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Unique ionotropic receptors for D-Aspartate are a target for serotonin-induced synaptic plasticity in *Aplysia californica*★

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Abstract

The non-L-glutamate (L-Glu) receptor component of D-aspartate (D-Asp) currents in *Aplysia californica* buccal S cluster (BSC) neurons was studied with whole cell voltage clamp to differentiate it from receptors activated by other well-known agonists of the *Aplysia* nervous system and investigate modulatory mechanisms of D-Asp currents associated with synaptic plasticity. Acetylcholine (ACh) and serotonin (5-HT) activated whole cell excitatory currents with similar current voltage relationships to D-Asp. These currents, however, were pharmacologically distinct from D-Asp. ACh currents were blocked by hexamethonium (C6) and tubocurarine (d-TC), while D-Asp currents were unaffected. 5-HT currents were blocked by granisetron and methysergide (MES), while D-Asp currents were unaffected. Conversely, while (2S,3R)-1-(Phenanthren-2-carbonyl)piperazine-2,3-dicarboxylic acid (PPDA) blocked D-Asp currents, it had no effect on ACh or 5-HT currents. Comparison of the charge area described by currents induced by ACh or 5-HT separately from, or with, D-Asp suggests activation of distinct receptors by all 3 agonists. Charge area comparisons with L-Glu, however, suggested some overlap between L-Glu and D-Asp receptors. Ten minute exposure to 5-HT induced facilitation of D-Asp-evoked responses in BSC neurons. This effect was mimicked by phorbol ester, suggesting that protein kinase C (PKC) was involved.

Keywords

patch clamping; electrophysiology; NMDA; plasticity; 5-HT; protein kinase C

★This paper is based on a presentation given at the *5th Aquatic Annual Models of Human Disease* conference: hosted by Oregon State University and Texas State University-San Marcos, and convened at Corvallis, OR, USA September 20-22, 2010.

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1. Introduction

Molluscs use a variety of small molecules for excitatory synaptic transmission. Among the receptors activated by small molecule neurotransmitters are the cys-family of ligand-gated ion channels. This family includes nicotinic acetylcholine (nACh) and 5-HT₃ receptors. Receptors for both ACh (Kehoe, 1972; Nierop et al., 2005; Nierop et al., 2006) and 5-HT₃ (Walcourt-Ambakederemo and Winlon, 1995; Green et al., 1996; Clemens and Katz, 2001) have been identified in the molluscan nervous system. A complete characterization of D-Asp receptor physiology in the *Aplysia* nervous system includes investigation of the ability of D-Asp to activate these receptors.

D-Asp is present at multiple receptor sites in the *Aplysia* nervous system (Zhao and Liu, 2001). Our related study (Fieber et al., 2010) documented that the buccal S cluster (BSC) neurons have a high preponderance of D-Asp-evoked responses. Receptors activated by D-Asp may overlap with the well-characterized neurotransmitter L-Glu, including N-methyl-D-aspartate receptors (NMDARs) and excitatory amino acid transporters (EAATs), but D-Asp also activates channels independently of L-Glu (Errico et al., 2010; Fieber et al., 2010). Given the unconventional nature of D-isomers as neurotransmitters, with NMDA, D-Ser and D-Asp the sole examples, other ligand-gated channels provide a reasonable starting point for the investigation of the non-L-GluR actions of D-Asp. We hypothesized that the non-L-GluR component of D-Asp whole cell currents may be characterized by activation of cys-family ion channels.

Since the buccal ganglion receives extensive serotonergic innervation (Weiss et al., 1978; Schwartz and Shkolnik, 1981), we further hypothesized that D-Asp currents may be subject to modulation by 5-HT. 5-HT-induced facilitation of sensorimotor synapses (Brunelli et al., 1976) is one of the fundamental processes associated with learning and memory formation in *Aplysia* (reviewed in Glanzman, 2008). At the postsynaptic membrane, sustained exposure to 5-HT activates metabotropic 5-HT receptors inducing a facilitation of L-Glu-evoked responses (Chitwood et al., 2001). This facilitation occurs due to an increase in AMPAR trafficking via exocytosis of vesicular receptor reserves, as well as local, postsynaptic protein synthesis (Li et al., 2005; Villareal et al., 2007). 5-HT appears to produce facilitation via activation of protein kinase C (PKC; Villareal et al., 2009). As D-Asp receptors may be related to ionotropic L-GluRs, we tested D-Asp current modulation by 5-HT. Our results suggest a role for D-Asp specific receptors in synaptic plasticity in *Aplysia*.

