AVT is involved in the regulation of ion transport in the intestine of the sea bream (*Sparus aurata*)

J.A. Martos-Sitcha[1,2,3], S.F. Gregório[1], E.S.M. Carvalho[1], A.V.M. Canario[1], D.M. Power[1], J.M. Mancera[2], G. Martínez-Rodríguez[3] & J. Fuentes[1]

(1) Centre of Marine Sciences (CCMar), CIMAR - Laboratório Associado, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

(2) Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, E-11510 Puerto Real (Cádiz), Spain

(3) Instituto de Ciencias Marinas de Andalucía, Consejo Superior Investigaciones Científicas (ICMAN-CSIC), E-11510 Puerto Real (Cádiz), Spain

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Author for correspondence:
Juan Fuentes (jfuentes@ualg.pt)
CCMar, CIMAR - Laboratório Associado,
Universidade do Algarve
Campus de Gambelas,
8005-139 Faro, Portugal

ABSTRACT

The intestine of marine fish plays a crucial role in ion homeostasis by selective processing of ingested fluid. Although arginine vasotocin (AVT) is suggested to play a role in ion regulation in fish, its action in the intestine has not been demonstrated. Thus, the present study investigated *in vitro* the putative role of AVT in intestinal ion transport in the sea bream (*Sparus aurata*). A cDNA encoding part of an AVT receptor was isolated and phylogenetic analysis revealed it clustered with the V1a2-type receptor clade. *V1a2* transcripts were expressed throughout the gastrointestinal tract, from esophagus to rectum, and were most abundant in the rectum regardless of long-term exposure to external salinities of 12, 35 or 55 p.p.t. Basolateral addition of AVT (10^{-6} M) to the anterior intestine and rectum of sea bream adapted to 12, 35 or 55 p.p.t. mounted in Ussing chambers produced rapid salinity and region dependent responses in short circuit current (Isc), always in the absorptive direction. In addition, AVT stimulation of absorptive Isc conformed to a dose-response curve, with significant ~~threshold~~ effects achieved at 10^{-8} M, which corresponds to physiological values of plasma AVT for this species. The effect of AVT on intestinal Isc was insensitive to the CFTR selective inhibitor NPPB (200 μM) applied apically, but was completely abolished in the presence of apical bumetanide (200 μM). We propose a role for AVT in the regulation of ion absorption in the intestine of the sea bream mediated by an absorptive bumetanide-sensitive mechanism, likely NKCC2.

Keywords: osmoregulation, arginine vasotocin, *V1a2* receptor, sea bream, salinity, short circuit, water absorption.

INTRODUCTION

The intestine of marine fish plays a key role in ion regulation. The ionic disequilibrium of marine fish with their surrounding environment requires high rates of water ingestion (drinking) as part of the osmoregulatory process to compensate the dehydrating effect of seawater in the gills (Evans et al., 2005). In this context, ion assimilation from the ingested fluid is required to drive water absorption, making the role of the intestine vital to maintain extracellular homeostasis. Water absorption seems to rely on Cl^- uptake, which is mediated by an apical $Na^+/K^+/2Cl^-$ co-transporter (Musch et al., 1982) or apical Cl^-/HCO_3^- anion exchangers (Grosell, 2006, 2011), both mechanisms are active in the sea bream intestine (Carvalho et al., 2012; Gregorio et al., 2013). A basolateral Na^+,K^+-ATPase generates the electrogenic potential to facilitate apical Cl^- absorption (Ferlazzo et al., 2012). Accordingly, higher intestinal Na^+,K^+-ATPase activity is detected in seawater compared to freshwater trout (Fuentes et al., 1997) and is consistent with higher ATPase subunit expression in seawater compared to freshwater eels (Kalujnaia et al., 2007).

Arginine vasotocin (AVT) is a peptide secreted from the neurohypophysis of non-mammalian vertebrates, whereas arginine vasopressin (AVP) is the structural and functional counterpart in mammals (Acher, 1993). The AVT/AVP family exerts its endocrine action by binding specific plasma membrane receptors and has diverse physiological actions ranging from behavior, stress, reproduction, and vascular control to osmoregulation (Balment et al., 2006; Goodson, 2008; Kulczykowska, 2007; Mancera et al., 2008). Three AVP receptors (AVPRs) are described in mammals and retain a specific and well-established tissue distribution, e. g. the AVPR type V1a, is associated with vascular smooth muscle; the AVPR V1b, is in pituitary corticotrophs; and the AVPR V2 is in the kidney and linked to renal hydrosmotic actions (Mahlmann et al., 1994; Warne, 2001). In contrast, AVTRs in teleost fish have a broader tissue distribution. Thus, AVTR V1 or V2 subtypes have been described in the central nervous system and in tissues such as gill, kidney, gonads or the gastrointestinal tract (Konno et al., 2009; Lema, 2010).

It seems likely that the prevailing action of the AVT/AVP system is directed to preservation of blood volume and osmolality (Warne et al., 2002). Thus, AVP stimulates for instance tubular Na^+ transport by activation of Na^+ channels present in the apical membranes of the kidney tubule (Mordasini et al., 2005; Schafer et al., 1990). Yet, in other epithelia, such as the bronchial epithelium, AVP stimulates Cl^- (and the resulting fluid) secretion via an NPPB-sensitive, likely CFTR dependent mechanism (Bernard et al., 2005). Other

electrophysiological studies have demonstrated that AVP alters NaCl absorption with K^+ requirement in the mouse kidney through Na-K-2Cl (NKCC) co-transporter (Hebert and Andreoli, 1984; Sun et al., 1991), an action that seems to be mediated by recruitment of co-transporter to the apical membrane (Molony et al., 1987).

Several studies have demonstrated the importance of AVT in ion and water regulation in fish (Balment et al., 2006; Kulczykowska, 1997, 2001; Warne and Balment, 1995). Most of these studies focused on the effects of AVT in ion regulation and demonstrated it parallels AVP actions in mammals. For instance, in trunk kidney preparations of rainbow trout (Amer and Brown, 1995; Warne et al., 2002) and dogfish, *Scyliorhinus canicula* (Wells et al., 2002), AVT decreased urine output, an action probably linked to vascular effects. Additionally, a regulatory action of AVT on Cl^- secretion occurs in cultured branchial pavement cells from sea bass (*Dicentrarchus labrax)* (Avella et al., 1999; Guibbolini and Avella, 2003), and the effect is DPC-sensitive and likely mediated by CFTR. In fish, there is also indirect indication for a putative action of AVT in the intestine. Thus, intracerebroventricular injections of AVT caused a reduction in water intake in eels kept in seawater (Kozaka et al., 2003). Additionally, the presence of AVT receptors in the gastrointestinal tract of the rainbow trout was inferred from the action of AVT on contraction of intestinal strips *in vitro (Conklin et al., 1999)* .

