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Chemotactic activities of vasoactive intestinal peptide, neuropeptide Y and substance P in *Leishmania braziliensis*

Michelle Giammarressi, Oriana Vanegas, Anthony Febres, Adrián Silva-López, Emilia Diaz López, Alicia Ponte-Sucre *

Laboratory of Molecular Physiology, Institute of Experimental Medicine, School of Medicine Luis Razetti, Faculty of Medicine, Universidad Central de Venezuela, Caracas, Venezuela

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ABSTRACT

Cell-cell interaction and active migration (and invasion) of parasites into skin host-cell(s) are key steps for successful infection by *Leishmania*. Chemotaxis constitutes a primordial chapter of *Leishmania*-host cell interaction, potentially modulated by neuropeptides released into the skin due, for example, to the noxious stimuli represented by the insect bite. Herein we have evaluated *in vitro* the effect of sensory (Substance P, SP) and autonomic (Vasoactive Intestinal Peptide, VIP, and Neuropeptide Y, NPY) neuropeptides on parasite taxis, and investigated the potential modulatory effect of SP on *Leishmania* (*Viannia*) *braziliensis*-macrophage interaction. We demonstrated that VIP (10^{-10} M) and NPY (10^{-9} M) are chemorepellent to the parasites, while SP (10^{-8} M) produces a chemoattractant response. SP did not affect macrophage viability but seems to impair parasite-macrophage interaction as it decreased promastigote adherence to macrophages. As this effect is blocked by ([D-Pro 2, D-Trp7,9]-Substance P (10^{-6} M), the observed action may be mediated by neurokinin-1 (NK_1) transmembrane receptors. VIP and NPY repellent chemotactic effect is impaired by their corresponding receptor antagonists. Additionally, they suggest that SP may be a key molecule to guide promastigote migration towards, and interaction, with dendritic cells and macrophage host cells.

1. Introduction

Leishmaniasis is caused by flagellated protozoa of the genus *Leishmania*, transmitted by sand flies of the genus *Lutzomyia* and *Phlebotomus* (Steverding, 2017). Pathologies caused by *Leishmania* can be classified as tegumentary and visceral leishmaniasis. Tegumentary leishmaniasis comprise cutaneous and mucocutaneous disease manifestations that are caused by at least 13 dermotropic species of the genus *Leishmania*, most of which are prevalent in the New World, being *Leishmania (Viannia) braziliensis* the most prevalent species (Zerpa et a. 2018).

Parasite (gender, subgenus and species) and host (nutritional status, immune system response) impact the type of disease. For example, infection by *L.* (*V.*) *braziliensis* may eventually lead to mucocutaneous leishmaniasis (MCL), characterized by the destruction of the walls of oral–nasal and pharyngeal cavities, potentially evolving to disfiguring lesions. It may appear years after the onset of cutaneous leishmaniasis (Zerpa et al., 2018).

The inoculation of (metacyclic) promastigotes into the skin (human or mammalian reservoirs) by the vector bite initiates the infection. Invading parasites are recognized as alien agents that trigger physiological responses and guide host actions including activation of inflammatory and immunological cells and molecules, among them neuropeptides, cytokines, etc. that would impact the final result; i.e., disease installation (with parasite survival), or (if the host can control the infection) variable rates of spontaneous healing (Diaz and Ponte-Sucre, 2018).

Macrophages play a key role during *Leishmania* infection as a host-cell for parasite replication and as an effector-cell by participating in its elimination. That is, once the parasite enters the macrophage, it differentiates into amastigote and replicate in the phagolysosome; intracellular amastigotes inhibit macrophage lysosomal enzymes, avoiding cellular killing. However, through the production of reactive oxygen species, nitric oxide, N-hydroxy-L -arginine, pro-inflammatory cytokines, etc., the macrophage favors parasite elimination (Kedzierski and Evans, 2018).

Leishmania promastigotes have a well-developed flagellum,

* Corresponding author.
E-mail addresses: aiponte@gmail.com, alicia.ponte@ucv.ve (A. Ponte-Sucre).

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fundamental for feeding, development and reproduction. Once *Leishmania* detects extracellular –chemical- signals, and being a motile cell, it directs its translation following (or evading) the gradients by the process called "chemotaxis". *Leishmania* parasites are large cells (10–20 μm long), that detect gradients of chemotactic agents and respond to minimal changes in their concentration, processing temporal and surrounding spatial information, and thus migrating correspondingly. Membrane receptors and intracellular signaling pathways allow them to shape the physiological response; that is, chemo-attractant and -repellent substances activate cell receptors and trigger a chain of events that results in migration (Diaz and Ponte-Sucre, 2018).

Modern dermatology deals with the so-called "Endocrine Cutaneous Neuro-immune System, ECNI" that describes how cutaneous nerve fibres, and immune and skin cells interact in the presence of specific stimuli (Choi and Di Nardo, 2018). Precise neuroanatomical connections between the central nervous system and the skin validate molecular interaction and communication between nerves and the immune system through molecules that originate in both organizations, the neuropeptides (Choi and Di Nardo, 2018). Thus, the skin reacts to endogenous and exogenous stimuli by perceiving and integrating environmental stimuli ending up in a response.

In the case of leishmaniasis, parasite and cutaneous nervous system roles for successful macrophage-promastigote interaction and initiation of inflammatory processes in cutaneous leishmaniasis have been investigated *in vitro* by immunohistochemical and radioimmunoassay techniques. The intimate link between sensory (substance P, SP; calcitonin gene-related peptide, CGRP; and somatostatin, SOM), and autonomic (neuropeptide Y, NPY; and vasoactive intestinal peptide, VIP) neuropeptides, and macrophage function has for example been evaluated in a *Leishmania (Leishmania) major*-macrophage model (Ahmed at al, 1998, 2001).

In this model, macrophage migration is inhibited by SOM at high (10^{-6} M) concentrations and SP at low (10^{-9} M and 10^{-10} M), and high (10^{-6} M) concentrations; additionally, the autonomic neuropeptide NPY prevents macrophage migration in a concentration-dependent manner, with a maximal inhibitory effect at 10^{-10} and 10^{-9} M, while VIP stimulates *L. (L.) major* induced macrophage migration at 10^{-9} M and 10^{-5} M. These results suggest that neuropeptides may modulate initial steps of host-parasite interaction and thus, influence disease development by affecting promastigote-induced macrophage migration (Ahmed at al, 1998; 2001).

