the flow and the d values would be artifacts.

we previously used in order to quantify a contraction of the whole worm body [9]. As shown in Fig. 6A, nicotine did not produce any effect on this parameter. In order to assess quantitatively the loss of motility, we devised a new parameter of analysis, the "displacement index" (d), described in detail in Fig. 1, and applied it to the experiments with nicotine. According to this analysis, the value of d approaches zero in case of paralysis since the worm occupies nearly the same position in the Cartesian plan in consecutive 1-min time intervals (Figs. 5F, G and 6B). The values of d in the sixth to eighth minutes of observation in each condition were averaged for significance testing. These results lead us to conclude that the new methodology was able to reproduce the nicotine effect ($P < 0.05$, $n = 12$ worms). We also tested nicotine in a concentration 10 times lower and this effect was also significant ($P < 0.05$; $n = 9$ worms) (Fig. 6C).

Considering that our binding assays raised the possibility that (+)-MK-801 could bind to nAChRs in *S. mansoni*, we decided to verify if MK-801 could block the in vivo effect of nicotine on worm motility. After a 10-min pre-incubation period in saline solution in order to establish the basal value of d, the solution was changed to one containing 1 mM (+)-MK-801. We did not observe significant changes in the value of d, indicating that the drug had no

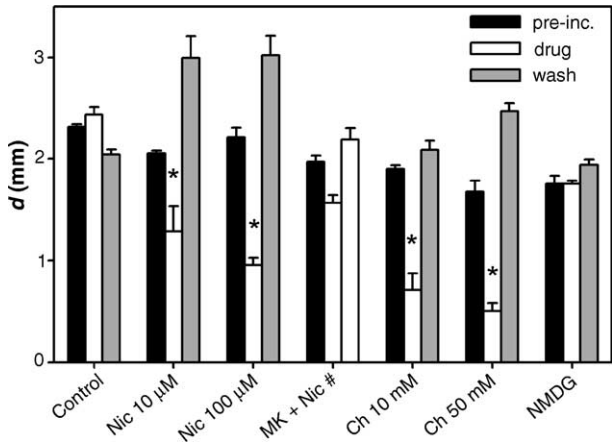

Fig. 7. Nicotinic modulation of *S. mansoni* motility. Each bar represents grand mean ± S.E.M. of the 3-min averaged *d* during the pre-incubation, exposure or washout periods for the indicated drugs (Nic, (−)-nicotine; MK, (+)-MK-801; Ch, choline). Significant differences in motility between the drug exposure and the pre-incubation periods were assessed by one-way repeated measurements ANOVA ($^{*}P < 0.05$). (#) For the bars labeled "MK + Nic", the middle bar represents the exposure to the antagonist MK-801 (1 mM) alone, and the third bar represents the subsequent exposure to MK-801 plus (−)-nicotine (100 μM), beginning at time = 20 min (the washout period is not shown). The number of worms evaluated in each set of experiments, from left to right (control to NMDG), was: 9, 9, 14, 12, 9, 6, and 3.

effect by itself in the worm's motility (Fig. 7). Next, we changed the solution to a new one containing 100 μM (−)-nicotine along with 1 mM (+)-MK-801. The addition of nicotine did not reduce the motility, indicating that co-incubation with MK-801 was able to protect the worm against the paralyzing effect of nicotine (Fig. 7).

Besides (−)-nicotine, we tested choline, a specific nicotinic agonist for the nicotinic receptor that contains α7 subunits of mammals [22,23]. At the concentrations of 10 and 50 mM, choline also was able to inhibit the motility of the worms, as seen by a significant reduction of the parameter *d* after addition of the drug (Fig. 7). The effect of high concentrations of choline could not be attributed to changes in osmolarity or ionic strength, since the addition of 50 mM *N*-methyl-D-glucamine (NMDG) was not able to inhibit the worm's motility (Fig. 7).

4. Discussion

The evidence for the presence of a glutamatergic neurotransmission in the trematode *S. mansoni* [7–9] led us to investigate the presence of NMDA receptors using [^{3}H]MK-801 as a tool. The specific binding of [^{3}H]MK-801 occurred mainly in the P_1 fraction, where 70% of the binding sites were recovered, coinciding with kainic acid binding sites that have been previously detected only in this fraction [9]. The pharmacological profile of [^{3}H]MK-801 binding was very different from that expected for a "classical" binding site at the NMDA receptor, based on what is known from mammalian studies. Mammalian NMDA receptors bind MK-801 in a "use-dependent" manner, so that binding is promoted by activation of the receptor channel [10,11]. The lack of stimulation of [^{3}H]MK-801 binding by glutamate, glycine and NMDA could be interpreted as indicative of the presence of endogenous agonists in our preparation of worm tissue [11]. However, in this case we should expect a decrease of [^{3}H]MK-801 binding after addition of AP5 and kynurenic acid, competitive antagonists at the agonist (glutamate) and co-agonist (glycine) site, respectively [12]. Because AP5 and kynurenic acid had no effect, we conclude that either these drugs are not effective as antagonists of the (possibly unique) NMDA receptors of *S. mansoni*, or that [^{3}H]MK-801 labels a receptor different from the NMDA receptor, in this worm.

