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Characterization of D-Aspartate Receptor Currents in Aplysia californica

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CHARACTERIZATION OF D-ASPARTATE RECEPTOR CURRENTS IN *APLYSIA CALIFORNICA*

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D-Aspartate (D-Asp) is an endogenous compound found in the central nervous system (CNS) of a variety of organisms. Despite its prevalence, however, relatively little understood of its physiological role. The prevailing theory is that D-Asp is an alternate agonist of N-methyl-D-aspartate receptor (NMDAR) channels. The goal of this work was to characterize the currents activated by D-Asp in neurons *Aplysia californica*, focusing on cells of the buccal S cluster (BSC).

First, a general electrophysiological characterization was carried out, examining ion permeability, agonist dose-response, and the kinetics of activation, inactivation, and desensitization. D-Asp activated non-specific cation currents characterized by permeability to Na^+ and K^+. D-Asp-induced currents shared similar current-voltage relationships and time courses of activation and inactivation with L-glutamate (L-Glu)-induced currents. D-Asp currents, however, were subject to prolonged desensitization. Additionally, D-Asp activated currents independently of L-Glu, the known agonist of NMDAR channels, suggesting a non-NMDAR-dependent role of D-Asp.

Next, select antagonists were used in an effort to pharmacologically characterize D-Asp receptor channels. These experiments suggested that D-Asp whole cell currents may be characterized by activation of multiple receptor sites, including NMDARS,

excitatory amino acid transporters (EAATs), and a putative non-L-Glu D-Asp receptor. Furthermore, bath-applied D-Asp attenuated L-Glu-activated currents.

Finally, D-Asp currents were compared to those evoked by acetylcholine (ACh) and serotonin (5-HT) in BSC cells. Results suggested that D-Asp activated receptor channels independently of ACh and 5-HT. Ten minute bath application of 5-HT was found to potentiate D-Asp current responses, likely through activation of a protein kinase C (PKC)-dependent mechanism, suggesting that D-Asp induced currents may be subject to synaptic plasticity associated with learning.

While the identity of the putative D-Asp receptor remains elusive, the current work has advanced our understanding of the role D-Asp may play in the nervous system. These results should provide the groundwork for future studies aimed at identifying this unknown receptor channel, as well as investigation of the potential relationship of D-Asp receptor modulation to learning and memory in *Aplysia*, which may have relevance in higher organisms.

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Introduction

1.1 Background

As recently as 50 years ago, it was widely believed that D-amino acids do not occur in living organisms. In recent decades, however, D-isomers of several amino acids have been discovered in various tissues of a number of organisms, ranging from microorganisms to higher organisms including both invertebrates and vertebrates. These molecules are found in some instances in sufficient quantities that important physiological roles have been suggested. D-Ser and D-Asp are two amino acids which in particular occur in relatively high concentrations in the central nervous system (Fuchs et al., 2005).

D-Asp has been discovered in the nervous systems of a number of different animal phyla. It has been found in the retina of various cephalopods where a role in vision has been proposed (D'Aniello et al., 2005), in ganglia of the lobster *Jasus lalandii* (Okuma and Abe, 1994), in *Aplysia limacina* (Spinelli et al., 2006) and *Aplysia fasciata* (D'Aniello et al., 1992), in *Ciona intestinalis* (D'Aniello et al., 2003) as well as higher vertebrates such as mice (Morikawa et al., 2001), rats (D'Aniello et al., 1993), chickens (Neidle and Dunlop, 1990), and humans (D'Aniello et al., 1998). In many experiments, D-Asp has been found in amounts equivalent to major excitatory neurotransmitters such as L-Glu and L-Asp. Despite its prevalence in the nervous system of so many organisms, we know relatively little about its actual role *in vivo*.

One proposed role of D-Asp is as an important regulator in development. D-Asp undergoes transient rises in concentration during embryonic development (Furuchi & Homma, 2005). After this initial spike in concentration, D-Asp levels in most tissues decrease to minimal levels (i.e. low nmol/g range). Due to its transient increase during development, D-Asp has been suggested to play a role in differentiation and maturation.

Evidence has also accumulated suggesting a neuroendocrine role for D-Asp (D'Aniello, 2006). Experiments investigating the effects of injected D-Asp showed that it accumulated in the adenohypophysis and led to significant increases in hormones including prolactin (D'Aniello et al, 2000a), growth hormone, and luteinizing hormone (D'Aniello et al, 2000b) in rats. D-Asp also is believed to promote testosterone production in Leydig cells (Nagata et al., 1999a). These investigators hypothesized that D-Asp acts through cholesterol translocation into the inner mitochondrial membrane where it is converted to pregnenelone, the precursor for testosterone. They suggested that it does this through increased steroidogenic acute regulatory protein (StAR) gene expression (Nagata et al., 1999b). StAR protein is a regulatory protein in this rate-limiting step of testosterone formation.

Of the two D-amino acids that occur in the CNS, D-Ser has received much more attention regarding its physiological purpose at the synapse. Numerous studies have implicated D-Ser as a substitute for Gly as a co-agonist at NMDA receptors (Fuchs et al., 2005). Conversely, the role of D-Asp in the CNS remains ambiguous. The physiological purpose of such a widespread molecule requires further investigation.

1.2 Possible Neurotransmitter Role

There are five criteria that a molecule must meet in order to be considered a neurotransmitter. The compound must exist freely within the tissue, the components for its synthesis and degradation need to be found near the site of action, there must be a method of transport to and release from the presynapse, and finally it must produce a physiological action. With the exception of the last, all of these criteria have been well-established for D-Asp.

As mentioned above, D-Asp is found in the nervous tissue of a wide variety of organisms (reviewed in D'Aniello 2007). A recent study by Miao et al. (2005) investigated the distribution of D-Asp in neurons of *Aplysia californica* at the subcellular level. Using capillary electrophoresis along with laser-induced fluorescence they found up to millimolar concentrations of D-Asp in the soma and synaptic processes. Using antibodies specific to D-Asp Spinelli et al. (2006) also found D-Asp localized both in the soma, and in dendrites and synaptic varicosities of neurons of *Aplysia limacina*.

Numerous studies have helped to elucidate the mechanisms through which D-Asp is created and broken down (Yamada et al., 2006). The L-isomer of aspartate is converted into the D-isomer by the enzyme aspartate racemase. This enzyme has been found colocalized with relatively large concentrations of D-Asp in tissues of various organisms. It has been found in areas of the rat brain, for example, where D-Asp concentrations are high, such as in the developing subventricular zone and cortical plate (Wolosker et al., 2000). D-Asp concentrations decrease in the retina and optic lobes of the cephalopod

Sepia officinalis when the animal is left in the dark, correlating with a decrease in aspartate racemase activity (D'Aniello et al., 2005).

The enzyme D-aspartate oxidase (DAO) selectively breaks down D-Asp to NH_3, H_2O_2, and α-oxaloacetate (D'Aniello et al., 1993). Immunohistochemistry experiments in rat brains have found an inverse relationship between D-Asp levels and DAO levels (Schell et al., 1997). One study investigating targeted knockout of DAO in mice found significantly increased levels of D-Asp in nervous and endocrine tissues of animals with non-functional genes for the enzyme (Huang et al., 2006). This would suggest that D-Asp oxidase acts as a natural regulator of D-Asp levels *in vivo*.

There is evidence that D-Asp uptake can occur via L-Glu and L-Asp transporters (Albus and Habermann, 1983). D-Asp has been shown to accumulate in artificial synaptomosomal preparations made from astrocytes and neurons containing these transporters (Waagepetersen et al., 2001). Release of D-Asp due to cell depolarization in the presence of high extracellular K^+ concentrations has also been documented. This release possibly occurs either through the exocytosis of synaptic vesicles (Savage et al, 2001) or through glutamate transporters (Waagepetersen et al., 2001). K^+ stimulation could account for either release mechanism, as the increased K^+ gradient could initiate action potentials to cause synaptic vesicle release, or cause the glutamate transporter to work in reverse as glutamate is normally counter transported into the cell in exchange for K^+.

A reproducible physiological action, the final criterion for D-Asp to be considered a neurotransmitter, has received relatively little attention. Dale and Kandel (1993) claimed that it was a partial agonist at L-Glu excitatory channels in *Aplysia* sensory

neurons, due to the smaller current amplitude in voltage clamp experiments in response to D-Asp application compared to L-Glu. Experiments in our lab using higher concentrations (1 mM as opposed to 100 uM in the Dale and Kandel study) have shown that D-Asp elicits currents comparable to those of L-Glu at the same concentrations, indicating that it is as effective an agonist, and has been shown to stimulate action potential producing currents in sensory neurons of the BSC and pleural VC cluster (Fieber, Carlson, et al. 2010). Another study (Kiskin et al., 1990) observed activation of NMDA receptors in rat hippocampal neurons by D-Asp. A more recent study by Miao et al. (2006) investigated the electrophysiological responses of D-Asp in *Aplysia* neurons of the abdominal bag cell cluster and cerebral F cluster. These investigators found that D-Asp induced depolarizations similar to L-Glu in some neurons examined.

A mouse model has been established to investigate the effects of increased levels of D-Asp (Errico et al. 2008a,b; Errico et al. 2010). Using a targeted deletion of the DAO gene and oral administration of D-Asp, a non-physiological level of the molecule was established. An increase in D-Asp was found to increase long-term potentiation via increased NMDAR activity. Furthermore, using antagonists targeting the different subunit types of NMDARs, D-Asp was found to activate currents through an interaction with NR2A-D receptor subunits. Thus it would appear that D-Asp might function as an endogenous, alternate agonist of NMDARs. However, D-Asp also activated currents independently of NMDARs, i.e. in the presence of NMDAR antagonists (Errico et al. 2010), suggesting the existence of a separae D-Asp receptor.

1.3 Modulation of Known Channels

D-Asp may have other actions rather than being a neurotransmitter in its own right. It may be an endogenous modulator of channels activated by other means, such as ligand-gated or voltage-gated channels. It could possibly exert modulatory actions on other channels through such mechanisms as block, allosteric modulation, or activation of second messenger systems.