2. Materials and Methods

2.1 Cell culture

California sea hares, *Aplysia californica*, (~300-500g; 6-8 months of age and sexually immature) were obtained from the University of Miami NIH National Resource for *Aplysia* in Miami, FL, USA. Primary cultures of BSC cells were prepared according to the methods in Fieber (2000). Animals were anesthetized for 1 h in a 1:1 mixture of natural seawater from the culture facility and 0.366 M MgCl₂. Ganglia were then dissected out and each placed in a 5 ml solution containing 18.75 mg dispase (Boehringer Mannheim), 5 mg hyaluronidase (Sigma), and 1.5 mg collagenase type XI (Sigma) and placed on an elliptical shaker at 114 revolutions/min for ~24 hours at room temperature (~22°C). Cells from specific ganglia or areas of ganglia were then dissociated onto 35 mm diameter polystyrene culture plates (Becton Dickinson, Falcon Lakes, NJ) coated with poly-D-lysine (Sigma, St. Louis, MO, USA). Cells were incubated at 17°C until used in experiments 24 h later.

2.2 Electrophysiology

Whole cell voltage clamp and current clamp measurements were made using glass patch electrodes pulled from thick-walled 1.5 mm diameter borosilicate glass capillaries using a Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA, USA). Voltage and current data were collected and whole-cell capacitance and series resistance compensations were made using an Axopatch 200B clamp amplifier with a capacitance compensation range of 1-1000 pF, connected to a PC and Digidata 1200 A/D converter using pCLAMP software to record data and issue voltage and current commands (Molecular Devices, Sunnyvale, CA, USA). Solutions were flowed onto cells during recording via a 6-bore gravity-fed perfusion system that dispensed solutions from 1 μ L micropipettes ~200 μ m away from the cell at a rate of ~0.4 ml/min. The environment around the cell was adjusted to a new solution within 500 ms of switching on the flow of the relevant gravity pipette. Solutions containing agonist were briefly applied to the cell via a micropipette attached to a picospritzer powered by N₂ adjustable for pressure and duration (Parker Hannifin, Cleveland, OH, USA). The picospritzer pipette tip was aimed at the cell, and positioned at an angle of ~45° from the perfusion flow but closer to the cell, ~30 μ m from the cell body. Unless otherwise noted, in all experiments D-Asp was applied via the picospritzer for 100 ms at a concentration of 1 mM. The effective concentration of agonist was estimated to reach the cell surface <25 ms after the start of the 100 ms pulse of agonist. Unless otherwise noted, in all experiments agonist (D-Asp, L-Asp, NMDA, AMPA, L-Glu, ACh, or 5-HT) was applied via the picospritzer for 100 ms at a concentration of 1 mM, and whole cell current figures are presented at a holding potential of -30 mV.

2.3 Multiple agonist application experiments

For multiple agonist application experiments, an estimate of the net charge passing across the cell membrane in response to activation of D-Asp receptors or receptors activated by another agonist was compared to that produced in response to application of both agonists simultaneously. Whole cell currents in individual cells were elicited by each agonist separately, then both agonists were applied simultaneously using a dual pico-spritzer pipette mount (Narishige, Japan). The resulting whole cell currents were analyzed using Axograph. Currents were reset to baseline to adjust for leak. The area under the curve from current activation to full inactivation was measured to estimate net charge across the cell membrane. The area described by the current resulting from application of two agonists simultaneously was compared to the area of the current obtained from adding responses to each agonist individually.

2.4 Solutions

Unless otherwise noted, reagents were from Sigma-Aldrich (St. Louis, MO, USA). Extracellular solution (ECS) normally consisted of artificial seawater (ASW) containing (mM) 417 NaCl, 10 KCl, 10 CaCl₂ (2 H₂O), 55 MgCl₂ (6 H₂O), 15 HEPES-NaOH, pH 7.6 (~physiological pH, unpublished observations). Control intracellular solutions (ICS) contained (mM) 458 KCl, 2.9 CaCl₂ (2 H₂O), 2.5 MgCl₂ (6 H₂O), 5 Na₂ATP, 10 EGTA, 40 HEPES-KOH, pH 7.4.