The physiological actions of AVT in the intestine of marine fish have received little attention despite its potential and intrinsic importance in ion uptake/secretion. Therefore, the present study determined AVT function in the gilthead sea bream (*Sparus aurata*) intestine by establishing: i) the distribution of AVTR V1a2-type, ii) the response of AVTR to modified external salinity and; iii) the effect *in vitro* of AVT on ion absorption/secretion.

MATERIALS AND METHODS

Peptides and Chemicals

Arginine vasotocin ([Arg8]-Vasotocin acetate), 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and Bumetanide (Bum) of the highest grade were purchased from Sigma-Aldrich (Madrid, Spain).

Animals

Sea bream (*Sparus aurata*) juveniles were obtained from commercial sources (Cupimar S.A., Cádiz, Spain). Fish were quarantined for 60 days in Ramalhete Marine Station (University of Algarve, Faro, Portugal) in 1000 L tanks with running seawater at a density <5 kg/m^3 and

handfed twice daily to a final ration of 2% body weight, with a commercial sea bream diet (Sorgal, Portugal). Fish were acclimated for at least 1 month before experimentation in flowing seawater (salinity 35 p.p.t.; water temperature 16-20ºC) under natural photoperiod for spring in the Algarve, Portugal. In all experiments, food was withheld for 36 h before sacrifice and tissue collection to ensure the absence of undigested food in the intestine.

For salinity adaptation juvenile sea bream (n=90; 20-30 g body weight) were separated into three equal groups and transferred to 250 L tanks in 3 independent closed water circuits with biological filters maintained at final salinities of 12, 35 or 55 p.p.t., temperature of 21°C and a 14:10 L:D photoperiod. Increase in salinity was achieved by adding Instant Ocean sea-salts to control seawater (35 p.p.t.), and decrease in salinity was achieved by dilution of full seawater with dechlorinated tap water. Fish were kept at different salinities for two months before tissue collection and were considered to be fully adapted (Laiz-Carrion et al., 2005). No mortality was registered during the trial and fish fed normally.

All animal manipulations were carried out in compliance with the Guidelines of the European Union Council (86/609/EU) and Portuguese and Spanish legislation for the use of laboratory animals. All animal protocols were performed under a “Group-I” licence from the Direcção-Geral de Veterinária, Ministério da Agricultura, do Desenvolvimento Rural e das Pescas (Portugal).

Cloning of AVT Receptor

For cloning of the AVT Receptor degenerate primers were designed (Table I) against highly conserved cDNA sequences of piscine species: *Platichthys flesus*: (GenBank accession number AF184966); *Astatotilapia burtoni*: (acc. no. AF517936); *Catostomus commersoni*: (acc. no. X76321); *Cyprinodon variegatus* (*V1a1*; acc. no. GU120189); *Cyprinodon variegatus* (*V1a2*; acc. no. GU120190); *Cyprynodon nevadensis amargosae* (*V1a1*; acc. no. GU014233); *Cyprynodon nevadensis amargosae* (*V1a2*; acc. no. GQ981413). Tissues of unfed fish were collected into RNA Later (Sigma-Aldrich, Madrid, Spain) incubated for 24 hours at 4ºC and stored at -20ºC until RNA extraction (within two weeks). Total RNA was isolated from brain, liver, kidney and gills using RNeasy® Plus MiniKit (Quiagen, Hidlen, Germany). Genomic DNA (gDNA) was eliminated with gDNA Eliminator spin columns (Quiagen, Hidlen, Germany). After reverse transcription of 500 ng total RNA (Super Script III, Invitrogen™ ™ , Paisley, UK) PCR amplifications were carried out with Platinum® Taq DNA Polymerase (Invitrogen) using the following PCR conditions: 94ºC, 1 min and 35 cycles

of 94ºC, 30 sec; 50ºC, 30 sec; 72ºC, 1 min and finalized with 72ºC, 10 min. PCR products were cloned into TA Vectors (TOPO TA Cloning® Kit; Invitrogen™, Paisley, UK), sequenced and identity confirmed by interrogating NCBI databases using the blastn protocol (Altschul et al., 1990).

To establish receptor identity/subtype amino acid sequences were retrieved from the NCBI protein database (www.ncbi.nlm.nih.gov, accessed in January 2013) and phylogenetic analysis of AVTR/AVPR translated sequences was performed using MEGA5 software (Tamura et al., 2011) with the Close-Neighbor-Interchange algorithm, based on amino acid differences (p-distances) and pairwise deletions. Reliability of the phylogenetic tree was assessed using bootstrap values (1,000 replicates).

AVTR V1a2 in the sea bream intestine

For AVTR V1a2-type expression analysis, specific primer pairs were designed using the software Primer3 (available in http://frodo.wi.mit.edu/) to amplify a 149 bp of the AVTR V1a2-type (Table I). Total RNA was isolated using the E.Z.N.A. Total RNA Kit (OMEGA Bio-tek, Norcross, GA, USA) following the manufacturer's instructions, and the quantity and quality assessed (Nanodrop 1000, Thermo Scientific, Barrington, IL, USA). Total RNA was treated with DNase using an RNAse-free DNase kit (Ambion, Life Technologies, Paisley, UK). Total RNA (500 ng) was reverse transcribed (RevertAid™ First Strand cDNA Synthesis Kit, #K1622, Fermentas, Thermo Scientific, Barrington, IL, USA)

Real-time qPCR amplifications were performed in duplicate in a final volume of 10 □l with 5 □l SsoFast EvaGreen Supermix (Bio- Rad, Hercules, CA, USA) as the reporter dye, 200 ng cDNA, and 0.5 pM of each forward and reverse primers. Amplifications were performed in 96-well plates using the *One-step Plus* sequence detection system (Applied Biosystems, Foster City, CA, USA) with the following protocol: denaturation and enzyme activation step at 95°C for 2 min, followed by 40 cycles of 95°C for 5 sec and 60°C for 10 sec. After the amplification phase, a temperature-determining dissociation step was carried out at 65°C for 15 s, and 95°C for 15 s. For normalization of cDNA loading, all samples were run in parallel using 18S ribosomal RNA (*18S*). To estimate efficiencies, a standard curve was generated for each primer pair from 10-fold serial dilutions (from 10 to 0.001 ng) of a pool of first-stranded cDNA template from all samples. Standard curves represented the cycle threshold value as a function of the logarithm of the number of copies generated, defined arbitrarily as one copy for the most diluted standard. All calibration curves exhibited correlation coefficients