While investigating excitatory transmission in *Aplysia* sensory neurons, Dale and Kandel (1993) discovered that bath-applied D-Asp, as well as NMDA, appeared to block L-Glu-activated currents. Another possibility, however, is that it is not actually a block of the currents that is occurring. Rather than physically occluding the channel or occupying the L-Glu-binding site without activating a current, low levels of bath-applied D-Asp may activate and then desensitize the channels. This would result in no currents observed in response to application of L-Glu. The suggestion by Dale and Kandel that D-Asp also acts as a partial agonist at the L-Glu-activated channels supports this hypothesis.

In a more recent study Gong et al. (2005) investigated the effects of D-Asp and NMDA on AMPA receptors in hippocampal neurons of rats. They found that both D-Asp and NMDA inhibited kainate-induced AMPA receptor currents in these neurons. Using preparations designed to alter the effects on NMDA receptors, such as the presence of NMDA receptor channel blockers, removal of glycine or addition of glycine had no effect on the block, indicating that it may be independent of any NMDA receptor activity. Furthermore, inhibition occurred in a concentration-dependent manner. L-Asp, on the other hand, did not inhibit these currents, indicating that inhibition may be stereoselective. It is also possible, however, that the L- isomer of aspartate simply has a

lower affinity for a putative inhibitory site, as the authors did not report concentration dependence of block as was done with D-Asp. The authors concluded that D-Asp must inhibit AMPA receptor currents by directly competing with the agonist-binding site. It is possible that D-Asp functions as an endogenous modulator of AMPA receptor channels in the rat hippocampus. This same type of endogenous block of AMPA or other receptor channels may occur in *Aplysia* neurons. Investigation into this possibility should provide insight into the role of D-Asp *in vivo*.

1.4 Aplysia as a Model for Study of the Role of D-Asparate in the CNS

Aplysia californica provides a useful system for the study of D-Asp *in vivo*. Numerous studies have confirmed the widespread presence of D-Asp in each of the ganglia of the *Aplysia* CNS in multiple species of *Aplysia* such as *A. fasciata* (D'Aniello et al., 1993), *A. limacina* (Spinelli et al., 2006), and *A. californica* (Liu et al. 1998; Miao et al., 2005; Miao et al., 2006). L-Glu receptors, the proposed site of action of D-Asp, are found throughout the *Aplysia* nervous system, including the well-studied NMDA (Ha et al., 2006) and AMPA-type receptors that activate channels (Antzoulatos & Byrne, 2004), as well as the invertebrate-specific L- Glu-activated Cl^- channels (King & Carpenter, 1989). *Aplysia* provides a simplified and well-studied model nervous system, with a number of identified cells and cell clusters in which D-Asp-induced responses can be studied. Therefore in this dissertation I set out to characterize the D-Asp receptor current in *Aplysia*. In chapter 2 I describe the general electrophysiological properties of D-Asp currents including permeant ions and times courses of activation, inactivation, and desensitization, with general comparisons made to L-Glu-elicited currents. In chapter 3 I

attempt to pharmacologically characterize D-Asp receptor channels using known blockers of L-Glu receptors in vertebrates. In Chapter 4 I investigate select potential alternate agonists of D-Asp channels, as well as provide the groundwork for investigation into a method of modulation of D-Asp currents by 5-HT. Chapter 5 closes the study with insights and speculations on the role of the D-Asp receptor in the physiology of this model organism.

Chapter 2

Electrophysiological characterization of D-Asp currents

2.1 Summary

D-Asp activates an excitatory current in neurons of *Aplysia californica*. While D-Asp is presumed to activate a subset of L-Glu channels, the identities of putative D-Asp receptors and channels are unclear. Whole cell voltage and current clamp were applied to neurons from primary cultures of *Aplysia* BSC cells to characterize D-Asp-induced ion channels. Both D-Asp and L-Glu evoked currents with similar current-voltage relationships, amplitudes, and relatively slow time courses of activation and inactivation when agonists were pressure applied. D-Asp-induced currents, however, were faster and desensitized longer, requiring 40 s to return to full amplitude. Of cells sequentially exposed to both agonists, 25% had D-Asp- but not L-Glu-induced currents, suggesting a specific receptor for D-Asp that was independent of L-Glu receptors. D-Asp channels were permeable to Na^+ and K^+, but not Ca^{2+}, and were subject to voltage-dependent Mg^{2+} block like vertebrate NMDAR channels. D-Asp may thus activate both NMDARs and non-L-Glu receptors in *Aplysia* neurons.

2.2 Background

D-Asp has been known to have physiological actions in neurons since the 1960's (Curtis & Watkins, 1963; Davies & Johnston, 1979). Decades later, after the discovery of free D-amino acids in the tissues of several organisms, the relevance of D-amino acids was recognized (reviewed in Fuchs et al., 2005; D'Aniello, 2007). D-Ser and D-Asp in

particular occur in relatively high concentrations in the CNS and evidence now suggests that D– amino acids may play key roles in neurotransmission.

While D-Ser has been the subject of much research regarding its role in the nervous system, there have been comparatively few studies of D-Asp. D-Ser acts as an agonist at the glycine binding site of NMDARs, while the precise function of D-Asp remains unclear. D-Asp undergoes a transient increase in concentration in the CNS during embryonic development of vertebrates. Levels rapidly decrease during the postnatal period, due to a rise in the concentration of DAO, the enzyme breaking down of D-Asp (Furuchi & Homma, 2005). While there has been considerable research on an endocrine role for D-Asp (D'Aniello, 2007), there has been less published on a possible neurotransmitter or neuromodulatory role, despite the presence of D-Asp and enzymes for its synthesis, release, uptake and breakdown in nervous tissue of a variety of organisms (D'Aniello, 2007) and in the synaptic processes of *Aplysia* (Miao et al., 2005; Spinelli et al., 2006). D-Asp is formed by conversion of L-Asp by an aspartate racemase, and is degraded by DAO (Homma, 2006; Yamada et al., 2006). D-Asp is released upon electrical stimulation (Savage et al., 2001) or stimulation with ionomycin or high extracellular KCl (Wolosker et al., 2000). D-Asp activates excitatory amino acid transporters effecting its reuptake into cells (Waagepetersen et al., 2001). Thus the main outstanding criterion for a neurotransmitter role is the physiological and molecular description of the ion channels activated by D-Asp.

Owing to their structural similarity, D-Asp and L-Glu both purportedly activate ionotropic glutamate receptors (Olverman et al., 1988; Kiskin et al., 1990; Huang et al., 2005; Errico et al., 2010). L-Glu-activated ion channels include subtypes preferentially

activated by NMDA, AMPA, or kainate. The majority of evidence gathered so far would suggest that D-Asp is a ligand for NMDA receptors. D-Asp binds to NMDARs with a 10-fold lower binding affinity than L-Glu (Olverman et al., 1988). NMDA receptors in rat hippocampal neurons are activated by D-Asp (Kiskin et al., 1990). Huang et al. (2005) found in rat hippocampal CA1 pyramidal cells that a photolabile analogue of D-Asp activated an L-Glu transporter as well as NMDARs, but did not activate AMPA/kainate or metabotropic glutamate receptors.

Miao et al. (2006) investigated electrophysiological responses to D-Asp in *Aplysia* neurons of the abdominal ganglion bag cell cluster and cerebral ganglion F cluster. They found that D-Asp typically induced depolarizations similarly to L-Glu. In some neurons, however, D-Asp responses had opposite polarity from L-Glu responses. This suggested that, while D-Asp may substitute for L-Glu at NMDARs, it may also have actions independent of L-Glu and its target receptors.

In addition to acting as a neurotransmitter, D-Asp may also have neuromodulatory actions, having little or no effect on its own, but acting in conjunction with a conventional transmitter. Evidence for this exists in several species. Bath-applied D-Asp blocked currents gated by L-Glu in *Aplysia* sensory neurons and it was proposed that D-Asp was a partial agonist at these receptors (Dale & Kandel, 1993). D-Asp inhibited AMPAR currents in rat hippocampal neurons and in *Xenopus* oocytes expressing recombinant AMPARs (Gong et al., 2005), and the authors suggesting that D-Asp directly competes for the agonist-binding site of L-Glu at these receptors. Brown et al. (2007) found that, while it did not elicit currents when applied alone, D-Asp slowed the time course of activation and inactivation of an L-Glu receptor cloned from squid. The

exact mechanism of modulation was speculated to be due to direct competition for the binding site or allosteric modification of the receptor.

While several investigators have detected D-Asp-induced membrane currents presumed to be carried via NMDAR channels, there has yet to be a thorough characterization of these currents. The goal of the experiements in this chapter was to provide an electrophysiological characterization of D-Asp-activated currents in neurons of *Aplysia*, focusing on the S cluster neurons of the buccal ganglion (BSC neurons). To this end, I have described the electrophysiological properties of D-Asp current responses, with general comparisons made to L-Glu-activated currents, in an effort to characterize the receptor-channels activated by D-Asp.

2.3 Materials and Methods

Cell culture

California sea hares, *Aplysia californica*, (~6-8 months of age) were obtained from the University of Miami NIH National Resource for Aplysia in Miami, FL. Primary cultures of BSC cells were prepared according to the methods in Fieber (2000). Animals were anesthetized for 1 hour in a 1:1 mixture of seawater from the culture facility and 0.366 M $MgCl_2$. 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 a shaker set to low speed for ~24 hours at room temperature (~22°C). Cells were then dissociated onto 35 mm diameter polystyrene culture plates (Becton Dickinson, Falcon Lakes, NJ) coated with poly-D-

lysine (Sigma, St. Louis, MO) and stored at 17°C until used in experiments 24 hours later.

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). 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). Solutions were flowed onto cells during recording via a 6-bore gravity-fed perfusion system that dispensed solutions from 1 µl micropipettes ~200 µm away from the cell. 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 N_2 adjustable for pressure and duration (Parker Hannifin, Cleveland, OH). 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 µm 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. Using brief puffs of agonist, the effective concentration of agonist was estimated to reach the cell surface <25 ms after the start of the 100 ms pulse of agonist.