Solutions containing D-Asp, L-Asp, NMDA, AMPA, or L-Glu were prepared from frozen 1 M stocks, diluted to 1 mM with the addition of ASW or experimental ECS. ACh and 5-HT solutions were prepared fresh and diluted to working concentrations (1 mM for ACh; 1 mM or 20 μ M for 5-HT) in ASW or experimental ECS.

MES was prepared fresh daily at the working concentration of 1 mM in ASW. Other antagonists were diluted in ASW from frozen stocks. Stocks of C6 (1M), d-TC (100 mM), and granisetron (100 mM; Tocris, St. Louis, MO, USA) were made in water. Stocks of Bis

(500 μ M; CalBiochem, La Jolla, CA) and phorbol 12-myristate 13-acetate (PMA; 25 nM) were made in DMSO. The stock of PPDA (Tocris; 50 mM) was made in 100 mM NaOH (aq.).

2.5 Statistical tests

Data are presented as mean $\pm$ SD. Significant differences for pharmacology, agonist co-application, and potentiation experiments were assessed via one-way within subjects (repeated measures) ANOVA with Scheffé post hoc test or Friedman's repeated measures with Dunn's multiple comparisons post hoc test. Comparison of the charge areas and of the effects of 5-HT +/- Bis on D-Asp currents was assessed using Student's paired t test. Analyses were performed using Data Desk software (version 6.2; Data Description, Inc., Ithaca, NY, USA) and GraphPad (GraphPad Software, Inc., LaJolla, CA, USA). Differences at $p < 0.05$ were accepted as significant.

3. Results

3.1 L-GluR agonists

Agonists of the subtypes of L-Glu receptors were tested in BSC neurons for comparison to D-Asp. NMDA elicited whole cell currents in 1/3 of 15 cells that responded to D-Asp (Fig. 1A). NMDA current responses averaged $< 1/3$ the amplitude of D-Asp currents in the same cells (e.g. at -30 mV, NMDAR current = -49.5 ± 49.7 pA; D-AspR current = -168 ± 181 pA; $n=5$). No cells responded to pressure application of AMPA (1 mM; $n=5$), while the same cells responded to D-Asp (Fig. 1B). Similarly, no cells responded to pressure application of L-Asp (1 mM; $n=6$), while half of the same cells responded to D-Asp (Fig. 1C).

3.2 ACh and 5-HT

ACh and 5-HT activated whole cell currents in BSC cells with I-V relationships and reversal potentials similar to D-Asp currents (Fig. 2). Therefore known antagonists of ACh and 5-HT receptor currents were tested on D-Asp currents in order to pharmacologically differentiate these currents (Fig. 3 and 4). Because the NMDAR blocker PPDA, selective for NR2C/NR2D subunit containing NMDARs was the most potent blocker of D-Asp currents in BSC neurons tested so far (Carlson & Fieber, unpublished), block by PPDA was compared to known antagonists of ACh and 5-HT receptors observed in response to all 3 agonists.

ACh activated currents were significantly reduced in the presence of the neuronal nicotinic receptor blocker C6 (Fig. 3A; 1 mM; mean decrease $25 \pm 29\%$; $p < 0.05$, repeated measures ANOVA with Scheffé post hoc test; $n=9$) and the nicotinic blocker d-TC (Fig. 3B; 1 mM; mean decrease $58 \pm 28\%$; $p < 0.05$, repeated measures ANOVA with Scheffé post hoc test; $n=5$), while D-Asp induced currents were unaffected. Conversely, PPDA (50 μ M) significantly reduced D-Asp currents (Fig. 3C; mean decrease $46 \pm 15\%$; $p < 0.05$, repeated measures ANOVA with Scheffé post hoc test; $n=6$), while ACh currents were unaffected.