158 R^2>0.98, and the corresponding real-time PCR efficiencies were >99%. Following this
159 general method AVTR V1a2-type mRNA distribution in the gastrointestinal tract was
160 analyzed in esophagus, stomach, pyloric caeca, anterior intestine, mid intestine and rectum of
161 sea bream adapted to 35 p.p.t. In addition, the response of AVTR V1a2-type mRNA to
162 external salinity was analyzed in the anterior intestine and in the rectum in sea bream adapted
163 long-term to 12, 35 and 55 p.p.t. external salinity.
164

165 **Short-circuit current (Isc) measurements**

166 The anterior intestine and rectum were collected, isolated and mounted in Ussing chambers as
167 previously described (Gregorio et al., 2013). Briefly, tissue was placed on a tissue holder of
168 0.71 cm^2 and positioned between two half- chambers containing 2 mL of physiological saline
169 (NaCl 160mM; $MgSO_4$ 1mM; NaH_2PO_4 2mM; $CaCl_2$ 1.5mM; $NaHCO_3$ 5mM; KCl 3mM;
170 Glucose 5.5mM; HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-
171 Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)) 4mM), at a pH of 7.80. During the
172 experiments the tissue was bilaterally gassed with 0.3% CO_2 + 99.7 O_2 and the temperature
173 maintained at 22°C. Short circuit current (Isc, $\mu A/cm^2$) was monitored by clamping of
174 epithelia to 0 mV. Epithelial resistance (Rt, $\Omega.cm^2$) was manually calculated (Ohm's law)
175 using the current deflections induced by a 2 mV pulse of 3 sec every minute. Voltage
176 clamping and current injections were performed by means of a DVC-1000 voltage clamp
177 amplifier (WPI, Sarasota, USA) or a VCCMC2 (Physiologic Instruments, San Diego, USA).
178 Bioelectrical parameters for each tissue were recorded after the tissue achieved a steady state,
179 which occurred between 30-40 min after mounting.
180 The response to a single dose of 10^{-6} M AVT was analyzed in preparations of anterior
181 intestine and rectum collected from sea bream adapted long-term to 12, 35 and 55 p.p.t.
182 external salinity. To test if AVT effects on intestinal Isc conform to typical dose-response
183 effects, rectal tissue from fish adapted to 55 p.p.t. salinity were collected and mounted in
184 Ussing chambers as described. After an initial period of Isc stabilization, tissues were treated
185 at 45 min intervals with doses of 10^{-10} to 10^{-6} M AVT at increased 10-fold intervals covering
186 the range of circulating plasma values of AVT in the sea bream (Kleszczyska et al., 2006;
187 Mancera et al., 2008).
188 Apical NPPB (200 μM) or Bumetanide (200 μM) were applied alone or in combination with
189 10^{-6} M AVT in rectal tissue of fish adapted to 35 or 55 p.p.t. to identify the AVT-responsive

mechanism. Concentration of specific blockers were selected to guarantee 100% inhibitory effects on a putative CFTR (NPPB) or a NKCC (Bumetanide)

Statistics

Results are presented as means ± SEM unless otherwise stated. After normality and homogeneity of variance were checked, comparison between groups was analyzed as appropriate using the Student's t-test or One-way analysis of variance (ANOVA) followed by the post-hoc Bonferroni test. All statistical analysis was performed with Prism 5.0 (GraphPad Software for Macintosh) and groups were considered significantly different at $p<0.05$.

RESULTS

cDNA for AVT Receptor

A cDNA for a partial AVT receptor spanning 392 bp of the coding sequence was isolated and clusters with the AVTR V1a2-type as shown by phylogenetic analysis (Figure 1).

AVTR V1a2 is expressed throughout the intestinal tract: esophagus, stomach, pyloric caeca, anterior intestine, mid intestine and rectum (Figure 2A). Furthermore expression increased in more distal portions of the intestinal tract, with highest expression in the rectum when compared to all other regions (Figure 2B).

After acclimation of sea bream to external salinities of 12, 35 and 55 p.p.t., AVTR V1a2 transcript abundance in the anterior intestine paralleled decreases/increases of external salinity with significantly lower expression levels at 12 p.p.pt. (Figure 3). In addition, the transcript abundance of AVTR V1a2 was significantly higher in the rectum compared to the anterior intestine at all salinities.

AVT intestinal electrophysiology

Basal values of short circuit current (Isc, mA/cm^2) and tissue resistance (Rt, $\Omega.cm^2$) are shown in Table II. Positive Isc recorded show secretory currents while absorptive currents are shown by negative values. Control preparations sustained constant Isc and Rt for the duration of the experimental periods (up to 3.5 hours). In general the effects of salinity followed the pattern previously described for the sea bream intestine (Gregorio et al., 2013). In the anterior intestine a small absorptive current was observed in fish at 35 and 55 p.p.t., while at 12 p.p.t. a secretory current was recorded (Table 2). In contrast the rectum of fish acclimated to 35 and

55 p.p.t. showed a secretory current, which in low salinity adapted fish was highly variable but averaged a small absorptive current.

The basolateral application of 10^{-6} M AVT to preparations of anterior intestine and rectum from fish at 55, 35 and 12 p.p.t did not affect tissue resistance (Rt, data not shown). In contrast, AVT evoked a rapid stimulatory action on Isc, (Figure 4) in the absorptive direction in all intestinal regions (the current became either more negative or less positive). The effects of AVT on Isc were rapid, with an onset between 3-5 min of application and reached maximal effects within 25-30 min after application (Figure 4A). Interestingly, the effect of AVT in the anterior intestine was higher in fish adapted to 12 p.p.t., while in the rectum it was higher in fish adapted to 55 p.p.t.

To test whether AVT effects on Isc conform to a typical dose-response curve AVT (10^{-10} - 10^{-6} M) was sequentially applied to rectal tissue of fish adapted to 55 p.p.t. (as the highest responder to AVT). The dose-response effects of AVT on Isc were linear, with no apparent plateau, ~~and a threshold~~ with significant effects ~~occurred~~ at concentrations of 10^{-8} M AVT with Isc decreases of -6.2 $\mu A/cm^2$ and a maximal effect of -13.2 $\mu A/cm^2$ ~~was~~ achieved with 10^{-6} M AVT.