To determine the time constants of activation and inactivation whole cell currents from five cells with comparable current amplitudes were fit using a double exponential Simplex/SSE algorithm (Axograph). Time constants for these fits were averaged (±SD) across cells.

Desensitization experiments were performed using a two-pulse protocol with the interpulse interval varied from 2 to 44 s. Current amplitude of the second pulse was expressed as a fraction of the initial current. The plot of the normalized average current amplitude as a function of the interpulse duration was fitted using a single exponential equation:

$$I_t = [I_{max} -(I_{max} - I_0)\exp(-t/\tau)]$$

where I_t is the current at time t, I_{max} is the current amplitude at infinite time, I_0 is the current at 0 time (directly after the first pulse), and τ is the time constant.

Dose-response data were expressed as a fraction of the current amplitude in response to 10 mM D-Asp. Data were fit using the equation:

$$I = I_{max}/[1 + (EC_{50}/[agonist])^n]$$

where n is the Hill slope coefficient (Verdoorn and Dingledine, 1988). Curves and graphs for densitization and dose-response data were plotted using GraphPad Prism (Version 5.03, GraphPad Software, San Diego, CA).

Solutions

All reagents were from Sigma-Aldrich (St. Louis, MO). Extracellular solution (ECS; Table 2.1) usually consisted of artificial seawater (ASW) containing (mM) 417 NaCl, 10 KCl, 10 $CaCl_2$ (2 H_2O), 55 $MgCl_2$ (6 H_2O),15 HEPES-NaOH, pH 7.6 (~physiological pH [unpublished observations]). Control intracellular solutions (ICS)

contained (mM) 458 KCl, 2.9 $CaCl_2$ (2 H_2O), 2.5 $MgCl_2$ (6 H_2O), 5 Na_2ATP, 1 EGTA, 40 HEPES-KOH, pH 7.4. Ion replacement experiments for determination of reversal potential shifts and permeability determinations were executed in ASW and in substituted solutions listed in Table 2.1, with appropriate pH above, according to the ion under investigation.

For ion substitution experiments, currents for determining the reversal potential were first recorded for an individual cell for one condition (control or ion-substituted). The bathing solution was then switched for the alternate solution and a new I-V relationship determined in that cell. For K^+ replacement experiments, after currents in one solution were recorded, the cell was patched again with a new pipette containing the alternate intracellular solution, and 10 minutes was allowed for the new solution to dialyze the cell interior before current measurements were made. For other ions the intracellular solution remained the same throughout the experiment.

Statistical tests

Significance of within-group comparisons was assessed using Student's paired t test, and the unpaired (two sample) t test was used for between group comparisons. Analyses were performed using Data Desk software (version 6.2; Data Description, Inc., Ithaca, NY). Differences at $p<0.05$ were accepted as significant. Data are presented as mean ± SD.

2.4 Results

Electrophysiological responses of Aplysia *neurons to D-Asp*

Initial investigations into the presence of D-Asp-induced currents in ganglia of *Aplysia* demonstrated 54.1% current responses in cultured buccal S cluster neurons (n=37), compared to 28.6% in non-BSC buccal neurons (n=7), 18.8% in abdominal ganglion bag cell neurons (n=16), 40.0% in unidentified cerebral ganglion neurons (n=25), 45.8% in pleural ventral caudal neurons (n=59), and 31.3% in unidentified neurons of the pleural ganglion (n=16). In some neurons pressure application of D-Asp elicited an action potential under current clamp conditions (Fig. 2.1) that corresponded to an inward current near the resting potential under voltage clamp (Fig. 2.1 inset), whereas D-Asp elicited only subthreshold depolarizations in others (data not shown). Pressure application of ASW was without effect on buccal S cluster neurons (data not shown), indicating excitatory responses were due to the presence of D-Asp.

Under voltage clamp conditions, some BSC neurons responded to both D-Asp and L-Glu in alternate applications of these agonists. Whole cell currents induced by D-Asp and L-Glu were inward at negative voltages, e.g. -60 mV and -30 mV, and outward at depolarized voltages, e.g. 60 mV (Fig. 2.2A and B). D-Asp- and L-Glu-activated currents possessed a similar current-voltage relationship. L-Glu-activated currents reversed at –0.4±9.3 mV (n=7), while D-Asp-activated currents reversed at 7.7±9.0 mV (n=37). There was no significant difference in reversal potential between D-Asp and L-Glu-activated currents. Furthermore, currents induced by D-Asp displayed a slight outward rectification when compared to L-Glu (Fig. 2.2A and B). It is important to note that in 71 cells in which both agonists were tested only 18 (25.4%) responded to both L-Glu and D-Asp,

while 35 (49.3%) responded only to L-Glu and 18 (25.4%) responded only to D-Asp. Chi-square Goodness of Fit test revealed that these responses were not randomly distributed ($p<0.05$).

Currents activated by L-Glu also displayed a slower time course of activation and inactivation when compared to those activated by D-Asp (Fig. 2.3A and B). Once D-Asp was applied, currents reached peak activation at 67.9±28.8 ms (n=22), while L-Glu-elicited currents peaked slower at 121±74.7 ms (n=21). Activation of L-Glu- and D-Asp-induced currents was significantly different ($p<0.05$, two sample t test; n=21). In response to a brief, 5 ms pulse of agonist, D-Asp currents spontaneously inactivated after 401±223 ms (n=16), while L-Glu currents inactivated after 657±462 ms (n=15). Steady-state inactivation times in response to 3 s pulses of agonist were not significantly different than spontaneous inactivation times for each agonist, at 497±314 ms for D-Asp-activated currents (n=12) and 595±399 ms for L-Glu-activated currents (n=6). When L-Glu- and D-Asp-activated current inactivation were compared, spontaneous and steady-state inactivation times were significantly different ($p<0.05$, two sample t test; n=15 and n=6, respectively).

Time constants for activation and inactivation reflected the differences in the kinetics of D-Asp- and L-Glu-evoked currents. The fast and slow activation time constants, τ_F and τ_S, of D-Asp currents were 2.9±0.9 ms and 12.7±9.9 ms (n=5), respectively, and for L-Glu currents were 6.2±2.5 ms and 38.1±12.3 ms (n=5), respectively (Fig. 2.3B). Both τ_F and τ_S of activation for L-Glu were significantly longer than those for D-Asp ($p<0.05$ for τ_F and $p<0.01$ for τ_S, two sample t test; n=5). τ_F and τ_S

for inactivation in response to a 100 ms application of agonist were 7.5±4.2 ms and 80.4±46.6 ms, respectively, for D-Asp, and 21.8±11.6 ms and 171±49.4 ms, respectively, for L-Glu currents. τ_F and τ_S of inactivation for L-Glu were significantly longer than those for D-Asp ($p<0.05$ for both, two sample t test; n=5).

Additional important distinctions between currents elicited by D-Asp and those elicited by L-Glu were the vulnerability of D-Asp currents to desensitization and their recovery from desensitization. D-Asp-activated currents rapidly desensitized during repeated applications of agonist, while L-Glu-activated currents recovered from desensitization within a few seconds and potentiated over the same time course that produced desensitization in D-Asp currents (Fig. 2.3C). D-Asp currents recovered from desensitization and returned to maximal amplitude at interpulse intervals of approximately 40 s (Fig. 2.4A). $V_{1/2}$ for recovery was 13 s while τ was 18.79 s. To avoid desensitization, ~1-2 minutes was allowed between applications of D-Asp to individual cells.

Dose-response data were obtained using concentrations of D-Asp between 3 μM and 10 mM (Fig. 2.4B). Currents were half maximal at a concentration of ~42 μM D-Asp. The Hill slope coefficient for the fitted curve was 0.67. Current amplitude was maximal at ≥1 mM D-Asp.

Ion permeability of D-Asp-activated channels

Each of the major ions in solution was systematically replaced with a larger, theoretically impermeant ion in order to determine which ions contributed to whole cell D-Asp-induced currents. Significant shifts in the reversal potential under these conditions indicate channel permeation for that ion. Figs. 2.5 and 2.6 summarize these results.

Sodium in the ECS was replaced with N-methyl-D-glucamine (NMDG) which demonstrated a significant negative shift in reversal potential of 47 mV ($p<0.01$, Student's paired t test; n=7), indicating that Na^+ was permeant (Fig 2.5A). Potassium in the intracellular solution was also replaced with NMDG and revealed a positive shift in reversal potential of 34 mV (Fig. 2.6A; $p<0.01$, Student's paired t test; n=7), indicating that K^+ was permeant. Thus, D-Asp appeared to activate a non-specific cation channel.

External Ca^{2+} was replaced in the ECS with NMDG, in addition to adding 1 mM EGTA to the ECS to chelate any residual Ca^{2+} (Fig. 2.5C). Replacing Ca^{2+} did not result in a shift in reversal potential from currents in Ca^{2+}-containing ASW (n=12). Because this apparent absence of a reversal potential shift may have been due to the small amount of Ca^{2+} being excluded (ASW contains 10 mM), Ca^{2+} permeability was further investigated by replacing external Mg^{2+} with additional Ca^{2+}. Addition of 55 mM extra Ca^{2+} did not cause a shift in reversal potential (Fig. 2.5D; n=6). The absence of a shift when Ca^{2+} was either excluded or amended in the ECS indicated D-Asp channels were not permeable to Ca^{2+}. Thus, D-Asp activated a non-specific cation channel that excluded Ca^{2+}.

Magnesium was also replaced with NMDG to test for voltage dependent block or permeation through D-Asp channels (Fig. 2.5B). Replacing external Mg^{2+} with NMDG did not cause a significant shift in reversal potential (n=10). Removing Mg^{2+} caused an increase in current amplitude across the voltage range. This difference in amplitude was significant at voltages ranging from -60 to –15 mV (mean increase of 188-306% compared to control; $p<0.05$, Student's paired t test; n=10). Current amplitudes at positive voltages in Mg^{2+}-free conditions were not significantly different from those in ASW that contained 55 mM Mg^{2+}. Thus, D-Asp-activated currents appeared to possess

voltage-dependent block by Mg^{2+} similar to that of NMDA receptor channels, but lack the Ca^{2+} permeability of NMDA channels.