Based on these results of Ahmed et al. (1998, 2001) and to further explore the role of neuropeptides in host-parasite inraction modulation we evaluated herein the potential chemotactic effect of VIP, NPY and SP *in vitro* using the "capillary-two chambers technique" in *L. (V.) braziliensis*. We also evaluated the effect of SP -at chemotactic concentrations-on parasite-macrophage interaction as a previous step to the establishment of the infection by *Leishmania*. Our results suggest that specific neuropeptides exert chemorepellent (VIP, NPY) and chemoattractant (SP) actions towards *Leishmania (V.) braziliensis*. Additionally they indicate that SP, modulates promastigote taxis and migration although impair parasite adhesion to macrophages, thus disturbing the instauration of the disease and opening a new horizon in the search of potential ways to control the ailment by minimizing the successful communication of parasites with their host-cell.

2. Materials and methods

2.1. Materials

Vasoactive Intestinal Peptide (VIP), Neuropeptide Y (NPY) and Substance P (SP), as well as the (non-selective) VIP receptor antagonist (VIP-peptide-fragment 6–28), VIP 6–28, the selective NPY antagonist Y1 GR 2323118 (also known as 1229U91 and GW1229) Dumont and Quirion, 2000), and the selective NK_1 receptor antagonist [D-Pro^2, D-$Trp^{7,9}$]-Substance P acetate, as well as analytical grade materials used to grow cells and perform the different biological experiments were purchased to Sigma-Aldrich Co (St. Louis, MS, USA).

2.2. Strains and culture conditions

2.2.1. Parasites

The reference strain *L. (V.) braziliensis* (MHOM/BR/LTB300) was kindly provided by Dr Noris Rodríguez (Institute of Biomedicine, Central University of Venezuela). The cells were cultured at 26 °C in semisolid blood agar supplemented with glucose–NaCl medium until stable growth (late log growth phase metacyclic parasites). Promastigotes were collected by centrifugation at 700×*g* for 10 min at room temperature (RT). The medium was decanted and the cells were suspended in the appropriate buffer according to the experiment to be performed (Alcázar et al., 2014).

2.2.2. Macrophages

Murine macrophages (J774) were donated by Dr Concepción Hernández (Institute of Experimental Biology, Central University of Venezuela). Cells were grown in RPMI medium supplemented with FCS 10%, L-glutamine 2 mM, and streptomycin 0.16 mg mL^{-1} (RPMI-c) at 37 °C, 5% CO_2. Cells were harvested by a cryogenic-mechanical technique. That is, cell culture bottles were located over a cold gel (4 °C) on an orbital shaker for 30 min. Later on, the culture bottles were hit on the culture hood surface to take afloat non-adherent cells. Cells were collected by centrifugation at 1500×*g*, for 10 min, at 4 °C, to avoid adherence with the plastic. The cells were suspended in RPMI-c to reach the desired density according to the experiment to be performed (Alcázar et al., 2014). Cell density was estimated with a hemocytometer.

2.3. Solutions

VIP, NPY and SP (10^{-10} to 10^{-5} M) and VIP_{6-28} (10^{-8} M), GR2323118 (10^{-7} M) and [D-Pro^2, D-$Trp^{7,9}$]-SP (10^{-6} M) dilutions were freshly prepared for each experiment in a buffer slightly different, Buffer A1 (NaCl 135 mM; KCl 5 mM; CaCl2 1 mM; MgSO4 1 mM Hepes 20 mM. 292 mOsm/Kg, pH 7.4) from the original Buffer A described previously (Díaz et al., 2011). Buffer A1 preserves the integrity of the neuropeptides herein used.

2.4. Chemotactic effect of VIP, NPY and SP on L. (V.) braziliensis

The chemotactic response of promastigotes to VIP, NPY and SP was determined by a modification of the vertical two-chamber capillary assay (Köhidai et al., 1995; Díaz et al., 2011). The reproducibility, quality and validity of the two-chamber capillary assay system as a good model to evaluate chemotaxis has been standardized for cell density and incubation time, as well as for concentration and osmotic gradients (Díaz et al., 2011, Díaz et al., 2013).

Briefly, the tips of an eight-channel micropipette were used as the inner chamber (IC) and the wells of a 96-well plate were used as the outer chamber (OC) of the two-chamber system. Tips were filled with the agonist solution at increasing concentrations (10^{-10} to 10^{-5} M). Tip number one was used as control (untreated) and was filled with Buffer A1. The wells were filled with a *Leishmania* suspension (200 μL) at 4 × 10^7 cells mL^{-1} in Buffer A1. The cells were incubated for 30 min at RT to measure chemotactic and not chemokinetic behavior of parasites (Díaz et al., 2011, Díaz et al., 2013). At the end of the incubation time, the cells that migrated into the IC were fixed in formaldehyde 2% in PBS (phosphate buffer 0.05 M, pH 7·2; NaCl 0.9 M) and counted in a hemocytometer.

2.5. Potential participation of neuropeptide receptors in chemotactic response

To determine the potential role of VIP, Y1 and tachykinin receptors

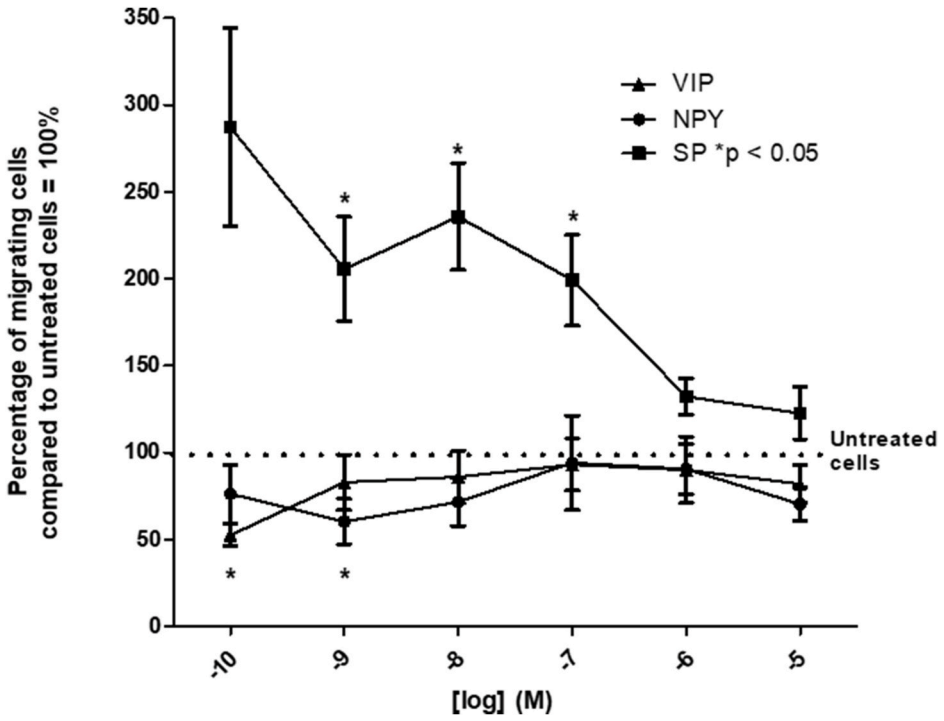

Fig. 1. Chemotactic responses of *L. (V) braziliensis* to VIP, NPY and SP. *p < 0.05. The figure illustrates the percentage of migrating cells at increasing concentrations of VIP (▲), NPY (●) and SP (■) comparing in each case with the percentage of mgrating cells in untreated control conditions.