Higher responses to AVT were obtained in tissues with a positive short circuit current presumably secretory (Table 2). To test the dependence of these currents on a putative CFTR, a selective inhibitor NPPB (200 μM) was applied apically to preparations of rectum collected from fish adapted to 35 and 55 p.p.t. NPPB produced a significant fall in the Isc recorded i.e. -4 $\mu A/cm^2$ in the rectum of 35 p.p.t. acclimated fish and -14 $\mu A/cm^2$ in the rectum of 55 p.p.t. acclimated fish (Figure 6).

To test whether the effects of AVT on Isc were mediated by regulation of the secretory or absorptive pathways, rectal tissues of fish acclimated to 35 and 55 p.p.t. were tested in Ussing chamber in the presence or absence of apical NPPB (200 μM) or bumetanide (200 μM). NPPB did not modify the response to 10^{-6} M AVT of rectal tissue from fish acclimated to 55 or 35 p.p.t seawater (Figure 7). In contrast, apical bumetanide (200 μM) completely abolished the epithelial response to AVT (Figure 7) in 35. p.p.t. fish (no fish acclimated to 55 p.p.t. were available for testing)

DISCUSSION

In keeping with the proposed role for AVT in ion and water balance in teleosts (Balment et al., 2006; Kulczykowska, 1997, 2001; Warne and Balment, 1995), the present study

established the intestine of marine teleosts as a target for a physiological role for the vasotocinergic system. We show the heterogeneous distribution and salinity dependence of the expression of a V1a2-type AVT receptor in the gastrointestinal tract and characterized the involvement of AVT on ion movements in Ussing chambers. The effect of AVT on ion transport in the intestine seems to be mediated by a bumetanide sensitive mechanism, likely a NKCC co-transporter.

The sea bream cope with large changes in environmental salinity ranging from 5 to 60 p.p.t. (Laiz-Carrión et al., 2005), but are unable to withstand freshwater (Fuentes et al., 2010a). Acclimation to salinity occurs through water absorption and ion balance mechanisms in the gills and intestine (Fuentes et al., 2006; Fuentes et al., 2010b; Gregorio et al., 2013; Raldúa et al., 2008). Furthermore, the gastrointestinal tract of the sea bream is a target for endocrine regulation of ion transport (Ferlazzo et al., 2012; Fuentes et al., 2010a; Fuentes et al., 2006; Fuentes et al., 2010b). Previous studies in vertebrates have demonstrated the widespread distribution of receptors for AVP/AVT in the intestine of birds (Tan et al., 2000), amphibians (Hasunuma et al., 2010) and mammals (Lolait et al., 1992; Morel et al., 1993). In fish, the intestinal expression of AVT receptor V1a-type and V2-type have been demonstrated in the African lungfish, *Protopterus annectens* (Konno et al., 2009). In addition, in the Amargosa River pupfish (*Cyprinodon nevadensis amargosae*) three different types of AVT receptors i.e. V1a1-type, V1a2-type and V2-type, are described (Lema, 2010). Our analysis of the sea bream partial AVT receptor indicates that it corresponds to an AVTR V1a2-type, one of the subtypes of the AVTR V1a described in teleosts (Figure 1), although the subtype V1a of the AVTR/AVPR has been associated with vascular smooth muscle (V1a1/V1) or preferentially described in the pituitary corticotrophe cells (V1a2/V1b) (Mahlmann et al., 1994; Verbalis, 2002). The expression of the AVTR V1a2-type receptor throughout the gastrointestinal tract of the sea bream highlights a potential role for the vasotocinergic system in the intestine. Furthermore, expression of AVT V1a2 is high in the esophagus, and there is an apparent antero-posterior increase from pyloric caeca to rectum, where it is expressed at the highest level (Figure 2). Additionally, salinity seems to act as regulatory trigger for AVT receptor expression in the Amargosa River pupfish (Lema, 2010), although, the only receptor that seems to sustain higher branchial expression in response to salinity is the V1a2-subtype, at least at 20 h post challenge (Lema, 2010). In the sea bream, we show that a long-term challenge with varying external salinity impacts the expression of V1a2-type AVT receptor in the intestine (Figure 3). It is tempting to suggest that the regionalization and transcriptional

response to salinity of the V1a2-type AVT receptor are both related to functional specificity in ion regulation of discrete regions of the intestine in sea bream as has previously been described (Gregorio et al., 2013).

The intestine of fish maintains both absorptive and secretory pathways. Cl^- uptake via NKCC co-transporters (Musch et al., 1982) or apical anion Cl^-/HCO_3^- exchangers (Grosell, 2011; Grosell et al., 2005) act to produce the driving force for water absorption. Additionally, the intestinal epithelium of *Fundulus heteroclitus* mounted in Ussing chambers can switch from net Cl^- absorption to net Cl^- secretion as shown by directional changes of the tissue short circuit current (Marshall et al., 2002). The sea bream intestine has preferential Cl^- absorption or secretion depending on external salinity and intestinal region (Carvalho et al., 2012; Gregorio et al., 2013). In the present study we describe how changes in external salinity and target tissue condition the response to a single dose of AVT in the anterior intestine and the rectum of the sea bream (Figure 4A). A consistent feature of the responses to AVT by the sea bream intestine *in vitro* is the consistency of the effect, always in the absorptive direction. AVT makes Isc less positive (or more negative) in all cases either by decreasing secretion or by increasing absorptive currents. However, the magnitude of absorption stimulation is different in different regions of the sea bream intestine. Interestingly, preparations from tissues with positive currents i.e. secretory (Isc>0) have the highest response to AVT and this is the case of the anterior intestine from fish maintained at low salinity (12 p.p.t.) and the rectum of fish kept in hyperosmotic conditions i.e. 35 and 55 p.p.t. (Figure 4B). In the latter we show that apical application of NPPB, a potent chloride channel blocker specific to CFTR (McCarty, 2000), results in decreases of the basal current of -3.5 $\mu A/cm^2$ in fish at 35 p.p.t. and -14 $\mu A/cm^2$, in fish at 55 p.p.t. demonstrating the presence of a functional CFTR.

AVT has a stimulatory action on Cl^- secretion in cultured pavement cells of the sea bass gill via a DPC-sensitive mechanism, likely CFTR (Avella et al., 1999; Guibbolini and Avella, 2003). Surprisingly, the effect of AVT on Isc in the intestine of the sea bream paralleled in direction and magnitude the inhibitory effect of NPPB on basal currents specifically in the rectum, that could be the reflection of a pharmacological effect of the high concentration of AVT (10^{-6} M) in the Ussing chamber. However, the effects of AVT followed a linear dose-response in the range of 10^{-10}-10^{-6} M and the effect of AVT has no apparent plateau up to the highest concentration of AVT used (10^{-6} M). This is in keeping with the previous report of heterologous expression of *P. flesus* AVT receptor in *Xenopus* oocytes where the response to AVT did not reach a plateau at concentrations in a range of 10^{-14} to 10^{-10} M (Warne, 2001).