External Cl^- was replaced to determine if this anion contributed to whole cell D-Asp-induced currents (Fig. 2.6B). Sodium-chloride, the major ionic constituent of the ECS was replaced with Na-gluconate. There was no significant shift in reversal potential under these conditions, indicating that Cl^- was not permeant through D-Asp-activated channels (n=9).

2.5 Discussion

D-Asp activated an excitatory, non-specific cation current in BSC neurons of *A. californica* and was capable of eliciting an action potential in isolated cells in short term tissue culture. D-Asp-induced current amplitude was half maximal at ~42 μM D-Asp via pressure ejection, and saturated at ~1 mM D-Asp. D-Asp channels were permeable to both Na^+ and K^+; however, Ca^{2+} was not a major current carrier through these channels.

The Hill coefficient for D-Asp binding was close to 1, indicating non-cooperativity, or independent binding of D-Asp to its target receptors. These results are consistent with those observed for various agonists, including D-Asp, at NMDA and non-NMDA receptors expressed in *Xenopus* oocytes (Verdoorn & Dingledine, 1988). Given the likely similarity of D-Asp receptor channels with those activated by L-Glu, which have a Hill coefficient closer to 2, a 2 ligand-binding model might instead have been expected. For L-Glu channels, however, a slope of 2 is typically observed only at lower agonist concentrations (Patneau & Mayer, 1990). Thus, it is possible that dose-response profiles of D-Asp in the low micromolar range might reveal a two-site binding model.

$V_{1/2,}$ or EC50, for D-Asp at its receptor is in the micromolar range, comparable to that observed for L-Glu-activated NMDA and non-NMDA receptors in vertebrate models (Dingledine et al., 1999).

Similarities between the whole cell currents induced by D-Asp and L-Glu in BSC cells were their activation ranges, current amplitudes, and relatively slow activation and inactivation in contrast with, for example, excitatory voltage-gated currents such as Na^+ or Ca^{2+} currents (Fieber, 1995). Currents for both agonists decayed independently of duration of agonist application between 0.1-3 s, suggesting a rapid transition to a desensitized state (Lester & Jahr, 1992) despite the continued presence of agonist. In contrast with L-Glu-induced whole cell currents, currents activated by D-Asp had a faster time course of activation and inactivation, an outward rectification of the I-V relationship, and a prolonged desensitization upon repeated applications of agonist. L-Glu-induced currents recovered from desensitization quickly and potentiated under the same conditions. Of the characterized L-Glu receptor channels, the relatively slow currents kinetics of both D-Asp and L-Glu-activated currents observed in this study coincided most closely with the kinetics of NMDAR channels (Dingledine et al., 1999), the purported site of action of D-Asp (Olverman et al., 1988; Kiskin et al., 1990; Huang et al., 2005).

NMDAR channels have a number of characteristics that distinguish them from non-NMDAR channels, most conspicuously high Ca^{2+} permeability that causes large shifts in reversal potential of the current when external [Ca^{2+}] is altered (Mayer & Westbrook, 1987). Interestingly, the ions permeant through *A. californica* D-Asp channels share more in common with non-NMDA receptors than with those specific for

NMDA. No shifts in reversal potential were detected in BSC D-Asp-activated currents upon removal of external Ca^{2+}, nor with a more than six-fold increase in external Ca^{2+}. NMDAR subunits have been detected in BSC cells with in situ hybridization (Ha et al., 2006); therefore, although NMDARs are present, D-Asp may not act as an agonist at NMDARs in these cells.

NMDAR channels also possess a constitutive block by Mg^{2+} resulting in small amplitude currents at voltages hyperpolarized to the reversal potential (Ascher & Nowak, 1988). This is important in the physiology of the associated channels as the channel acts as a coincidence detector, requiring depolarization to relieve Mg^{2+} block and allow for full NMDAR activation (Bliss & Collingridge, 1993). This characteristic is believed to be fundamental to the neurophysiology underlying learning mechanisms such as long-term potentiation. Voltage-dependent Mg^{2+} block has also been observed in *Aplysia* NMDAR-like channels (Dale & Kandel, 1993). While D-Asp currents possess a voltage-sensitive Mg^{2+} block similar to NMDAR channels, the absence of significant Ca^{2+} permeation argues against D-Asp activation of NMDARs. While the permeability of the channels observed in this study, i.e. current carried mostly by Na^{+} and K^{+}, coincides with AMPA or kainate receptors, these channels are not known to be sensitive to voltage-dependent Mg^{2+} block. It is possible that the currents observed here represent a novel receptor type, sharing some physiological properties with the well-characterized NMDAR channel.

The contention that D-Asp activates non-L-Glu channels is supported by the observation that approximately one quarter of BSC cells responded to both L-Glu and D-Asp, while a quarter more responded only to D-Asp and not to L-Glu. These data are consistent with previous results from our lab in pleural ganglia (Fieber et al., 2010). The

existence of currents activated by D-Asp, but not L-Glu, however, contrasts with earlier findings that D-Asp is an alternate agonist of L-Glu channels in vertebrate brain (Olverman et al., 1988; Kiskin et al., 1990). Our results are consistent with findings of Errico et al., (2010), however, in mouse hippocampal neurons, in which D-Asp activated NMDA receptors, but D-Asp generated excitatory postsynaptic potentials persisted under pharmacological conditions that completely blocked NMDA receptors. It is likely that, while D-Asp and L-Glu may be activating the same receptors in some BSC cells, each agonist appears to target distinct receptors in other cells. Thus, it is possible that the current responses observed in this study represent a mixed population of receptor channels, with D-Asp activating a combination of NMDAR channels and another non-L-Glu channel. This could confound interpretation of results involving D-Asp-induced currents. The observations reported in this study, however, such as the prolonged desensitization of receptors for D-Asp to this agonist and permeability to Na^+ and K^+ but not Ca^{2+}, were consistent with a uniform population of receptor-channels distinct from, at least, L-Glu-activated NMDA receptors. Pharmacological antagonists targeting different L-Glu receptor subtypes should elucidate the identity of channels activated by both L-Glu and D-Asp.

While L-Glu responses recovered from desensitization within a few seconds, D-Asp currents required ~40 s for current amplitude to fully recover. The fast recovery from desensitization observed for L-Glu channels observed in BSC cells was consistent with previous studies of L-Glu desensitization, and was much faster than that observed for D-Asp. For example, L-Glu receptors in outside-out patches from *Drosophila* muscle fully recovered from desensitization in 1-2 s (Heckmann & Dudel, 1997), while recombinant

NMDARs transfected into mammalian HEK-293 cells displayed similar fast recovery from desensitization (Vicini et al., 1998). Recovery was somewhat slower for cells expressing NR2B subunits, compared to those expressing NR2A; however, this recovery was still on the order of a few seconds. Desensitized kainate receptors in rat hippocampal neurons had a recovery time course most similar to that observed in this study (Wilding & Huettner, 1997), requiring ~50 s to recover to 90% of control and >100 s to return to full amplitude in response to application of either L-Glu or kainate. It should be noted that the higher affinity agonist, kainate, showed a longer time course of recovery than L-Glu at these receptors, suggesting that different agonists at the same receptor have different binding kinetics that affect inactivation of the channel.

This idea is corroborated by another study investigating the relationship of desensitization recovery kinetics and agonist potency of AMPARs expressed in the tsA201 cell line (Zhang et al., 2006). While the recovery time courses observed were faster than those observed here for D-Asp, there was a direct relationship between recovery time and agonist potency. So, (S)-2-amino-3-[3-hydroxy-5-(2-methyl-2H-5-tetrazolyl)-4-isoxazolyl]propionic acid (2-Me-Tet-AMPA), the most potent agonist examined, had the longest recovery time, while L-Glu, the least potent of the agonists used by these authors, had the shortest recovery time. Channels activated by L-Glu recovered on the order of ~100 ms, while those activated by 2-Me-Tet-AMPA recovered fully by 10-12 s. This was suggested to be due to increased stability of the closed-cleft conformation when a high affinity ligand was bound to the receptor.

According to this model, desensitization recovery times observed for D-Asp currents in BSC cells may be owing to D-Asp acting as a high affinity agonist at its target

receptor. This could explain what was described as the modulatory actions of D-Asp in previous studies (Dale & Kandel, 1993; Gong et al., 2005; Brown et al., 2007). It is possible that D-Asp binds with high affinity to, but without activating, L-Glu receptors, and thus directly competes with the native agonist. Such an action for D-Asp could represent an endogenous mechanism of synaptic modulation.

It is likely that the long desensitization time of D-Asp currents is important in the physiology of the channel. Ultimately, desensitization will alter the excitatory post-synaptic currents (Trussel & Fischbach, 1989). The strong binding of the closed-cleft conformation of the channel could represent an endogenous mechanism for protection from excitotoxicity. Over-activation of L-Glu receptors is a well-known characteristic of neuropathies such as stroke and Alzheimer's disease. Binding of D-Asp to these channels, and the resulting desensitization, could prevent cell death, and ultimately tissue damage resulting from excitotoxicity. If there is cross-activation of D-Asp with L-Glu receptors in mammalian systems, this could represent a promising target for drugs treating these diseases. That free D-Asp levels are shown to be lower in patients with Alzheimer's disease would support this hypothesis (D'Aniello et al., 1998).

While it would appear that D-Asp acts as an excitatory neurotransmitter in the CNS, the data would suggest that, similar to L-Glu, D-Asp may possess multiple sites of action, some of which likely overlap with L-Glu. If so, elucidation of the precise role D-Asp plays at the synapse will be difficult. This is confounded by the fact that, in addition to apparently acting as a neurotransmitter in its own right, D-Asp may also be an endogenous modulator of L-Glu channels. Further investigation is needed to address the potential multiple functions of D-Asp. However, the results in this chapter suggest that

D-Asp performs actions independent of L-Glu in the nervous system of *Aplysia* and may activate a novel receptor type.

Table 2.1. Ion concentrations in recording solutions (mM).