-respectively- in the chemotactic response exerted by VIP, NPY and SP on *Leishmania* parasites, the cells were pre-incubated for 12 min in a fixed antagonist concentration [VIP_{6-28} (10^{-8} M), GR2323118 (10^{-7} M) and [D-Pro^2, D-$Trp^{7,9}$]-SP (10^{-6} M)] and afterwards the chemotactic response induced by VIP (10^{-10} M), NPY (10^{-9} M) and SP (10^{-8} M) was evaluated. Cells pre-incubated in Buffer A1 were used as control. Next steps were followed according to the above-described assay.

2.6. Effect of VIP, NPY and SP on L. (V.) braziliensis morphology

The effect of chemotactic concentrations of VIP (10^{-10} M), NPY (10^{-9} M) and SP (10^{-7} M and 10^{-10} M) on the morphology of *L. (V.) braziliensis* promastigotes was determined by a modification of a previously used protocol incubating an aliquot of parasites (4×10^7 cell mL^{-1}) for 30 min with the corresponding neuropeptide. Untreated cells, considered as control, were incubated in Buffer A1. For each sample a 10 μl aliquot was placed on a slide, allowed to dry at RT, fixed for 5 min with 100% methanol (Sigma- Aldrich Co.) and stained under standard conditions in a 10% Giemsa solution (Sigma-Aldrich Co.) (Diaz et al., 2015). The slides were then washed with distilled water and left on the bench until dry. One hundred parasites of each stained slide were analyzed by immersion oil light microscopy at 1000 × magnification (Axioskop 40 microscope; Zeiss). As *Leishmania* promastigotes have a fusiform body and a flagellum, the measured dimensions were: body length excluding the length of the free flagellum (outer membrane distance from the anterior to the posterior end), kernel-level maximum (outer membrane distance through the center of the karyosome) and length of the free flagellum (from the end attached to the parasite body to its free end).

2.7. Potential role of NK_1 receptors in parasite adherence to macrophages

Macrophages ($M_Ø$) were isolated and seeded as described herein. Suspension of $M_Ø$ (200 μL, 2×10^6 cell mL^{-1}) was seeded in each of an eight-chamber slide (Lab-Tek® Chambered Coverglass w/Cvr). After 30 min incubation at 37 °C, 5% CO_2, non-adherent cells were removed, washing with RPMI. The cultured macrophages were exposed to *L. (V.) braziliensis* promastigotes at a 10:1 ratio parasite/macrophage and incubated with RPMI-c (**B_1**, untreated); or with DMSO 0.5% (B_2) or Buffer A1 (B_3), as solvent controls; or $M_Ø$ exposed to *L. (V.) braziliensis* alone (**C_1**) as control, or with *L. (V.) braziliensis* and treated with amphotericin-B (AMB, 1 μM) (**C_2**) as a positive control; or $M_Ø$ with *L. (V.) braziliensis* and treated with SP (10^{-8} M) (**M_1**) as experimental-1, SP-NK_1 antagonist (10^{-6} M) (**M_2**) as experimental-2 or SP (10^{-8} M) and SP-NK_1 antagonist (10^{-6} M) as experimental-3 (**M_3**). In these experiments, the total incubation time was 120 min to avoid internalization of the parasites and better measure parasite adherence instead of macrophage internalization. The incubation was performed at 37 °C, 5% CO_2, cells were stained by Hemacolor® kit (Merck-Millipore, USA) using a standard protocol. Number of macrophages that showed promastigotes adhered to their surface were determined by manual counting of at least 300 macrophages using light microscopy (Alcázar et al., 2014).

2.8. Analysis of results

Statistical significance of differences between untreated cells and those exposed to VIP, NPY and SP for the macrophage adherence assays (data expressed as mean ± SEM, n = at least 4 experiments) was performed by the Student's *t*-test. For the area and flagellum length data, a population of 200 parasites (each for untreated and experimental conditions) were evaluated in three independent experiments performed for each experimental condition. Data are expressed as mean ± SEM. Statistical significance was evaluated by one-way ANOVA, Bonferroni posttest. Differences were considered significant if $p < 0.05$.

3. Results

3.1. Chemotactic effect of VIP, NPY and SP on L. (V.) braziliensis

The chemotactic effect of VIP, NPY and SP on *L. (V.) braziliensis* is illustrated in Fig. 1. VIP (10^{-10} M) and NPY (10^{-9} M) were chemorepellent to *Leishmania* parasites when compared to untreated cells. The