Furthermore, regardless of the concentration, AVT actions are not mediated by regulation of the secretory pathway *via* CFTR. On the contrary a consistent single stimulatory effect on the absorptive pathway was observed. This is also supported by the unchanged stimulatory effect of AVT on the absorptive current recorded in the presence or absence of apical NPPB (Figure 7). Remarkably, the hormone level at which a significant response to AVT occurred was 10^{-8} M, which falls within circulating plasma levels (2-30 nM) of AVT in the sea bream (Kleszczyska et al., 2006; Mancera et al., 2008). The present results in the sea bream contrast with previous reports in tilapia where AVT (2 μg/mL) was without effect on water, sodium and chloride transport in the anterior intestine either in freshwater- or seawater-adapted fish (Mainoya, 1985). However, our results support an important physiological role for AVT in the regulation of ion transport in the marine fish intestine *in vivo*. This role for AVT is likely more important during hyperosmotic adaptation and is probably linked with the functional specialization of the sea bream intestine, specially at high salinities (Gregorio et al., 2013). Interestingly, high salinity challenge results in coordinated increases/decreases of plasma AVT/pituitary storage, respectively (Balment et al., 2006; Kulczykowska, 1997, 2001; Warne and Balment, 1995). Therefore, the linear response of the sea bream intestine to increasing doses of AVT indicates that the receptor is not a limiting factor of the biological action in ion transport.

In rats AVP regulates the expression of secretory-type Na-K-2Cl co-transporter (NKCC1) mRNA and protein in a dose-dependent manner in the outer medullary-collecting duct (OMCD), and therefore promotes the secretory pathway (Wakamatsu et al., 2009). However, AVP also controls NKCC2, the absorptive form of the Na-K-2Cl co-transporter (Ares et al., 2011). It seems that the action of AVT in the intestine of the sea bream is achieved solely by its stimulatory effect on the absorptive pathway, which is preferentially mediated by the Na-K-2Cl co-transporter (Musch et al., 1982). The loop diuretic bumetanide is a specific inhibitor of the NKCC (Haas, 1994) and when applied apically to sea bream rectum preparations, abolished almost completely the stimulatory effect of AVT in the absorptive Isc (Figure 7), and indicates there is functional association, likely similar to that described in the thick ascending limb of the loop of Henle in terrestrial vertebrates (Ares et al., 2011). However, it remains to be established if these effects are achieved solely via the AVT V1a2-receptor type.

In conclusion, the present study provides strong evidence that AVT is involved in the regulation of ion transport in the intestine of the sea bream. At least one AVT receptor, which

355 is distributed throughout the gastrointestinal tract, is expressed in response to salinity
356 challenge in different intestinal regions. The intestinal of the sea bream *in vitro* responds to
357 stimulation with AVT, an effect that is region and salinity dependent. ~~Threshold~~ Lowest
358 effective doses for significant effects of AVT at concentrations similar to circulating plasma
359 levels of the hormone support the physiological relevance of this regulation, which is likely
360 achieved via apical NKCC2.
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533 **Table I**. Primers used for molecular identification of partial cDNA sequences of the AVT-R
534 and for qPCR expression analysis.
535

Degenerate primers	**Nucleotide sequence**
AVT-RFw	5'- AGCGTSCTGCTGGCSATG -3'
AVT-RRv	5'- GCAKATRAASCCGTAGCA -3'
AVT-Rnested-Fw	5'- AARCACCTSAGCCTBGCBGA -3'
AVT-Rnested-Rv	5'- GTBATCCAGGTGATGTASGC -3'
qPCR primers	
AVTRFw	5'- TTATCTCAACGTGGATGTGCAG-3'
AVTRRv	5'- TTACCAGGTGATGTAGGCCTTG -3'
18sFw	5'- AACCAGACAAATCGCTCCAC-3'
18sRv	5'- CCTGCGGCTTAATTTGACTC-3'

536

537 **Table II**. Bioelectrical properties of discrete regions of the intestine of the sea bream mounted
538 in Ussing chambers and short-circuited to Vt = 0 mV. Fish underwent long-term acclimation
539 to water salinities of 12, 35 and 55 p.p.t. The number of fish utilized for each portion of
540 intestine and each salinity is indicated in brackets. Isc (μAmp/cm^2) and Rt (Ω.cm^2) within the
541 same intestinal region displaying different letters were significantly different ($p<0.05$, One-
542 way ANOVA followed by Bonferroni post-hoc test). Asterisks represent significant
543 differences between intestinal regions at the same salinity ($p<0.05$, Student t-test).
544

		Isc (μAmp/cm^2)	**Rt (Ω.cm^2)**
Anterior intestine	**12 p.p.t. (n=11)**	4.62±2.36^{a}	72.29±13.00^{a}
	35 p.p.t. (n=12)	-6.99±1.77^{b}	75.57±10.71^{a}
	55 p.p.t. (n=13)	-4.06±2.11^{b}	113.24±12.75^{b}
Rectum	**12 p.p.t. (n=11)**	-1.37±1.78^{a}*	65.05±4.08 ^{a}*
	35 p.p.t. (n=12)	3.93±1.06^{b}*	64.81±4.55^{a}*
	55 p.p.t. (n=11)	16.35±2.26^{c}*	90.93±9.98^{b}*

545

546 **FIGURE LEGENDS**

547

548 **Figure 1**. AVP/AVT receptor phylotree inferred using the Maximum Parsimony (MP)
549 method and 1000 bootstrap replicates. The MP tree was obtained using the Close-Neighbor-
550 Interchange algorithm in MEGA5 using alignments generated by CLUSTALW. GenBank and
551 Ensembl accession numbers of amino acid sequences are as follows: *Oryzias latipes* V1a1
552 (BAL45623), V1a2 (BAL45624) and V2 (BAJ04637); *Thalassoma bifasciatum* V1a1
553 AFJ96998) and V1a2 (BAL70406); *Epineohelus adscensionis* V1a1 (AEI54996) and V1a2
554 (ADO33897); *Cyprinodon nevadensis amargosae* V1a1 (ACY07771), V1a2 (ACY07772)
555 and V2 (ACX85730); *Cyprinodon variegatus* V1a1 (ACY74366), V1a2 (ACY74367) and V2
556 (ACY74368); *Homo sapiens* V1a (AAD17891) and V2 (ACR39021); *Amphiprion ocellaris*
557 V2 (BAL70406); *Protopterus annectens* V2 (AB377532); and *Polypterus senegalus* V2
558 (BAJ04635)