Description	ECS *		ICS	
Control	NaCl	417	KCl	458
	KCl	10	$CaCl_2 \cdot 2H_2O$	2.9
	$CaCl_2 \cdot 2H_2O$	10	$MgCl_2 \cdot 6H_2O$	2.5
	$MgCl_2 \cdot 6H_2O$	55	Na_2ATP	5
	HEPES-NaOH	15	EGTA	10
			HEPES-KOH	40
Na^+ substitution	**NMDG**	**417**	KCl	458
	KCl	10	$CaCl_2 \cdot 2H_2O$	2.9
	$CaCl_2 \cdot 2H_2O$	10	$MgCl_2 \cdot 6H_2O$	2.5
	$MgCl_2 \cdot 6H_2O$	55	**K_2ATP**	**5**
	HEPES-**HCl**	15	EGTA	10
			HEPES-KOH	40
K^+ substitution	NaCl	417	**NMDG**	**458**
	NMDG	**10**	$CaCl_2$	2.9
	$CaCl_2 \cdot 2H_2O$	10	$MgCl_2 \cdot 6H_2O$	2.5
	$MgCl_2 \cdot 6H_2O$	55	Na_2ATP	5
	HEPES-**HCl**	15	EGTA	10
			HEPES-**HCl**	40
Ca^{2+} substitution	NaCl	417	KCl	458
	KCl	10	$CaCl_2 \cdot 2H_2O$	2.9
	NMDG	**10**	$MgCl_2 \cdot 6H_2O$	2.5
	$MgCl_2 \cdot 6H_2O$	55	Na_2ATP	5
	EGTA	**1**	EGTA	10
	HEPES- **HCl**	15	HEPES-KOH	40
Ca^{2+} addition	NaCl	417	KCl	458
	KCl	10	$CaCl_2 \cdot 2H_2O$	2.9
	$CaCl_2 \cdot 2H_2O$	**65**	$MgCl_2 \cdot 6H_2O$	2.5
	HEPES-NaOH	15	Na_2ATP	5
			EGTA	10
			HEPES-KOH	40
Mg^{2+} substitution	NaCl	417	KCl	458
	KCl	10	$CaCl_2 \cdot 2H_2O$	2.9
	$CaCl_2 \cdot 2H_2O$	10	$MgCl_2 \cdot 6H_2O$	2.5
	NMDG	**83**	Na_2ATP	5
	HEPES-**HCl**	15	EGTA	1
			HEPES-KOH	40
Cl^- substitution	**NaGluc**	**417**	KCl	458
	KGluc	**10**	$CaCl_2 \cdot 2H_2O$	2.9
	$CaCl_2 \cdot 2H_2O$	10	$MgCl_2 \cdot 6H_2O$	2.5
	$MgCl_2 \cdot 6H_2O$	55	Na_2ATP	5
	HEPES-NaOH	15	EGTA	10
			HEPES-KOH	40

*Control ECS is artificial seawater (ASW)
NMDG – N-methyl-D-glucamine
NaGluc – Na-gluconate
KGluc – K-gluconate
Bold entries are the components at variance with normal solutions

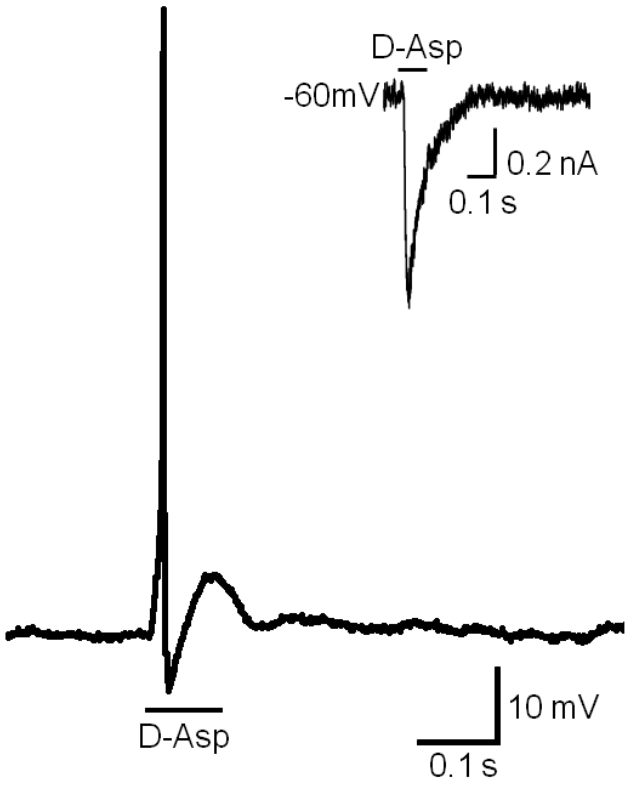

Fig. 2.1. Action potential in BSC neuron evoked by pressure application of D-Asp (1 mM; 100 ms). Inset: D-Asp-activated whole cell current in the same BSC cell at -60 mV.

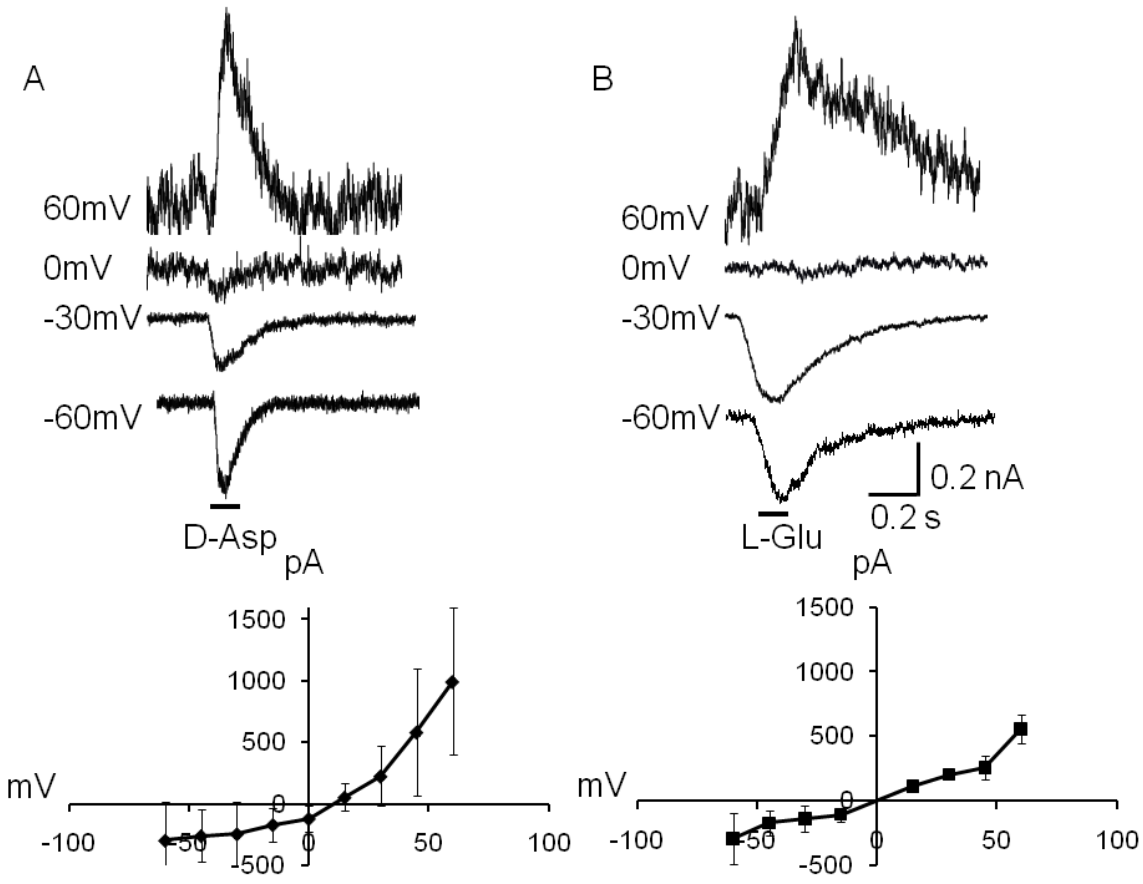

Fig. 2.2. BSC whole cell currents in response to pressure application of D-Asp and L-Glu.
A. D-Asp evoked currents in a single BSC cell at different holding potentials. 1 mM D-Asp, 100 ms (bar) and average current-voltage (I-V) relationship ±SD (n=55).
B. L-Glu Asp evoked currents in a BSC cell at different holding potentials. 1 mM L-Glu, 100 ms (bar) and average I-V relationship ±SD (n=8).