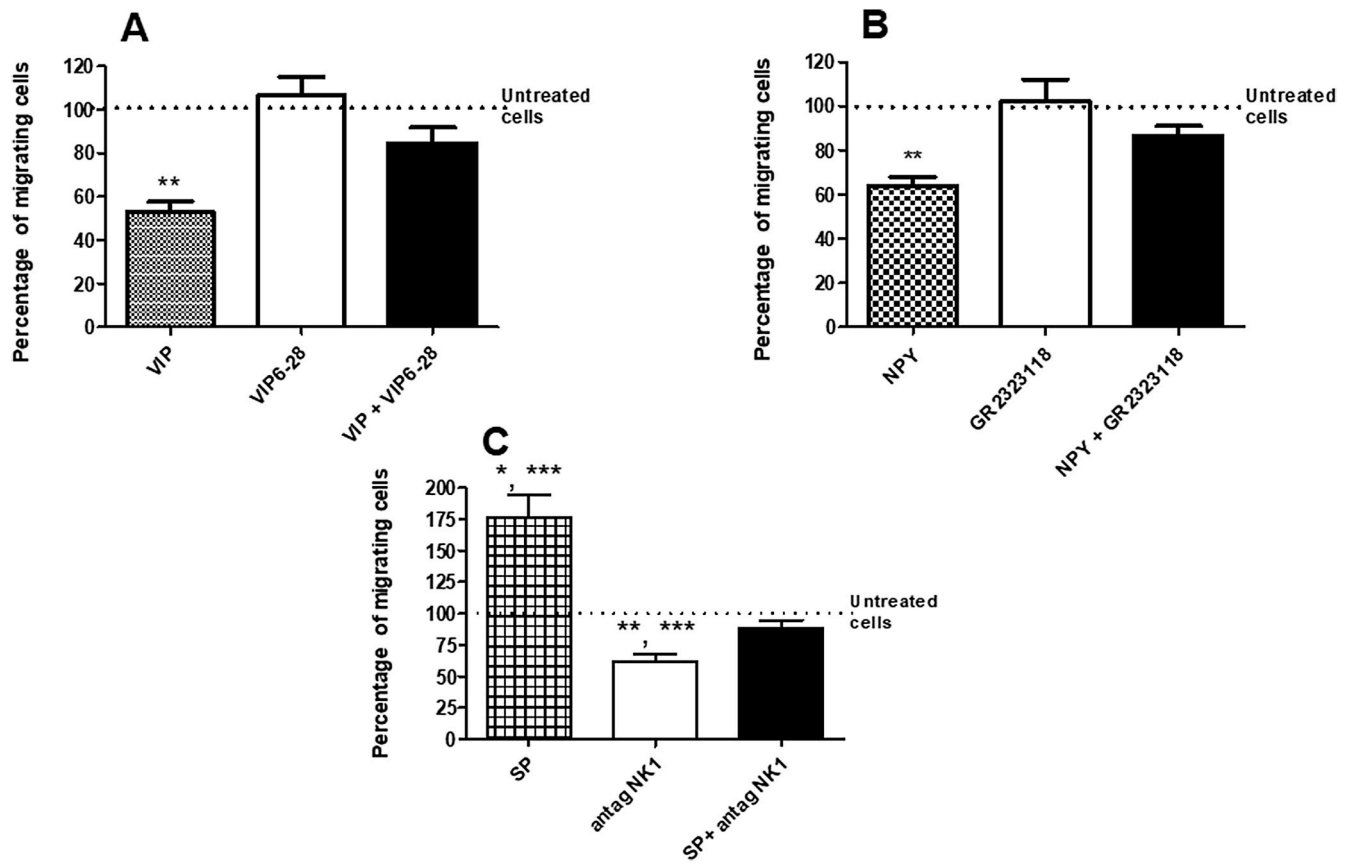

Fig. 2. Effect of the antagonists (A) VIP (VIP_{6-28}, 10^{-8} M), (B) NPY (GR2323118, 10^{-7} M) and (C) (D-Pro^2, D-$Trp^{7,9}$]-Substance P, (10^{-6} M), on the chemotactic response elicited by VIP (10^{-10} M, n= 5), NPY (10^{-9} M, n= 6) and SP (10^{-8} M, n= 7), respectively on L. (V.) braziliensis. For each panel the first column demonstrates the chemotactic/chemorepellent activity of VIP (**$p < 0.001$), NPY (**$p < 0.001$) and SP (*$p < 0.05$) comparing in each case with the percentage of migrating cells in untreated control conditions. The second column illustrates the effect of the corresponding antagonist on parasite migration; only [D-Pro^2, D-$Trp^{7,9}$]-Substance P elicited per se a chemo-repellent effect (**$p < 0.001$) when comparing with the percentage of migrating cells in untreated control conditions, and dramatically decreased the chemotactic effect elicited by SP (***$p < 0.0001$). The third column represents the effect of a 12 min pre-incubation of parasites with their corresponding antagonists on parasite migration when confronted with their corresponding chemotactic/chemorepellent agonist as the stimuli present in the inner chamber.

Table 1
Morphological changes elicited by neuropeptides VIP, NPY and SP on *L. (V.) braziliensis* promastigotes. Data are expressed as mean ± SEM. *p < 0,05; **p < 0,01; ***p < 0,0001. N = 200.

Peptides	Body (μm)			Flagellum (μm)
	Length (% change)	Wide (% change)	Area (μm^2) (% change)	Length (% change)
Untreated (Buffer A)	9.48 ± 0.16	1.51 ± 0.04	14.31 ± 0.16	10.41 ± 0.14
VIP (10^{-10} M)	**8.79 ± 0.18** ** **(-7%)**	**1.92 ± 0.05** *** **(+27%)**	**16.88 ± 0.18*** +18%)**	**9.78 ± 0.16** ** **(-6%)**
NPY (10^{-9} M)	9.04 ± 0.18	1.51 ± 0.04	13.65 ± 0.18	**9.88 ± 0.15** * **(-5%)**
SP (10^{-7} M)	9.39 ± 0.17	1.55 ± 0.04	14.55 ± 0.05	10.71 ± 0,19
SP (10^{-10} M)	9.36 ± 0.16	1.58 ± 0.04	14.78 ± 0.05	10.04 ± 0,16

figure demonstrates the wide-range chemoattractant character of SP (10^{-9} - 10^{-7}). Only physiological concentrations of SP produced effects on parasites migration, and this neuropeptide caused the most significant increase in promastigote migration of to the IC at 10^{-8} M.

3.2. Potential role of VIP (1–2), Y1 and NK_1 neuropeptide receptors

Subsequently, the potential role of receptors on the chemotactic effect of VIP, NPY and SP was evaluated. Fig. 2 illustrates the effect elicited by VIP_{6-28} (Fig. 2A, 10^{-8} M; non-selective antagonist of VPAC), GR2323118 (Fig. 2B, 10^{-7} M; antagonist Y_1 receptor) and [D-Pro^2, D-$Trp^{7,9}$]-SP, (Fig. 2C, 10^{-6} M; selective SP-NK_1 antagonist) on the chemorepellent response induced by VIP (10^{-10} M), NPY (10^{-9} M) and the chemoatractant response of SP (10^{-8} M) respectively. VIP_{6-28} (10^{-8} M) and GR2323118 (10^{-7} M) did not alter *per se* promastigote migration compared to untreated cells. On the other hand, the use of the selective SP-NK_1 antagonist (10^{-6} M), resulted in a statistically significant (*** $p<0.0001$) blockage of the chemoattractant response induced by SP (10^{-8} M, Fig. 2C); additionally, although non-statistically significant, this antagonist consistently promoted by itself a chemorepellent response, compared to untreated cells. In conclusion, Fig. 2C demonstrates that pre-incubation with the NK1 antagonist reduced significantly parasite migration towards the corresponding agonist SP, present in the inner chamber.