559

560 **Figure 2**. Distribution of AVT V1a2-type receptor in discrete sections of the gastrointestinal
561 tract of the sea bream adapted to seawater (35 p.p.t.). A, Ethidium bromide stained gels. B,
562 Relative expression of AVTR as measured by qPCR. Results are presented as average + SEM
563 (n=4). Columns displaying different letters are significantly different ($p<0.05$, One-way
564 ANOVA followed by Bonferroni post-hoc test). Key to the figure: Eso: oesophagus; Sto:
565 stomach; PC: pyloric caeca; Ant. intest.: anterior intestine; Mid intest.: mid intestine; -C:
566 negative control.

567

568 **Figure 3**. Relative expression of AVT V1a2-type receptor in the anterior intestine and the
569 rectum of the sea bream after long-term acclimation to 12, 35 and 55 p.p.t seawater. Results
570 are shown in arbitrary units (AVTR/18S) determined by qPCR. Each column represents the
571 average $\pm$ SEM (n=7). Within intestinal region columns displaying different letters are
572 significantly different ($p<0.05$, One-way ANOVA followed by Bonferroni post-hoc test).
573 Asterisks represent significant differences between intestinal regions at the same salinity
574 ($p<0.05$, Student t-test).

575

576 **Figure 4.** A. Original trace of short-circuit current (Isc, $\mu A/cm^2$) recorded after basolateral
577 application of AVT 10^{-6} M to the rectum from sea bream acclimated to 55 p.p.t. Vertical
578 current deflections are generated by ± 1 mV pulses to calculate Rt. B. Changes in the effects
579 of basolateral AVT 10^{-6} M in the short circuit (ΔIsc, $\mu Amp/cm^2$) in anterior intestine and

580 rectum of the sea bream. Fish underwent long-term acclimation to changing external salinity
581 12, 35 and 55 p.p.t. Each column represents the average + SEM (n= 6-8). Within intestinal
582 region columns displaying different letters are significantly different (p<0.05, One-way
583 ANOVA followed by Bonferroni post-hoc test).
584
585 **Figure 5**. Changes in short-circuit current (ΔIsc, μAmp/cm^2) measured in Ussing chambers in
586 response to consecutive basolateral addition of AVT (10^{-10}-10^{-6} M) to rectum from sea bream
587 acclimated to 55 p.p.t. Each point represents the average ± SEM (n=3). Asterisks represent
588 significant differences from controls (p<0.05, One-way ANOVA followed by Bonferroni
589 post-hoc test).
590
591 **Figure 6**. NPPB-sensitive short-circuit current (Isc, μAmp/cm^2) in the rectum of sea bream
592 adapted to 35 or 55 p.p.t. Basal values of Isc are shown for epithelia after a stabilization
593 period followed by apical addition of NPPB (200 μM). Results are shown as mean ± SEM
594 (n=5-6). Asterisks represent significant differences from basal values (p<0.05, Student's t-
595 test).
596
597 **Figure 7**. AVT-dependent short circuit current (ΔIsc, μAmp/cm^2) in the rectum of juvenile
598 sea bream acclimated to 35 or 55 p.p.t. meassured in Using chambers. AVT was tested alone
599 (AVT; 10^{-6} M) or in combination with apical NPPB (+NPPB, 200 μM) or bumetanide (+Bum,
600 200 μM). ND, Not determined. Results are shown as mean+SEM (n=6). Asterisks represent
601 significant differences from AVT alone (p<0.05, One-way ANOVA followed by Bonferroni
602 post-hoc test).