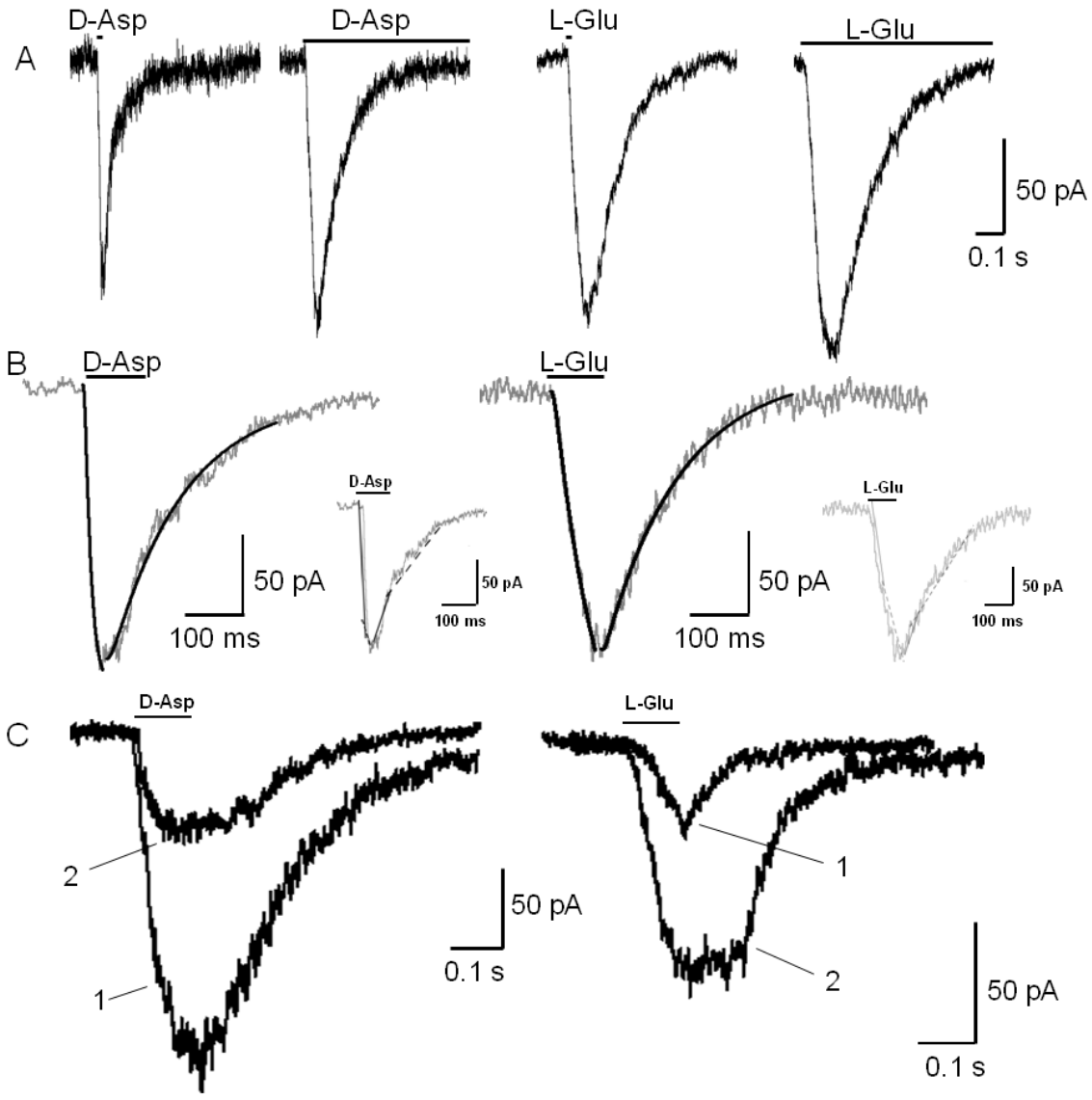

Fig 2.3. Activation and inactivation of D-Asp and L-Glu whole cell currents, and desensitization of D-Asp currents.
A. Example D-Asp (left) and L-Glu (right) currents in response to brief (5 ms) and sustained (3 s) agonist application.
B. Example time courses of activation and inactivation for D-Asp- and L-Glu-induced currents (1 mM, 100 ms). The data were fit using a double exponential Simplex/SSE algorithm (Axograph). Inserts: τ_F (solid lines) and τ_S (dashed lines) of D-Asp- and L-Glu-induced currents. D-Asp: Activation τ_F =4.2 ms, τ_S =12.4 ms, inactivation τ_F =8.8 ms τ_S=151.2 ms. L-Glu: Activation τ_F =6.63 ms, τ_S =40.5 ms, inactivation τ_F =15.0 ms τ_S =175 ms.
C. Superimposed currents in the same BSC cell elicited in response to two applications of D-Asp (left) and L-Glu (right). 1 and 2 are separated by 10 s.

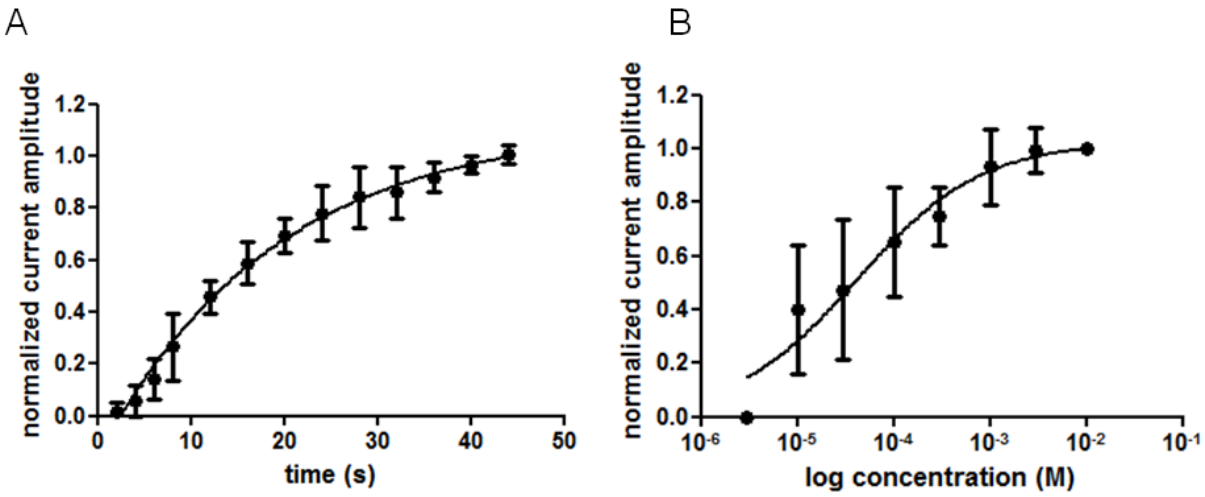

Fig. 2.4. Dependence of whole cell D-Asp current amplitude on frequency of agonist application and on concentration.
A. Normalized average current amplitude in response to a second application of D-Asp in a 2-pulse protocol as a function of the increasing interval between application over 2 – 44 s intervals, ±SD. Line fitted to single exponential equation (Robert and Howe, 2003): $I_t = [I_{max} -(I_{max} - I_0)\exp(-t/\tau)]$.) n=8; $V_{1/2}$ =13 s; τ = 18.79.
B. Average dose-response for D-Asp ±SD. Line fitted to Boltzman equation (Verdoorn and Dingledine, 1988): $I=Imax/[1 + (EC_{50}/[agonist])^n]$. n=6.

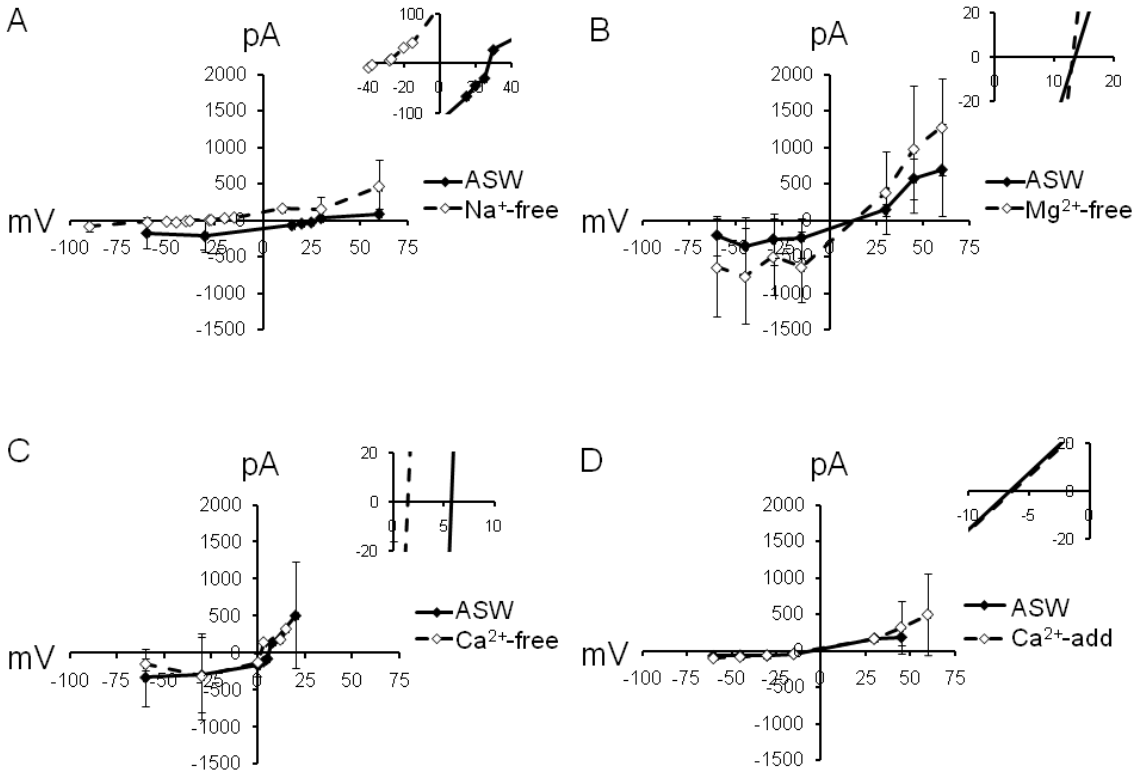

Fig. 2.5. Average I-V relationships in D-Asp currents with external cation replacement and higher resolution view of reversal potentials (insets).
A. Significant reversal potential shift of -47 mV after replacement of external Na with NMDG. ($p<0.05$; n=7).
B. Absence of shift in reversal potential shift after replacement of external Mg^{2+} with NMDG. (n=10)
C. Absence of shift in reversal potential after replacement of external Ca^{2+} with NMDG. (n=12)
D. Absence of shift in reversal potential after replacement of external Ca^{2+} and Mg^{2+} with NMDG. (n=6)

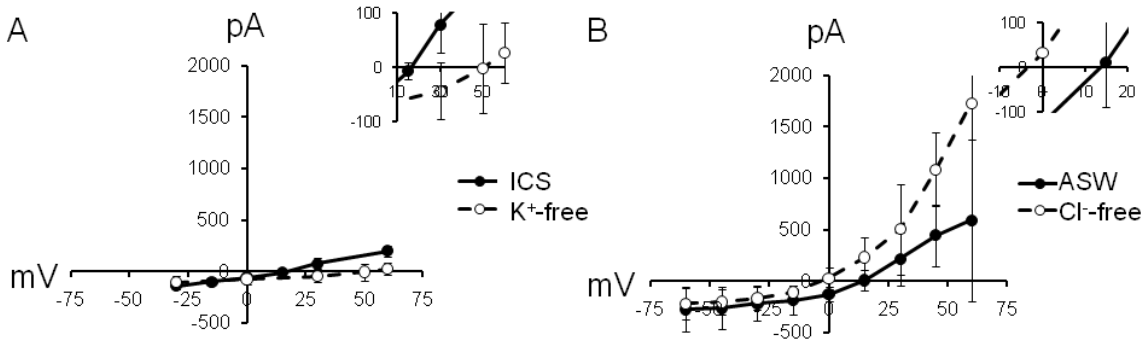

Fig.2. 6. I-V relationships of D-Asp currents with K^+ and Cl^- replacement and higher resolution view of reversal potentials (insets).
A. Significant reversal potential shift of 34 mV after replacement of internal K^+ with NMDG ($p<0.05$; n=7).
B. . I-Vs before and after replacement of external NaCl with Na-gluconate. (n=9)