3.3. Effect of VIP, NPY and SP on Leishmania spp. morphology

We further analyzed if, besides their chemotactic effect, neuropeptides influenced the morphology of parasites, especially the flagellum. The results are summarized in Table 1. The mean length and wide of untreated parasites were 9.48 ± 0.16 μm and 1.51 ± 0.04 μm

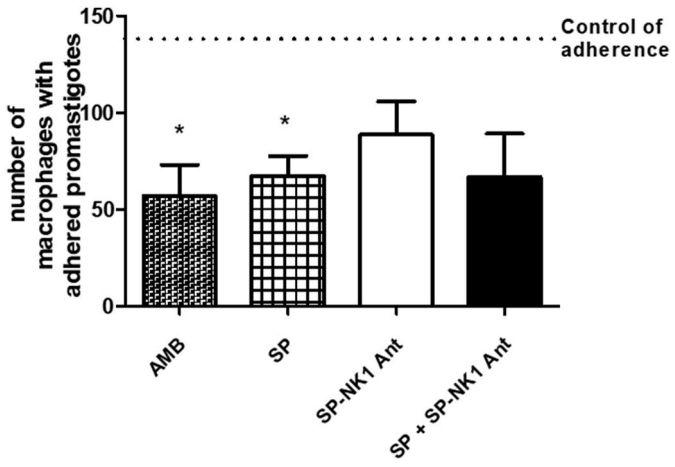

Fig. 3. Effect of the antagonists (A) NPY (GR2323118, 10^{-7} M), (B) VIP (VIP_{6-28}, 10^{-8} M) and (C) SP (D-Pro^2, D-$Trp^{7,9}$]-Substance P, (10^{-6} M) on parasite adherence to macrophages. *$p < 0.05$.

respectively, while the flagellum length was 10.410 ± 0.1420 μm. VIP (10^{-10} M) elicited significant changes on the area of promastigotes. That is, the mean wide increased, while body length decreased when compared to untreated parasites. As a result, the area of the cell increased around 18%. On the other hand, both VIP (10^{-10} M) and NPY (10^{-9} M) decreased the flagellum length of incubated parasites (around 5–6%) when compared to untreated cells. Interestingly, both neuropeptides produced a quite similar chemorepellent response in *Leishmania. (V.) braziliensis*. Finally, SP although being chemoattractant did not produced significant changes in parasite morphology at these chemoattractant concentrations of SP.

The previous results encouraged us to test if the chemotactic effect elicited by the peptides on *L. (V.) braziliensis* promastigotes was a result of changes in the parasite membrane potential (ΔVm), determined by a protocol (Padrón-Nieves et al., 2014) not described herein. The results (data not shown) suggest that in untreated parasites ΔVm is −175 mV (interval of confidence −177 mV to −172.7 mV) and that neither chemorepellent peptides (NPY or VIP) nor the chemoattractant peptide (SP), or their antagonists, induced significant changes in ΔVm compared to untreated cells.

3.4. Potential involvement of NK_1 receptors on parasite adherence to macrophages

The previous results suggest that SP elicited a chemoattractant effect on *L. (V.) braziliensis* parasites at physiological concentrations. To further dissect this response, we explored the potential involvement of NK_1 receptors on the capacity of macrophages to adhere parasites. Fig. 3 illustrates the effect of the experimental conditions on the number of macrophages that presented attached parasites after 120 min incubation. It can be observed that the number of macrophages with adherent parasites decreased significantly (67.00 ± 13.52 vs 138.00 ± 7.26%) in cell cultures incubated with SP at chemoattractant physiological concentrations (10^{-8} M) vs untreated cells. The results were similar to those obtained in cultures (57.00 ± 16.00 vs 138.00 ± 7.26%) incubated with AMB (1 μM), the positive control of the experiment, compared to untreated cells. In cultures incubated with the SP-NK_1 antagonist (SP-NK1-Ant, 10^{-6} M) alone, the number of macrophages that had promastigotes adhered to their surface did not change significantly with respect to untreated cells. A similar result was obtained when the antagonist was used simultaneously with SP (88.00 ± 17.00 vs 138.00 ± 7.26%). In these conditions that favored parasite adherence over internalization, the percentage of infected macrophages remained low as expected (8.51 ± 4.05 for control conditions) but still the effect of AMB 1 μM could be detected (2.125 ± 0.71).) In all the tested conditions, the average number of parasites per infected macrophage remained low between 1-5. All together these data suggest that although inducing parasite migration, SP decreases the number of macrophages that could adhere promastigotes, thus indicating that SP impairs parasite adhesion into macrophages. They also suggest that although not statistically significant, this effect could be blocked by the SP-NK_1 antagonist (SP-NK1-Ant). Additionally they suggest that NK_1 receptors might be involved in parasite- macrophage interaction.

4. Discussion

The skin is the place *par excellence* for host-environment interaction; perceived stimuli are processed and signaled to both the peripheral and central nervous systems. Neuropeptides -and their receptors-, are key-signaling elements mediating this interaction (Peters et al., 2006). By expressing neuropeptides and their receptors, neuronal cell endings, resident skin cells and infiltrating migratory immunocytes share a powerful "neuropeptide language", which enables an interdependent communication between the central nervous system and the skin immune system that guarantees a successful interaction with the environment but may also contribute to disease when dysregulation (Peters et al., 2006; Ahmed et al., 1999).

The mode of locomotion in *Leishmania*, is guided by the flagellum tip, at the forefront of parasite. This makes *Leishmania*-host cell interactions very peculiar. Some studies suggest that inert ellipsoid particles are only weakly phagocytosed as compared to conventional spherical beads by macrophages (Champion and Mitragotri, 2009). These results have been confirmed for *Leishmania* (Forestier et al., 2011). These authors demonstrated that phagocytosis of *Leishmania (L.) donovani* promastigotes by bone marrow macrophages is triggered by interaction between the parasite flagellum tip with the host cell. This implies the necessity of prior adhesion of the parasite to the macrophage surface membrane before internalization. Herein we concentrated in evaluating parasite adherence and not internalization to dissect this initial step of the process and the effect of SP.

The herein presented results suggest that skin neuropeptides modulate, at physiological concentrations, chemotactic responses in *Leishmania* and thus may influence skin invasion. In fact, VIP (10^{-10} M) and NPY (10^{-9} M) induce *in vitro* a chemorepellent response in *L. (V.) braziliensis* promastigotes. These "autonomic" neuropeptides trigger "an escape response" on promastigotes that "swim" away from the stimuli; as parasites and host-cells distance from each other, a "potential protection" of the host against infection develops. Similar data has been described in an *L. (L.) major*-(Raw 264.7)-macrophage model in which VIP and NPY decreased the percentage of infected macrophages suggesting an effect of these neuropeptides also on phagocytosis (Ahmed et al., 2001). Additionally, SP (10^{-9} - 10^{-7} M), a member of the tachykinin family of molecules, was chemoattractant to *L. (V.) braziliensis* promastigotes, increasing parasite migration.