5-HT activated currents were significantly reduced in the 5-HT₃ receptor antagonist granisetron (Fig. 4A; 100 μ M; mean decrease $79 \pm 28\%$; $p < 0.01$, Friedman repeated measures test with Dunn's multiple comparisons post hoc test; $n=5$) and the 5HT₁/5HT₂ receptor antagonist MES (Fig. 4B; 1 mM; mean decrease $82 \pm 27\%$; $p < 0.01$, Friedman repeated measures test with Dunn's multiple comparisons post hoc test; $n=5$). D-Asp currents were unaffected by both drugs. PPDA had no effect on 5-HT currents, while D-Asp currents were significantly reduced in PPDA (Fig. 4C; $44 \pm 20\%$; $p < 0.05$, repeated measures ANOVA with Scheffé post hoc test; $n=5$).

3.3 Agonist co-application

The charge area described by currents activated by D-Asp or another agonist (ACh, 5-HT, or L-Glu) applied separately was compared to that resulting from simultaneous application of D-Asp and the second agonist, in order to determine if the agonists activate a receptor with dual agonist binding and activity. There was no significant difference between the sum of the currents from individual applications of D-Asp or ACh compared to application of D-Asp and ACh together (Fig. 5A; $n=15$), indicating activation of a unique receptor-channel by each agonist. There also was no significant difference between the sum of the currents elicited in the same cells by D-Asp and 5-HT separately and that resulting from the agonists applied together (Fig. 5B; $n=8$), indicating activation of separate receptor-channels by D-Asp and 5-HT. The sum of individual D-Asp and L-Glu current charge areas in the same cells, however, was significantly greater than that obtained by application of D-Asp and L-Glu together (Fig. 5C; mean difference of $30\pm23\%$; $p<0.05$, Student's paired t test; $n=5$), suggesting some receptors associated with channels are activated by both D-Asp and L-Glu.

3.4 5-HT facilitation

5-HT was bath applied to BSC neurons at $20\text{ }\mu\text{M}$, a concentration shown to facilitate L-Glu-evoked responses in *Aplysia* (Villareal et al., 2009), for durations of 80 s or 10 min between applications of D-Asp to determine if 5-HT had a potentiating effect on D-Asp currents. Eighty s application of 5-HT had no effect on D-Asp current amplitude (Fig. 6A). Ten min application, however, significantly increased D-Asp current amplitude, and the potentiation was sustained after 10 min washout of 5-HT (Fig. 6B and 7A; mean increase over control $44\pm60\%$ and $40\pm50\%$, respectively; $p<0.05$, repeated measures ANOVA with Scheffé post hoc test; $n=8$).

PMA (0.5 nM), an activator of PKC, mimicked the potentiating effect of 5-HT. While 80 s bath application of PMA prior to eliciting D-Asp currents had no effect on D-Asp current amplitude (Fig. 6C), 10 min of PMA significantly increased current amplitude, and the potentiation was sustained after 10 min washout of PMA (Fig. 6D and 7B; mean increase over control $65\pm57\%$ and $51\pm59\%$, respectively; $p<0.05$; repeated measures ANOVA with Scheffé post hoc test).

Bis (500 nM), a PKC inhibitor, was tested for block of the potentiating effect that 10 min bath applied 5-HT had on D-Asp currents. When bath-applied alone for 80 s or 10 min, Bis had no effect on D-Asp current amplitude (Fig. 8A and B, respectively). Eighty second application of 5-HT + Bis also had no significant effect on D-Asp current amplitude (Fig. 8C). D-Asp currents increased significantly after 10 min bath applied 5-HT + Bis, suggesting that Bis did not block the potentiating effect of 5-HT (Fig. 8D; repeated measures ANOVA with Scheffé post hoc test; $p<0.05$; $n=5$).

4. Discussion

Free D-Asp is found in the nervous system of *Aplysia* (D'Aniello et al., 1993; Liu et al., 1998; Miao et al., 2005; Miao et al., 2006; Spinelli et al., 2006) and vertebrates (D'Aniello, 2007). D-Asp activates an excitatory, non-specific cation channel in BSC neurons (Carlson & Fieber, unpublished). While D-Asp currents show some similarities with L-Glu currents, D-Asp activates currents in many cells not responding to L-Glu (Fieber et al., 2010), suggesting independent target receptors. The present study extends our previous results by: first, further supporting the supposition that D-Asp activates some of the same receptor targets as L-Glu, second, exploring whether D-Asp has actions at other well known ligand gated receptors, and third, testing whether D-Asp responses are subject to the modulatory influences important to learning in this model system.