The affinities of both MK-801 and ketamine in worm tissue were extremely low, being 500 and 20,000 lower than those described for mammalian NMDA receptors, as reported by others [21] and measured by ourselves in rat brain, in exactly the same experimental conditions used for *S. mansoni* (Table 2). In mammalian tissues, another receptor known to bind ketamine and MK-801 with very low affinity is the nicotinic receptor. Mammalian NMDA receptors and nAChRs are not structurally related (NMDA and nAChR are formed by four and five subunits, respectively [24,25]), but they share certain pharmacological properties such as the noncompetitive inhibition by a number of different dissociative anesthetics, including ketamine, MK-801 and phencyclidine [26]. MK-801 has been reported to inhibit mammalian nAChRs of the muscle type at concentrations around 0.3 μM [27] and to bind to *T. californica* electric organs with low affinity characterized by K_d values of 5 μM (desensitized state) or 140 μM (resting state) [25]. The IC_{50} measured for MK-801 in *S. mansoni* (200 μM) is thus compatible with the binding to a nicotinic receptor in the resting state. The study of the stereoselectivity of MK-801 binding further supports this hypothesis since (+) and (−) isomers had essentially the same IC_{50} in *S. mansoni* (Fig. 4A), just like with human α7 nAChRs [18]. In contrast, (+)-MK-801 exhibits a five-fold higher affinity than its (−) isomer at the NMDA receptor [21], as was also observed in our experiments with rat brain, although the (+) isomer was only about twice as potent as the (−) isomer (Fig. 4B).

The second experimental approach used to investigate the nature and role of MK-801 binding sites and their putative relation to nicotinic receptors was the measurement of changes in motor activity of whole worms [28]. Classical studies have demonstrated that acetylcholine, at very high concentrations, induces a flaccid paralysis of *S. mansoni* [29,30]. The high concentration needed for such effect (10 mM) could be due to permeability barriers of the whole worm and factors such as metabolism [3,31]. Here, we showed that (−)-nicotine produced similar effects, at a relatively low concentration (10 μM, Fig. 6C). It must be noted that the spontaneous pattern of activity upon which the effect of (−)-nicotine was revealed requires the pre-

sence of 5-HT in the solution bathing the worms. 5-HT has an important role in the neuromuscular control of *S. mansoni*, acting probably as an excitatory modulator [32,33]. Therefore, the lack of effect of nicotine reported by Mellin et al. [29] could be due to the lack of 5-HT in their experiments. We also showed that MK-801 blocked the paralyzing effect of nicotine, which is in agreement with the reported non-competitive inhibition of vertebrate nAChRs by MK-801 [19,25].

The idea that the behavioral effects of nicotine in *S. mansoni* are mediated by specific receptors is supported by evidence of the expression of nAChRs in the worm, particularly in the tegument [34]. These tegumental receptors bind α-bungarotoxin and this binding is inhibited by both D-tubocurarine and nicotine. The high recovery of [^{3}H]MK-801 binding sites in the P_1 fraction of *S. mansoni* is compatible with a binding to a tegumental nAChR since this fraction has been shown to contain tegumental structures [15]. Regarding the physiological role of these nAChRs in *S. mansoni*, it is noteworthy that the parasite surface also concentrates a high amount of acetylcholinesterase [35]. This acetylcholinesterase is co-localized with the surface nAChR [36,37], confirming the existence of a regulatory pathway for acetylcholine which can modulate the uptake of glucose in response to circulating acetylcholine present in the host's blood stream [38]. Acetylcholinesterase and acetylcholine receptors have also been observed on the surface of several blood cells in vertebrates, e.g., T cells and erythrocytes, where they could play a similar role in nutrient transport [39]. The cholinesterases in mammalian blood cells show similar molecular form, membrane anchorage and kinetic characteristics to the worm's enzymes, suggesting a common function in phylogenetically distant species [36,39,40].

The recent cloning and molecular characterization of two putative nAChR subunits from *Schistosoma* [41] further supports the presence of a nicotinic cholinergic pathway in *S. mansoni*. The comparison of the α-type (ACh-binding) subunit found in *S. manso*ni with other sequences of nicotinic receptors indicated that they are very similar to the α7-like subunits identified in insects and nematodes, and also to the α7 and α8 subunits of vertebrates. The α-type subunit (SmAR1α) was localized exclusively to the surface structures of the parasite, while the non-α subunit (SmAR1β) was localized within the musculature and on discrete cell bodies within the connective parenchyma. While the function of these putative nAChRs remains to be shown, both the surface receptors, by modulating nutrient transport [38], and deeper receptors, by controlling neuromuscular function, could conceivably mediate the paralyzing effect of nicotine. The fact that the vertebrate α7 nAChR agonist choline also induced a flaccid paralysis might suggest a functional homology between the putative receptors in *S. mansoni* and their vertebrate counterparts.

The data presented in this work permit us to propose that the specific binding of [^{3}H]MK-801 to the subcellular P_1 fraction of *S. mansoni* possibly occurs on a nicotinic receptor and not on a NMDA glutamatergic receptor. This hypothesis is based on binding studies indicating a very low affinity for (+)-MK-801 and ketamine, the lack of modulation of [^{3}H]MK-801 binding by glutamatergic agonists and antagonists and the absence of stereoselectivity for the MK-801 isomers. The flaccid paralysis produced by the nicotinic agonists ((−)-nicotine and choline) and its prevention by (+)-MK-801 are also consistent with such hypothesis. The evidences for a low-affinity binding of (+)-MK-801 to nicotinic receptors in mammals and the recent characterization of three versions of nicotinic receptors in the transcriptome of *S. mansoni* [10] also support our proposal that [^{3}H]MK-801 binds to nicotinic receptors in the tegument of *S. mansoni*. As a conclusion, this work not only contributes to the basic knowledge of *S. mansoni* biology but also identifies a new putative target for the development of antiparasitic agents related to the neuromuscular system of this flatworm.

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