Chapter 3:

Pharmacological characterization of D-Asp currents

3.1 Summary

D-Asp activates a non-specific cation current of unknown identity independently of L-Glu in neurons of *Aplysia californica*. Whole cell voltage clamp studies were conducted using primary cultures of *Aplysia* BSC neurons in an effort to pharmacologically identify these receptor channels. The NMDAR co-agonist Gly potentiated D-Asp currents only at –30 mV, while D-Ser did not potentiate D-Asp currents at any amplitude. A portion of D-Asp currents were blocked by the L-Glu antagonists kynurenate, APV, PPDA, and TCS 46b, suggesting that L-Glu channels, particularly NMDAR channels, may partially contribute to D-Asp whole cell currents. The EAAT blocker TBOA also blocked a fraction of D-Asp currents, suggesting that currents associated with these transporters also contribute to D-Asp whole cell currents. Non-NMDA L-GluR antagonists that preferentially block AMPA/kainate receptors significantly increased D-Asp currents, suggesting a possible allosteric potentiating effect of these antagonists on D-Asp receptors. L-Glu induced currents were significantly reduced in the presence of bath-applied D-Asp, whereas bath-applied L-Glu had no effect on D-Asp induced currents. The mixed effects of these agents on D-Asp induced currents in *Aplysia* illustrate that the underlying channels are not uniformly characteristic of any known agonist associated channel type.

3.2 Background

D-Asp activates a non-specific cation channel, impermeable to Ca^{2+}, in BSC neurons of the *Aplysia* nervous system (Chapter 2). These channels are distinct from L-Glu channels, with D-Asp in many instances eliciting whole cell currents in cells lacking L-Glu-induced responses. Since D-Asp is believed to act as an alternate agonist at L-Glu channels, specifically NMDAR channels (Olverman et al., 1988; Kiskin et al., 1990; Huang et al., 2005), the identity of these non-L-Glu channels activated by D-Asp is unclear. Further, it is not known if D-Asp and L-Glu activate the same receptor channel in those cells responding to both agonists.

An important aspect of the physiology of NMDARs is the co-agonist binding site. In addition to the requirement that L-Glu bind in order for NMDAR channels to open, these receptors possess an obligatory co-agonist binding site that also must be occupied for full activation of the channel (Kleckner & Dingledine, 1988). This was originally described as potentiation by glycine (Gly; Johnson & Ascher, 1987). Shortly thereafter, it was discovered that occupancy of this site is obligatory, with Gly binding required for channel opening (Kleckner & Dingledine, 1988). In the same study it was discovered that D-Ser substitutes effectively at the glycine-binding site of NMDARs. While NMDARs are among the purported sites of action of D-Asp in the nervous system, it has not been established if these co-agonists are required for activation of D-Asp currents.

One of the inherent challenges in working with L-Glu receptors is that many neurons express multiple types of receptors, including NMDA, AMPA, and kainate receptors, and that these subtypes can be further subdivided based on variations in subunit composition (Dingledine et al., 1999). In recent decades, however, a number of

pharmacological agents have been developed that have facilitated isolation of currents associated with these channels in electrophysiological investigations (Kew & Kemp, 2005; Lodge, 2009). Indeed, many of the studies investigating the role of L-Glu in synaptic plasticity in *Aplysia* have relied largely on pharmacological evidence for identification of the receptors being studied (reviewed in Antzoulatos and Byrne, 2004). It is interesting then, that despite the professed role of D-Asp as an alternate agonist at NMDARs, pharmacological evidence supporting this hypothesis is limited to a single study Errico et al. (2010).

Errico et al. (2010) investigated electrophysiological responses of D-Asp in 13 to 15 day old C57BL/6J mice, a model used to study the effects of supraphysiological levels of D-Asp. Using NVP-AAM077, cis-PPDA, and Ro 25-6981, NMDAR antagonists selective for NR2A, NR2C/D, and NR2B subunits, respectively, the authors reported ~67% block of D-Asp-induced currents for each drug, approximating the degree of block of NMDA-induced currents in the same cells. When these three antagonists were applied together or when MK-801, a comprehensive NMDAR blocker, was applied, NMDA currents were completely blocked while D-Asp-activated currents were reduced 80%. These results suggested that while D-Asp activated currents in the hippocampus similar enough to NMDARs currents to be blocked by NMDAR blockers, it also activated a current clearly not due to NMDAR activation.

In addition to the proposed but poorly supported neurotransmitter role, there is considerable evidence that D-Asp plays a modulatory role at L-Glu-activated receptors. Antagonistic effects of D-Asp have been observed in L-Glu channels in *Aplysia* (Dale & Kandel, 1993) and in rat hippocampal neurons and *Xenopus* oocytes expressing AMPARs

(Gong et al., 2005). Further, D-Asp slowed the gating kinetics of a squid glutamate receptor (Brown et al., 2007). In none of these models, however, did D-Asp activate ion channels. It is thus unknown whether D-Asp acts in dual roles, both as a modulator of L-GluR channels and as a neurotransmitter at novel receptors.

The experiments in this chapter were designed to further elucidate the identity of channels activated by D-Asp. To achieve this I attempted a pharmacological characterization of the D-Asp-induced current in *Aplysia* neurons, with a focus on antagonists and co-agonists of L-Glu receptor channels.

3.3 Materials and Methods

Cell Culture/Electrophysiology

Preparation of primary cell cultures of *Aplysia* BSC cells and whole cell voltage clamp measurements were made as described in Chapter 2 Materials and Methods section.

Pharmacology

Pharmacology experiments were performed as a three-pulse protocol, with inhibition of current assessed as a proportion of maximal current amplitude. Due to the desensitizing nature of D-Asp-activated currents (Chapter 2) 80 s was allowed between each application of agonist. An initial control application of agonist from the picospritzer pipette in flowing ASW was followed by a switch of the bathing solution to one containing blocker. Eighty seconds later, a second pulse of agonist was applied. The bathing solution was then switched back to ASW and a third application of agonist was made to permit washout of the antagonist. Because memantine and MK-801 require

channel opening for block to occur, two applications of agonist were made during antagonist exposure before washout, and each application was compared to the control.

CTZ experiments were performed both under the conditions described above and under conditions designed to investigate block of desensitization. For desensitization experiments, cells were exposed to repeated applications of D-Asp both in ASW and in ASW + CTZ. Three applications of D-Asp were made: an initial, control application (t_0), an application at $t_{10} = t_0 + 10$ s, and a final application at $t_{20} = t_0 + 20$ s. Currents were normalized to the control current for each condition.

Solutions

Unless noted, all reagents were from Sigma-Aldrich. Normal ECS consisted of ASW containing (mM) 417 NaCl, 10 KCl, 10 $CaCl_2$ (2 H_2O), 55 $MgCl_2$ (6 H_2O),15 HEPES-NaOH, pH 7.6. Control ICS contained (mM) 458 KCl, 2.9 $CaCl_2$ (2 H_2O), 2.5 $MgCl_2$ (6 H_2O), 5 Na_2ATP, 1 EGTA, and 40 HEPES-KOH, pH 7.4.

For pharmacology experiments, antagonists were diluted in ASW from frozen stocks. Stocks of the following drugs were made in water, then diluted in ASW at 1:100 or greater, for their working concentrations: 4-Acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium (SITS; 50 mM), DL-kynurenic acid (kynurenate; 100 mM), DL-2-Amino-5-phosphonopentanoic acid (APV; 100 mM), memantine hydrochloride (Tocris; 100 mM), 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX; Tocris; 10 mM), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium (NBQX; Tocris; 50 mM), 6,7-dinitroquinoxaline-2,3-dione disodium (DNQX; Tocris; 100 mM), and (R)-(+)-3-amino-1-hydroxypyrrolidin-2-one (HA-966; Tocris,; 100 mM). Stocks of (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-

dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801; Tocris; 5 mM) and DL-threo-b-Benzyloxyaspartic acid (TBOA; Tocris; 45 mM) were made in ASW. Stocks of (S)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxybenzyl)pyrimidine-2,4-dione (UBP 302; Tocris; 50 mM) and cyclothiazide (CTZ; 20 mM) were made in DMSO. The 1,3-Dihydro-5-[3-[4-(phenylmethyl)-1-2H-benzimidazol-2-one (TCS 46b; Tocris; 50 mM) stock was made in ethanol. The stock of (2S,3R)-1-(Phenanthren-2-carbonyl)piperazine-2,3-dicarboxylic acid (PPDA; Tocris; 50 mM) was made in 100 mM NaOH (aq.).

Statistical tests

Data are presented as mean±SD. Differences in current amplitudes with treatments were assessed using Student's paired t tests. Differences in amplitude after desensitization were assessed using two-sample t-tests. Analyses were performed using Data Desk software (version 6.2; Data Description, Inc., Ithaca, NY). Differences at $p<0.05$ were accepted as significant.

3.4 Results

Gly/D-Ser

The amplitude of D-Asp currents was compared in the presence and absence of Gly (1 mM) and D-Ser (1 mM), and the current-voltage relationships plotted at voltages from –60 mV to +60 mV (Fig. 3.1). Current amplitude was significantly greater when D-Asp was co-applied with Gly near the resting potential of BSC cells at –30 mV (Fig. 3.1A, bottom; mean increase 24±34%; $p<0.05$, Student's paired t test, n=14), but not significantly different at any other voltages examined between -60 and +60 mV. There were no significant changes in current amplitude with D-Ser at any of the voltages

examined between -60 and +60 mV. HA-966 (100 μM), a Gly-site antagonist of NMDARs, was tested for block of D-Asp currents both at -30 and 60 mV, to accommodate the voltage-specific effects of Gly at NMDARs. No block of D-Asp currents by HA-966 was observed with or without Gly present, at either -30 or 60 mV (Table 3.1). When pressure-applied to cells in the absence of D-Asp, neither Gly nor D-Ser induced currents in BSC neurons.