NMDA activated small amplitude currents in only 33% of cells it was tested in, while D-Asp activated much larger currents in all these cells. While previous studies have established that D-Asp activates NMDARs (Olverman et al., 1988; Kiskin et al., 1990; Huang et al., 2005), D-Asp may have actions in *Aplysia* ganglia independent of NMDARs. Because BSC cells have high expression of NMDA-like receptors (Ha et al., 2006), the absence of NMDAR currents in 67% of BSC cells suggests either that some NMDA-like receptors are not ionotropic or that many NMDA-like receptors are not activated by NMDA.

The absence of an AMPA-induced current in *Aplysia* BSC neurons, while surprising given their broad distribution (Carroll et al., 2001), was consistent with previous studies in which *Aplysia* neurons, dissected and desheathed but otherwise intact within the ganglion, failed to respond to AMPA (Trudeau and Castellucci, 1993). The failure of BSC cells to respond to AMPA may reflect that it is not the diagnostic agonist for these receptors in *Aplysia*, rather than an absence of AMPA-like receptors. The block of synaptic transmission observed by Trudeau and Castellucci (1993) in the presence of the non-NMDA blockers 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 1-(4-chlorobenzoyl)-piperazine-2,3-dicarboxylic acid (CBPD) support the conclusion that AMPARs exist in *Aplysia*.

The L-isomer of Asp did not activate currents in *Aplysia* BSC neurons, attesting to the stereospecificity of D-Asp-activated receptors in these neurons. These results contrast with previous findings in *Aplysia* cerebral and abdominal ganglia in which L-Asp elicited depolarizations similar to D-Asp or L-Glu (Miao et al. 2006). It may be that BSC cells lack expression of a receptor activated by both D- and L-Asp and L-Glu observed in other ganglia. If so, BSC cells may be a good target for molecular studies aimed at characterization of a unique receptor activated by D-Asp.

The pharmacological evidence argues against D-Asp-induced activation of nicotinic or serotonergic receptor channels. The ACh currents likely represent activation of the molluscan homolog of vertebrate nAChR (Nierop et al., 2005), pharmacologically similar to those underlying excitatory post-synaptic potentials induced by ACh and blocked by C6 and d-TC (Kehoe, 1972). The activation of an ionotropic 5-HT receptor in *Aplysia* BSC cells is consistent with previous studies in molluscs (Walcourt-Ambakederemo and Winlon, 1995; Green et al., 1996; Clemens and Katz, 2001). 5-HT currents in BSC neurons are consistent with activation of a single ionotropic receptor type blocked by granisetron, and likely represent a homologue of the vertebrate 5-HT₃ receptor. The observation that the 5-HT₁-HT₂ receptor antagonist MES blocked BSC 5-HT-induced currents may have been due to the less selective nature of this antagonist at the concentration used here (Bradley et al., 1986).

The good agreement between the current areas described by co-application of ACh or 5-HT with D-Asp, and those in the same cells induced by D-Asp and ACh or 5-HT alone support the idea that ACh and 5-HT activate receptors distinct from D-Asp. In contrast, comparison of the current areas of D-Asp- and L-Glu-induced currents with that of their mixture suggested that D-Asp and L-Glu do, in part, activate the same receptors.

An alternative explanation for the non-additive nature of D-Asp and L-Glu currents, however, is that D-Asp blocked or desensitized L-GluRs. Previous studies have observed lesser amplitude L-Glu-activated currents in the presence of D-Asp (Dale and Kandel, 1993; Gong et al., 2005). Due to the poor efficacy of L-Glu receptor blockers in *Aplysia* (Dale and Kandel 1993) it is not possible to distinguish between these possibilities in voltage clamp studies, however, expression studies using channel mRNA may distinguish between them.