SP at physiological concentrations decreased signficantly parasite adherence to macrophages. Duboni and Park (2006) have demonstrated that SP (300 nM and overnight incubation) induces bone marrow-derived mesenchymal stem cells mobilization; an action that seems to be mediated by N-cadherin since exposing target cells to an N-cadherin blocking antibody (clone GC-4) inhibits SP-mediated cell migration. SP (10^{-8} M) also altered opsonin mediated phagocytic uptake of bacteria in *Moraxella catarrhalis* and *Haemophilus influenzae* (Augustyniak et al., 2012). Finally, physiological SP concentrations (10^{-8}, 10^{-10} M) reduce the adhesion of RAW264.7 macrophages subjected to mechanical stress (Muschter et al., 2019). These results suggest similar to what we have found herein, that SP action is in general associated with decreased adherence and increased migration.

The NK_1 antagonist (D-Pro^2, D-$Trp^{7,9}$) significantly blocked the SP dependent chemoattractant response and promoted -by itself-a non-statistically significant, chemorepellent response, a result that suggests

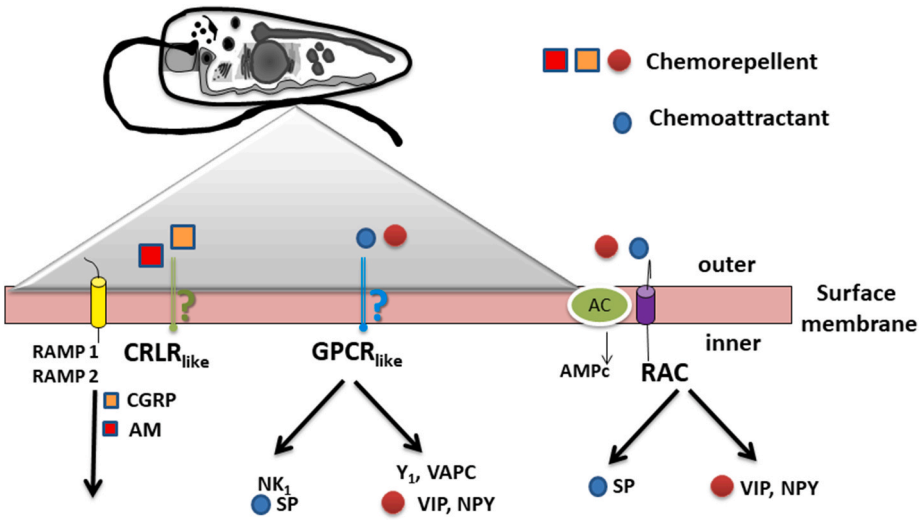

Fig. 4. Putative receptor model associated with neuropeptide responses in *Leishmania* (*V.*) *braziliensis* promatsigotes *in vitro*. The zoom detail depicts flagellum membrane organization including a scheme of potential transmembrane receptors that may be (NK1, Y1 and VAPC), G-protein (like), CRLR (like) coupled RAMP (1,2) receptors (Febres et al., 2018), and RAC receptors.

that this classic antagonist in vertebrates-, might be working as an inverse agonist (Berg and Clarke, 2018) at the potential NK1 *Leishmania* receptors, thus promoting (negative) chemotactic activity and inducing a (negative) migratory response in *Leishmania*.

In *Trypanosoma (b.) brucei* infected mice treated sub-curatively with the trypanocidal drug diminazene aceturate, the NK1 antagonist RP-67,580 has been successfully used to reduce the severity of the associated inflammatory response and meningoencephalitis (Kennedy et al., 1997). The inactive enantiomer RP-68,651 had no effect on the central nervous system inflammatory reaction. Although different parasites were used, taken together these results suggest that SP plays a key role during the instauration of these diseases and that NK1 receptors might be present in these ancient parasites.

Morphology and physiological behavior of *Leishmania* are dynamic. The parasites rapidly adapt to environmental changes imposed by their niches inside their hosts (Diaz and Ponte-Sucre, 2018). For example, the skin neuropeptide herein used VIP, exerts an antiparasitic effect (Delgado et al., 2009). This neuropeptide alters *T. (b.) brucei* morphology and transform them into swollen round parasites, with increased cell size and intracellular vacuolar-like structures, a detached flagellum and reduced mobility (Campos-Salinas and Gonzalez-Rey, 2009). In our hands, *L. (V.) braziliensis* promastigotes incubated in the presence of neuropeptides, evidenced an increased body area (VIP $\approx +$ 18%) and decreased flagellum length [VIP (−6%) and NPY (−5%)]. Parasites did not lost viability, a result probably explained by the fact that we used physiological (10^{-10}, 10^{-9} M respectively) instead of the pharmacological (12.5 μM) neuropeptide concentrations, and shorter incubation times (30 min vs. 4 h) used by Campos Salinas and colleagues (Campos-Salinas and Gonzalez-Rey, 2009). Antimicrobial properties of VIP (Campos-Salinas et al., 2014) may explain its chemorepellent effect, as promastigotes might detect this molecule as a noxious stimulus and "move away". In our hands, the chemoattractant neuropeptide SP (10^{-7} M - 10^{-10} M) did not elicit changes on *L (V.) braziliensis* morphology at physiological concentrations.

Although the classical GPCR-related signal transduction systems (e.g. heterotrimeric G-proteins, 7-transmembrane receptors, effector proteins, RAMPs) have not been described in *Leishmania* parasites, orthologs of some of these molecules (with reduced domains and function) have been designated in some members of the Trypanosomaitdae family (de Mendoza et al., 2014). Thus, the herein presented results encourage us to propose that the chemotactic effects stimulated by VIP, NPY and SP in *L. (V.) braziliensis* could be mediated by putative G-protein coupled receptors. These putative G-protein coupled receptors might be located at the flagellum membrane. Essential receptor domains may be conserved in these ancient parasites as has been detailed for VPAC1 receptors for which the N-terminal extracellular ectodomain is essential for VIP recognition (Couvineau et al., 1996).

Additionally, putative adenylate cyclase associated receptors (RAC) might be linked with the neuropeptide associated chemotactic responses observed herein. These catalytic (enzyme) receptors modulate intracellular phosphorylation and levels of second messengers. Eleven genes that share structural features with those encoded by *T. (b.) brucei* and *T. equiperdum* have been described in *Leishmania*: An extracellularly N-terminal, a transmembrane domain and a catalytic C-terminal that associates with intracellular enzymes (Handman et al., 2008; Sanchez et al., 1995).