Figure 1. Martos-Sitcha *et al*

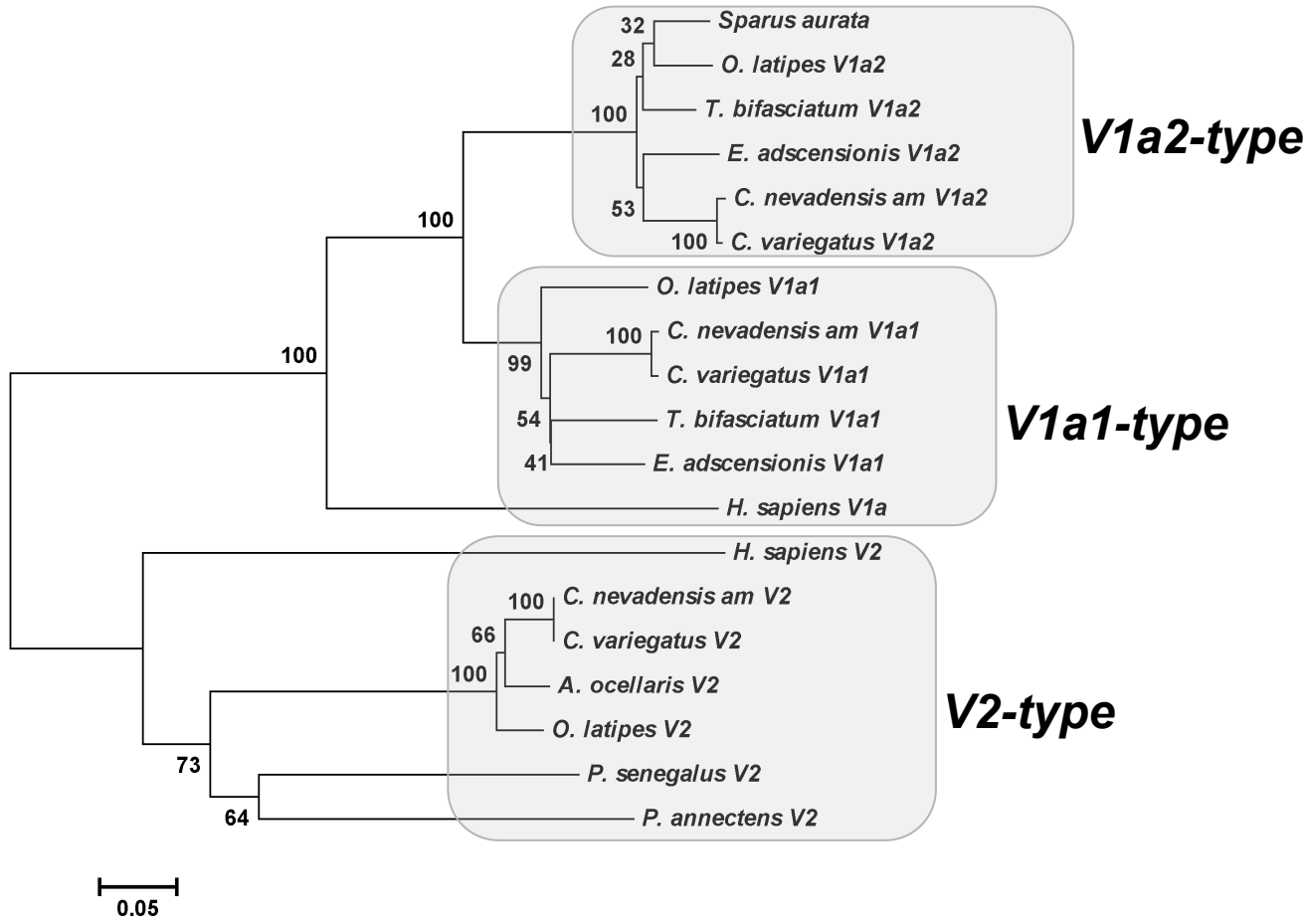

Figure 2. Martos-Sitcha *et al.*

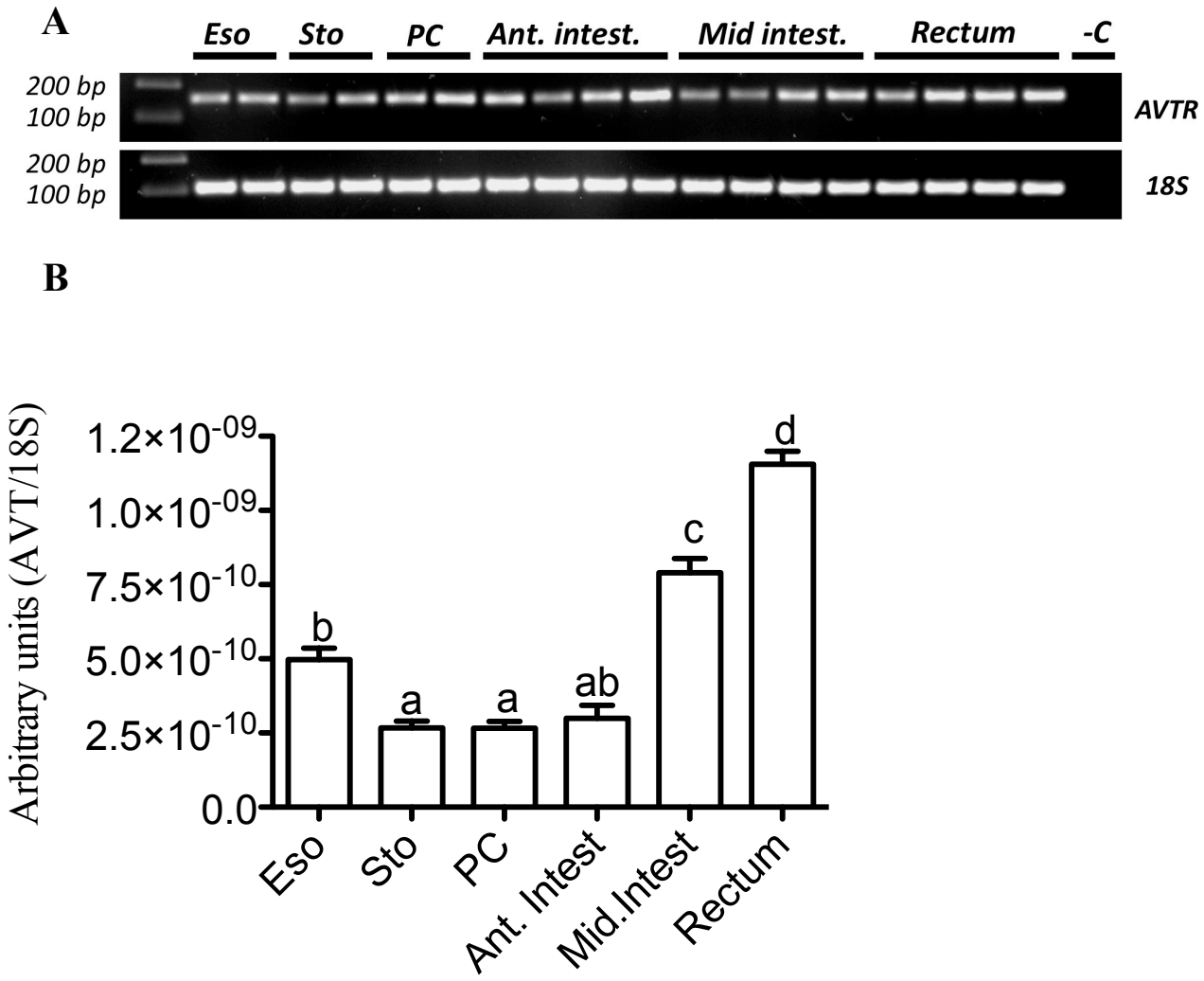

Figure 3. Martos-Sitcha *et al.*

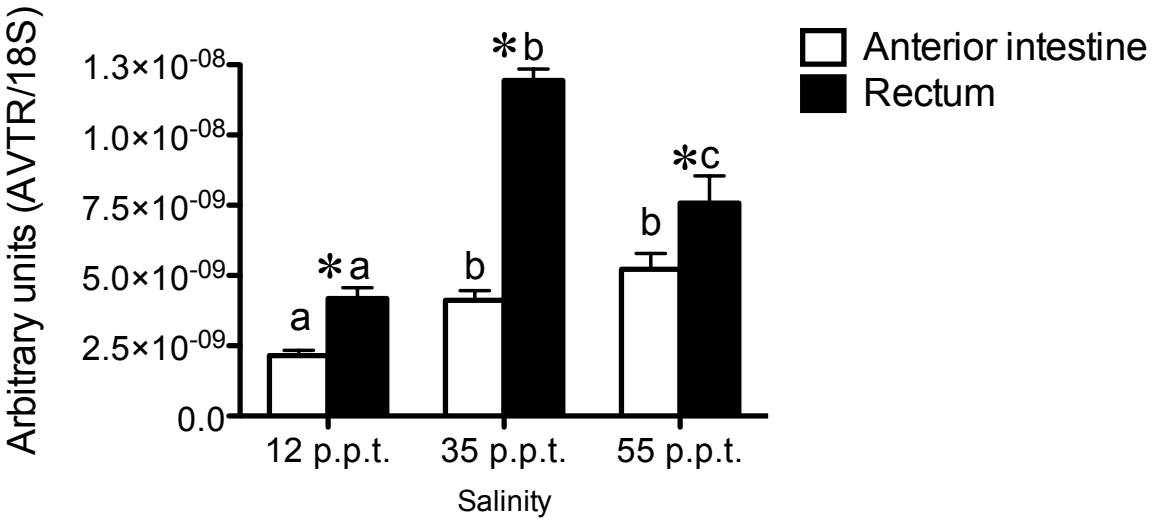