The enhancement of D-Asp currents after 10 min 5-HT exposure mirrors previous results showing an enhancement of L-Glu responses under the same conditions (Chitwood et al., 2001). The absence of D-Asp current facilitation during 80 s exposure to 5-HT suggests that

5-HT is likely acting through intracellular, second messenger pathways. The durability of the potentiation following wash out further implicates involvement of second messengers, as does its mimic by the phorbol ester PMA. The failure of the broad spectrum PKC inhibitor Bis to block these effects suggests that 5-HT may be acting on multiple second messengers, including PKC. Three isoforms of PKC have been identified in *Aplysia* - Apl I, Apl II, and Apl III (Kruger et al., 1991; Bougie et al., 2006). Activity of these enzymes has been linked to 5-HT-mediated facilitation of L-Glu responses (Villareal et al., 2009), and the D-Asp-induced facilitation similarly may employ them. The large amount of variation in facilitation of responses observed in this study may indicate heterogeneity of modulatory mechanisms within the BSC neurons.

The 5-HT-mediated enhancement of D-Asp responses suggests a new receptor susceptible to synaptic facilitation in *Aplysia*. Activation of PKC has been associated with learning in *Aplysia* (Sossin et al., 1994; Sutton and Carew, 2000; Sutton et al., 2004; Zhao et al., 2006; Hu et al., 2007). BSC cells possess receptive fields in the buccal mass and perioral region of the *Aplysia* mouth and have been observed to undergo sensitization in reduced preparations (Walters et al., 2004). Additionally, the control of feeding in *Aplysia* has been used as a model for more complex forms of learning such as classical and operant conditioning (Baxter and Byrne, 2006). As the buccal ganglion receives numerous serotonergic inputs (Weiss et al., 1978; Schwartz and Shkolnik, 1981), and lesioning of serotonergic neurons innervating the buccal ganglion disrupts feeding behavior (Rosen et al., 1989), the modulation observed in this study likely represents an endogenous mechanism of facilitating synaptic inputs associated with feeding. Thus, this system should provide an ideal model for the investigation of modulation of D-Asp responses related to learning. It is possible synapses in other areas of the nervous system of *Aplysia*, such as the abdominal or pleural ganglion and the associated gill- and tail-withdrawal reflexes, respectively, may utilize similar mechanisms of facilitation. Investigation of the link between modulation of D-Asp responses *in vitro* and behavior in intact animals may have implications for learning in the numerous systems in which free D-Asp is present. The identification of non-L-Glu channels activated by D-Asp will require molecular characterization.

5. Conclusions

D-Asp-induced excitation was subject to 5-HT-induced modulation that is similar to that involving AMPARs, but this modulation occurred at unique receptors for D-Asp. Where as the L-Glu current facilitation by 5-HT at sensorimotor synapses implicated an increase in the number of AMPARs at the post synaptic membrane (Chitwood et al., 2001), D-Asp receptors may substitute for AMPAR in this role in some *Aplysia* neurons.

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Abbreviations

Bis	bisindolylmaleimide
BSC	buccal S cluster
C6	hexamethonium
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione disodium
D-Asp	D-aspartate

DNQX	6,7-dinitroquinoxaline-2,3-dione disodium
d-TC	tubocurarine
EAAT	excitatory amino acid transporter
L-Glu	L-glutamate
MES	methysergide
PMA	phorbol 12-myristate 13-acetate
PPDA	(2S,3R)-1-(Phenanthren-2-carbonyl)piperazine-2,3-dicarboxylic acid

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Fig. 1.
BSC whole cell current responses induced by D-Asp, specific L-Glu receptor agonists, and L-Asp (1 mM; 100 ms).
A. Whole cell currents in a single cell induced by D-Asp (left) and NMDA (right).
B. Whole cell currents in a single cell induced by D-Asp (left) and AMPA (right).
C. Whole cell currents in a single cell induced by D-Asp (left) and L-Asp (right).

Fig. 2.

Currents and current-voltage (I-V) relationships of D-Asp currents and currents evoked by ACh and 5-HT.

A. top - D-Asp (left) and ACh (right) whole cell currents in a single cell at different holding potentials. bottom - average I-V $\pm$ SD of currents evoked by D-Asp and ACh (both 1 mM; 100 ms; n=9).

B. top - D-Asp (left) and 5-HT (right) whole cell currents in a single cell at different holding potentials. bottom - Average I-V $\pm$ SD of currents evoked by D-Asp and 5-HT (both 1 mM; 100 ms; n=5).