Our results suggest that the chemorepellent effect exerted by VIP associates with an increased area and shorter flagellum length, while that of NPY is only associated with a decrease in the flagellum length. On the other hand, SP being chemoattractant did not alter neither parasite morphology nor flagellum length. Altogether these results seem to point out that to "swim away" the parasite actively modifies physiological characteristics of the functional swimming propeller, the flagellum. In this regard, at least 15 genes have been identified in *Leishmania* that codes for MAP-kinases. Among them, MPK3, MPK13 and MPK9 are fundamental for changes in the flagellum length (Lopes et al., 2010). Additional results are needed to increase our knowledge in this regard.

VIP derivatives have been shown to be protective against CL by means of membrane-disrupting mechanisms that may associate with changes in ΔVm. Derivatives VIP51 and VIP51(6–30) seem to cover parasite surface in less than 5 min, induce a complete disorganization of the parasite structure (they become round), and cause leakage of the cellular content (Campos-Salinas et al., 2014). As mentioned, a more rounded body area was detected in our hands in the presence of VIP; being done with physiological (10^{-10} M VIP) instead of pharmacological (10^{-6} M VIP) neuropeptide concentrations, and shorter incubation times our experiments suggest the fundamental role that these neuropeptides may have during *Leishmania* skin incursion. Finally, in our hands, the chemotactic effect elicited by the peptides on *L.* (*V.*) *braziliensis* promastigotes gave no conclusive results on an effect on parasite ΔVm. Thus, to dissect whether or not initial processes (our case) or late processes [as evaluated by (Campos-Salinas et al., 2014)] result in ΔVm changes would need sophisticated methods probably not yet available.

In conclusion, the present study provides evidence that vasoactive,

sensory and autonomic neuropeptides exert modulating effects on parasite migration, suggesting a potential role neuropeptide could have on host-parasite interaction, modulating the natural course of the protozoan life cycle. The findings suggest that proteins and molecules potentially involved in the associated receptor cascade, with similar functions to those present in higher eukaryotes, signpost conservation of ancient signaling systems associated with unicellular responses, fundamental for cell survival, i.e., taxis and migration (Kennedy et al., 1997). Further research is needed to elucidate the intrinsic mechanisms of the herein described effects, especially for SP. This neuropeptide could be acting through a different process than the chemorepellent peptides. Progress in this field will potentially provide sound pathomechanistic concepts and novel therapeutic strategies for patients with chronic, and frustrating to treat CL.

As we have previously described the negative chemotactic effect exerted in *L. (V.) braziliensis* promastigotes by the vertebrate/human-type vasoactive molecules adrenomedullin (AM) and calcitonin gene-related peptide (CGRP) (Febres et al., 2018) and demonstrated by immunoblot and *in silico* tools, a putative Receptor Activity Modifier Protein-2 (RAMP-2) homolog in *Leishmania* we can also postulate the potential existence in *Leishmania*, of CRLR (associated) receptors. Fig. 4 summarizes our proposed model, highlighting essential receptors that may be conserved in these ancient parasites.

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Authors' contributions

MG, OV, AF and ADSL: performed the experimental procedures as well as the data analysis as medical students trained at the Laboratory of Molecular Physiology. EDL and APS: were responsible for the project and experimental design, as well as deeply involved in the writing of the manuscript as well as in preparing the figures and tables. APS: is the corresponding author of the article. All authors have approved the final article organization and writing.

CRediT authorship contribution statement

Michelle Giammarressi: Investigation, Methodology, Formal analysis, Data curation. **Oriana Vanegas:** Investigation, Methodology, Formal analysis, Data curation. **Anthony Febres:** Investigation, Methodology, Formal analysis, Data curation. **Adrián Silva-López:** Investigation, Methodology, Formal analysis, Data curation. **Emilia Diaz López:** Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Methodology, Software, Supervision, Validation. **Alicia Ponte-Sucre:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Software, Supervision, Validation, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no competing interest.

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Appendix A. Supplementary data

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References

Ahmed, A., Mutt, V., Nordlind, K., 1999. Modulating effects of sensory and autonomic neuropeptides on murine splenocyte proliferation and cytokine secretion induced by *Leishmania major*. Immunopharmacol. Immunotoxicol. 21, 507–526.

Ahmed, A., Wahb, A., Nordlind, K., 2001. Neuropeptides modulate a murine monocyte/macrophage cell line capacity for phagocytosis and killing of *Leishmania major* parasites. Immunopharmacol. Immunotoxicol. 23, 397–409.

Ahmed, A., Wahbi, A., Nordlind, K., Kharazmi, A., Sundqvist, K., Mutt, V., et al., 1998. In vitro *Leishmania major* promastigote-induced macrophage migration is modulated by sensory and autonomic neuropeptides. Scand. J. Immunol. 48, 79–85.

Alcázar, W., Silva López, A., Alakurtti, S., Tuononen, M.L., Yli-Kauhaluom, J., Ponte-Sucre, A., 2014. Betulin derivatives impair *Leishmania braziliensis* viability and host-parasite interaction by. Bioorg. Med. Chem. 22, 6220–6226.

Augustyniak, D., Jankowski, A., Mackiewicz, P., Skowyra, A., Gutowicz, J., Drulis-Kawa, Z., 2012. Innate immune properties of selected human neuropeptides against *Moraxella catarrhalis* and nontypeable *Haemophilus influenzae*. BMC Immunol. 13, 24.

Berg, K., Clarke, W., 2018. Making sense of pharmacology: inverse agonism and functional selectivity. Int. J. Neuropsychopharmacol. 21 (10), 962–977. https://doi.org/10.1093/ijnp/pyy071.

Campos-Salinas, J., Cavazzuti, A., O' Valle, F., Forte-Lago, I., Caro, M., Beverley, S., et al., 2014. Therapeutic efficacy of stable analogues of vasoactive intestinal peptide against pathogens. J. Biol. Chem. 289 (21), 14583–14599.

Campos-Salinas, J., Gonzalez-Rey, E., 2009. Autophagy and neuropeptides at the crossroad for parasites: to survive or to die? Autophagy 5 (4), 551–554. https://doi.org/10.4161/auto.5.4.8365. Epub 2009 May 6.

Champion, J.A., Mitragotri, S., 2009. Shape induced inhibition of phagocytosis of polymer particles. Pharm. Res. (N. Y.) 26, 244–249.

Choi, J., Di Nardo, A., 2018. Published in: Seminars in Immunopathol. Skin Neurogenic Inflammation, vol. 40. Springer Nature, pp. 249–259, 3.