Figure 4. Martos-Sitcha *et al.*

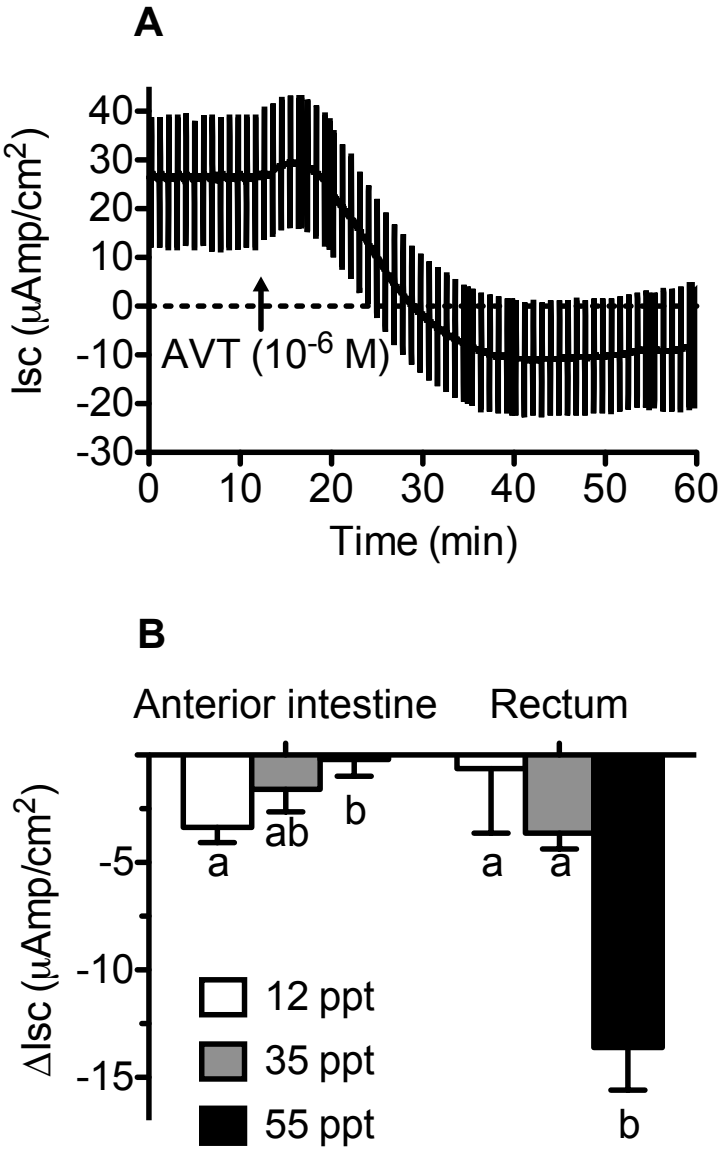

Figure 5. Martos-Sitcha *et al.*

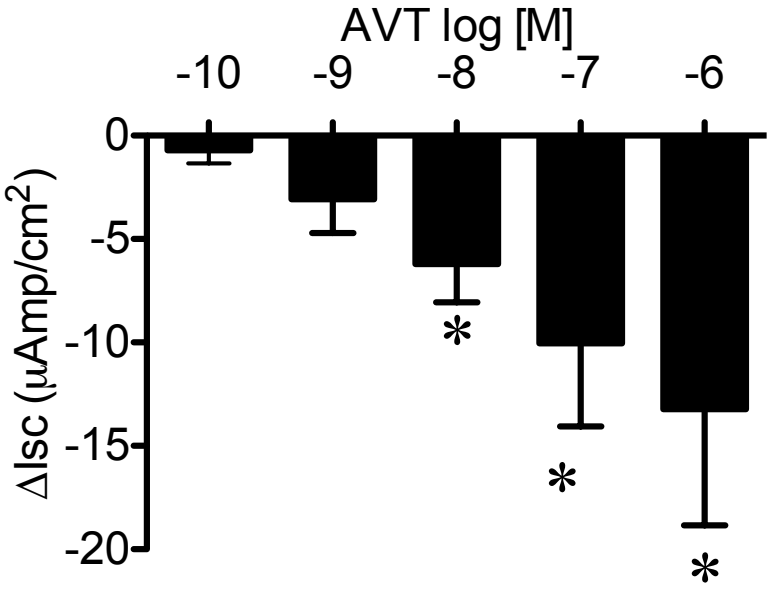

Figure 6. Martos-Sitcha *et al.*

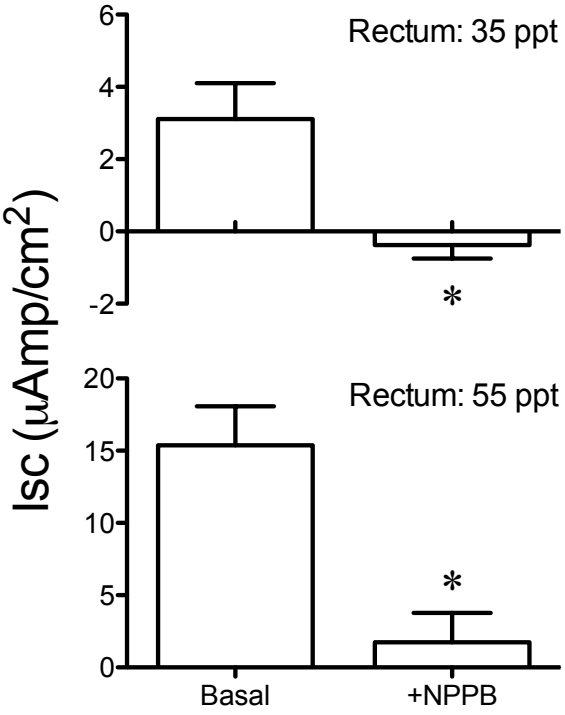

Figure 7. Martos-Sitcha *et al.*