Fig. 3.

Pharmacological differentiation of D-Asp and ACh currents.

A. Summary of effects of bath-applied C6 (1 mM) on D-Asp- and ACh-activated currents (left; $n=9$) and example control ACh induced currents and ACh currents in bath-applied C6 (right). *denotes significant difference between control/washout and application of blocker at $p < 0.05$; repeated measures ANOVA with Scheffé post hoc test.

B. Summary of effects of bath-applied d-TC (1 mM) on D-Asp- and ACh-activated currents (left; $n=5$) and example currents in response to ACh and ACh in bath-applied d-TC (right).

C. Summary of effects of bath-applied PPDA (50 μ M) on D-Asp- and ACh-activated currents (left; $n=8$) and example currents in response to D-Asp and D-Asp in bath-applied PPDA (right).

Fig. 4.

Pharmacological differentiation of D-Asp and 5-HT currents.

A. Summary of effects of bath-applied granisetron (100 μ M) on D-Asp- and 5-HT activated currents (left; n=5) and example control 5-HT induced currents and 5-HT currents in bath-applied granisetron (right). ** denotes significant difference between control/washout and application of blocker at $p < 0.01$; Friedman repeated measures with Dunn's post hoc test.

B. Summary of effects of bath-applied MES (1 mM) on D-Asp- and 5-HT-activated currents (left; n=5) and example currents in response to 5-HT and 5-HT in bath-applied MES (right).

C. Summary of effects of bath-applied PPDA (50 μ M) on D-Asp- and 5-HT-activated currents (left; n=5) and example currents in response to D-Asp and D-Asp in bath-applied PPDA (right). *denotes significant difference between control/washout and application of blocker at $p < 0.05$; Friedman repeated measures with Dunn's post hoc test.

Fig. 5.

Currents in response to individual and combined agonists.

A. Currents in response to pressure application (1 mM; 100 ms) of D-Asp (black), ACh (red), and D-Asp + ACh (purple). Mean difference $+3 \pm 28\%$.

B. Currents in response to pressure application (1 mM; 100 ms) of D-Asp (black), 5-HT (green), and D-Asp + 5-HT (blue). Mean difference $-5 \pm 26\%$.

C. Currents in response to pressure application (1 mM; 100 ms) of D-Asp (black), L-Glu (gold), and D-Asp + L-Glu (green). Mean difference $-30 \pm 23\%$; $p \leq 0.05$, paired T test ($n=5$).

Fig. 6.

Effects of exposure to bath-applied 5-HT and PMA on D-Asp-activated currents.

A. Effects of 80 s bath application of 5-HT on D-Asp currents (n=11).

B. Effects of 10 min bath applied 5-HT (20 μ M) on D-Asp currents. * denotes significant difference between control and 10 min application/10 min washout of 5-HT at $p \leq 0.05$, repeated measures ANOVA with Scheffé post hoc test (n=8).

C. Effects of 80 s bath application of PMA (0.5 nM) on D-Asp currents (n=6).

D. Effects of 10 min bath application of PMA on D-Asp currents (n=6).

Fig. 7.

Response of D-Asp currents to bath application of 5-HT and PMA.

A. D-Asp-activated currents: 1- before application of 5-HT. 2- after 10 min bath application of 5-HT (20 μ M). 3- 10 min after washout of 5-HT.

B. D-Asp-activated currents: 1- before application of PMA. 2- after 10 min bath application of PMA (0.5 nM). 3- 10 min after washout of PMA.

Fig. 8.

Effects of exposure to Bis or Bis + 5-HT on D-Asp-activated currents.

A. Summary of effects of 80 s bath application of Bis (500 nM) on D-Asp currents (n=6).

B. Summary of effects of 10 min bath application of Bis on D-Asp currents (n=5).

C. Summary of effects of 80 s application of Bis+5-HT (20 μ M) on D-Asp currents (n=5).

D. Summary of effects of 10 min bath application of Bis+5-HT on D-Asp currents (n=5). * denotes significant difference between control and 10 min application/10 min washout of 5-HT in the presence of Bis at $p \leq 0.05$, repeated measures ANOVA with Scheffé post hoc test.