Couvineau, A., Rouyer-Fessard, C., Maoret, J.J., Gaudin, P., Nicole, P., Laburthe, M., 1996. Vasoactive intestinal peptide (VIP)1 receptor. J. Biol. Chem. 271, 12795–12800.

de Mendoza, A., Sebé Pedrós, A., Ruiz-Trillo, I., 2014. The evolution of the GPCR signaling system in eukaryotes: modularity, conservation, and the transition to metazoan multicellularity. Genome Biol. Evol. 6, 606–619.

Delgado, M., Anderson, P., Garcia-Salcedo, J., Car, M., Gonzalez-Rey, E., 2009. Neuropeptides kill African trypanosomes by targeting intracellular compartments and inducing autophagic-like cell death. Cell Death Differ. 16, 406–416.

Díaz, E., Köhidai, L., Ríos, A., Vanegas, O., Ponte-Sucre, A., 2011. Ensayos de quimiotaxis in vitro en *Leishmania sp*. Evaluación de la técnica de los capilares-dos cámaras en promastigotes. Rev Fac Farmacia UCV 74, 31–40.

Díaz, E., Köhidai, L., Ríos, A., Vanegas, O., Silva, A., Szabó, R., Mező, G., Hudecz, H., Ponte-Sucre, A., 2013. *Leishmania braziliensis*: cytotoxic and chemotactic effects of branched chain polypeptide conjugates with poly [L-Lysine] backbone Experiment Parasitol, 135 (1), 134–141.

Diaz, E., Zacarias, A.K., Pérez, S., Vanegas, O., Köhidai, L., Padrón-Nieves, M., Ponte-Sucre, A., 2015. Effect of aliphatic, monocarboxylic, dicarboxylic, heterocyclic and sulphur-containing amino acids on *Leishmania* spp. Chemotaxis. Parasitology 142 (13), 1621–1630.

Diaz, E., Ponte-Sucre, A., 2018. Leishmaniasis, the biology of a parasite. Drug resistance. In: Ponte-Sucre, A., Padrón Nieves, M. (Eds.), Leishmania Parasites. Consequences, Molecular Mechanisms, and Possible Treatments, second ed. Springer Verlag Wien, ISBN 978-3-319-74185-7, pp. 1–19. ISBN 978-3-319-74186-4 (eBook).

Duboni, M.J., Park, K.S., 2006. The mechanisms of substance P-mediated migration of bone marrow-derived mesenchymal stem cell-like ST2 cells. Int. J. Mol. Med. 37, 1105–1111. https://doi.org/10.3892/ijmm.2016.2496.

Dumont, Y., Quirion, R., 2000. [125I]-GR231118: a high affinity radioligand to investigate neuropeptide Y Y1 and Y4 receptors. Br. J. Pharmacol. 129 (1), 37–46.

Febres, A., Vanegas, O., Giammarresi, M., Gomes, C., Díaz, E., Ponte-Sucre, A., 2018. Is the activity of CGRP and Adrenomedullin regulated by RAMP (-2) and (-3) in Trypanosomatidae? An in-silico approach. Infect. Genet. Evol. 61, 197.

Forestier, C.L., Machu, C., Loussert, C., Pescher, P., Späth, G.F., 2011. Imaging host cell-leishmania interaction dynamics implicates parasite motility, lysosome recruitment, and host cell wounding in the infection process. Cell Host Microbe 9, 319–330.

Handman, E., Goding, J., Papenfuss, A., Speed, T., 2008. *Leishmania* surface proteins. In: Fasel, N., Myler, P. (Eds.), *Leishmania*, after the Genome. Norflok. Caiser Academic Press, pp. 177–204.

Kedzierski, L., Evans, K., 2018. The role of the immune system in resistance to infection. Drug resistance. In: Ponte-Sucre, A., Padrón Nieves, M. (Eds.), Leishmania Parasites. Consequences, Molecular Mechanisms, and Possible Treatments, second ed. Springer Verlag Wien, ISBN 978-3-319-74185-7, pp. 109–142. 978-3-319-74186-4, (eBook).

Kennedy, P.G.E., Rodgers, J., Jennings, F.W., Murray, M., Leeman, S.E., Burke, J.M., 1997. A substance P antagonist, RP-67,580, ameliorates a mouse meningoencephalitic response to *Trypanosoma brucei brucei*. Proc. Natl. Acad. Sci. U. S.A. 94, 4167–4170.

Köhidai, L., Lemberkovics, E., Csaba, G., 1995. Molecule dependent chemotactic responses of Tetrahymena pyriformis elicited by volatile oils. Acta Protozool. 34, 181–185.

Lopes, A., Gomes, M., Dutra, F., Vermelho, A., Meyer-Fernandes, J., Silva-Neto, M., et al., 2010. Intracellular signaling pathways involved in cell differentiation in trypanosomatids. Open Parasitol. J. 4, 102–110.

Muschter, D., Beiderbeck, A.S., Späth, T., Kirschneck, C., Schröder, A., Grässel, S., 2019. Sensory Neuropeptides and their receptors participate in mechano-regulation of murine macrophages. Int. J. Mol. Sci. 20 (3), 503. https://doi.org/10.3390/ijms20030503.

Padrón-Nieves, M., Diaz, E., Machuca, C., Rodriguez, N., Cotrim, P., Ponte-Sucre, A., 2014. Correlation between glucose uptake and membrane potential in *Leishmania* parasites isolated from DCL-patients with therapeutic failure: a proof of concept. Parasitol. Res. 113 (6), 2121–2128.

Peters, E., Ericson, M., Junichi, H., Hordinsky, M., Seiffert, K., Ansel, J.C., et al., 2006. Neuropeptide control mechanisms in cutaneous. Biology: physiological and clinical significance. J. Invest. Dermatol. 126, 1937–1947.

Sanchez, M., Zeoli, D., Klamo, E., Kavanaugh, M., Landfear, M., 1995. A family of putative receptor-adenylate cyclases from Leishmania donovani. J. Biol. Chem. 270, 17551–17558.

Steverding, D., 2017. The history of leishmaniasis. Parasitol Vectors 10, 82–91. https://doi.org/10.1186/s13071-017-2028.

Zerpa, O., Padrón Nieves, M., Ponte-Sucre, A., 2018. American tegumentary leishmaniasis. Drug resistance. In: Ponte-Sucre, A., Padrón Nieves, M. (Eds.), Leishmania Parasites. Consequences, Molecular Mechanisms, and Possible Treatments, second ed. Springer Verlag Wien, ISBN 978-3-319-74185-7, pp. 177–193. ISBN 978-3-319-74186-4 (eBook).