Pharmacology of D-Asp receptors

Additional pharmacological data are summarized in Table 3.2 and Figs. 3.2-6. Unless otherwise noted, pharmacology experiments were performed at –30 mV. The Cl^- channel blocker SITS (100 μM) was tested for block of D-Asp currents. Since D-Asp currents are not carried by Cl^- ions (Chapter 2), this drug served as a negative control. There was no significant difference in current amplitude of D-Asp currents in the presence of SITS (Table 3.2; n=7).

D-Asp currents were significantly reduced in amplitude by 23±24% in the presence of kynurenate (1mM), a general L-Glu receptor antagonist (Fig. 3.2A; $p<0.01$, Student's paired t test; n=9). The NMDAR antagonist APV (100 μM) had mixed effects, causing a large, reversible increase in current amplitude in 4 of 16 cells examined (Fig. 3.3A; mean =160±56%; $p<0.05$, Student's paired t test), and a significant decrease in all other cells tested (Fig. 3.3B; mean=10±36%; $p<0.05$, Student's paired t test; n=12). There was no significant difference in current amplitude in APV compared to controls when all 16 cells exposed to APV were considered as a single sample. D-Asp currents were not blocked by either MK-801 (500 nM; n=14) or memantine (100 μM; n=6), potent

NMDAR antagonists, in either the first exposure to D-Asp or the second application designed to allow the channels to open for the blocker to act (Table 3.2).

TCS46b (50 μM), an antagonist at subunits NR1A and NR2B of NMDARs, caused a slight, significant reduction in current amplitude (Fig. 3.2C, Table 3.2; mean block of 16±16%; $p<0.05$, Student's paired t test; n=7). PPDA (50 μM), an NMDAR antagonist showing greater preference for NR2C and NR2D subunit-containing NMDARs, was the most effective blocker of D-Asp currents observed, at 40±20% block (Fig. 3.2D, Table 3.2; $p<0.01$, Student's paired t test; n=12).

The AMPA/kainate receptor antagonists CNQX (100 μM) and NBQX (5 μM) significantly potentiated D-Asp current amplitude (Fig. 3.3C-D, Table 3.2; mean increase 30±18%; $p<0.01$, Student's paired t test; n=8; and 15±14%; $p\leq0.05$, Student's paired t test; n=7, respectively), and the effect of each was reversible. DNQX (100 μM) and UBP302 (50 μM), non-NMDAR antagonists with higher specificity to kainate than AMPA receptors, did not block D-Asp currents (Table 3.2; n=8 and n=6, respectively).

TBOA (1 mM), a blocker of excitatory amino acid transporters (EAATs), significantly reduced D-Asp currents to a small degree (Fig. 3.2B; mean decrease 10±10%; $p<0.05$, paired t test; n=8).

D-Asp induced currents desensitized when agonist was repetitively applied at <40 s intervals (Chapter 2). CTZ prevents desensitization of AMPARs. Bath applied CTZ (200 μM) had no effect on the first D-Asp currents elicited in BSC neurons (Table 3.2; n=7). CTZ was further tested for its effects on D-Asp currents elicited on a time frame shorter than the normal interval of 40 s, when desensitization can be observed. The

amplitudes of D-Asp currents elicited 10 and 20 s after the first were normalized to the first current both in normal ECS and in ECS with CTZ (Fig 3.4). Compared to their respective controls there were no significant differences in the amplitudes of D-Asp-induced currents observed at 10 and 20 s in ECS versus in CTZ (when the second current occurred 10 s after the control, mean ECS/CTZ=24±22% of control/38±25% of control while at 20 s=19±21%/33±22%, respectively; n=8).

Effects of bath-applied agonists on L-Glu and D-Asp receptor currents

D-Asp was tested for its effects on L-Glu-activated currents in BSC neurons. In the presence of bath-applied 0.5 mM D-Asp, L-Glu-induced (1 mM) current amplitude was significantly reduced by 44±33% (Fig. 3.5A and B; $p<0.01$, Student's paired T test; n=24). This effect washed out with removal of D-Asp, indicating that the reduction in current amplitude was due to the presence of D-Asp. In contrast, bath-applied L-Glu (0.5 mM) had no effect on amplitude of D-Asp currents (Fig. 3.5C and D; n=6).

3.5 Discussion

One of the inherent difficulties in working with a molluscan model is that the majority of ion channel antagonists were developed for use in vertebrates, and often specifically mammalian models. Thus when working in an invertebrate model it is difficult to infer from a lack of block by a particular antagonist whether the receptor in question is not present or whether the antagonist simply does not block homologous channels in the model. While it may be more difficult to pharmacologically isolate a particular receptor channel in an invertebrate model, this difficulty is offset by the allure of a simple nervous system for the study of neurotransmitter actions of new agonists. In

addition, considering the effects of multiple agonists, antagonists and results from ion/substrate substitution experiments combined reduces the risk of misinterpretation of pharmacological observations.

In the previous chapter I described the D-Asp activated current in *Aplysia* as a non-specific cation channel that is permeable to Na^+ and K^+, but not Ca^{2+}. Similarly to NMDARs, the currents were blocked by Mg^{2+} at negative voltages. In many cells, however, D-Asp activated currents were not activated by L-Glu, suggesting activation of unique D-Asp receptors. D-Asp may have multiple receptor targets, some of which overlap with L-Glu.

EAATs are responsible for the re-uptake of L-Glu and D-Asp from the extracellular space. These transporters produce an electrogenic current via the uptake of substrate, in which $1H^+$, $3Na^+$, and one ligand (e.g. D-Asp or L-Glu) are co-transported into the cell, and one K^+ counter-transported out (Zerangue & Kavanaugh, 1996). Additionally, activation of EAATs initiates an uncoupled Cl^- conductance in some EAATs (Wadiche et al., 1995). This Cl^- conductance would be additive with D-Asp-activated non-specific cation currents across most of the voltage range (E_{Cl} = -4.7 mV, while $E_{D\text{-}Asp}$ = 7.7 mV [Chapter 2]). A number of studies investigating EAATs have utilized D-Asp as an agonist for these transporters (Davies & Johnston, 1972; Anderson et al., 1990; Balcar & Li, 1992; Apricò et al., 2007), and the EAAT blocker TBOA has been shown to be effective in blocking uptake of L-Glu in a transporter cloned from *Aplysia* (Collado et al., 2007). D-Asp currents in BSC neurons were slightly reduced in TBOA, supporting a small contribution of EAAT activation to D-Asp whole cell currents.

Kynurenate is a general L-Glu receptor antagonist in vertebrates (Stone 1993), and also was one of the first characterized antagonists of L-Glu-evoked currents in *Aplysia* (Dale & Kandel, 1993). One mM kynurenate was less potent at blocking D-Asp currents (~20%, this study) than L-Glu currents (~80%, Dale and Kandel, 1993). Block of a small percentage of current by kynurenate, PPDA, and TCS 46b suggests that activation of L-Glu receptors partially contributes to D-Asp whole cell currents, or that D-Asp may activate currents sharing similar pharmacology to L-Glu receptors.

While the permeability of D-Asp currents is most consistent with AMPA or kainate subtypes of L-Glu-activated receptors, the pharmacological data suggest that D-Asp activates a channel distinct from these receptors. The AMPA/kainate blockers UBP 302 and DNQX had no effect on current amplitude. While UBP 302 had not been tested in *Aplysia* or other invertebrates, DNQX has been shown to block serotonin-induced facilitation of a putative excitatory AMPAR-mediated response in *Aplysia* siphon motor neurons (Chitwood et al., 2001), as well as L-Glu-induced currents in mechanoafferent neuron B8 (Klein et al., 2000) and at sensorimotor synapses (Dale & Kandel, 1993; Armitage & Siegelbaum, 1998; Jin & Hawkins, 2003). Additional evidence that D-Asp does not activate AMPARs was the observation that CTZ did not prevent D-Asp current desensitization. CTZ has been shown to prevent desensitization at *Aplysia* sensorimotor synapses, presumably via acting at AMPARs (Antzoulatos et al., 2003). Thus, D-Asp likely does not activate AMPARs in *Aplysia* BSC cells.

Gly and D-Ser are obligatory co-agonists at NMDARs (Kleckner & Dingledine, 1988) and are not known to be voltage-specific. D-Ser had no effect on D-Asp induced currents, while Gly potentiated them only at –30 mV. Errico et al. (2010) working in a

mouse model suggested that D-Asp acts both at NMDARs and at receptors independent of NMDARs. It is possible that D-Asp-induced activation of NMDARs within the whole cell D-Asp current diluted the potentiating effect of Gly or D-Ser on the NMDAR fraction. Thus while D-Asp may activate NMDARs in these neurons, NMDARs probably are minor contributors to whole cell currents. At the high ionic strength of the solutions used in this study, as much as 100 nM contaminating Gly may be present even in Gly-free conditions, therefore we cannot rule out that the NMDA co-receptor site is already occupied in NMDARs on *Aplysia* neurons (Kleckner & Dingledine, 1988). The absence of block by the Gly-site antagonist HA-966, however, supports the conclusion that NMDARs, if present, are minor contributors to whole cell D-Asp induced currents.

It is interesting to note that the potent NMDAR channel blockers MK-801 and memantine had no significant effect on D-Asp-induced currents of BSC neurons. While MK-801 partially blocked D-Asp-induced currents in mice CA1 pyramidal neurons (Errico et al., 2010), these drugs do not appear in the literature of *Aplysia* pharmacology, possibly due to a lack of antagonism of NMDARs in this model. Our results confirm their lack of activity in *Aplysia*.

PPDA and TCS 46b are subunit-specific NMDAR antagonists with PPDA effective in blocking receptors containing NR2C/D subunits (Feng et al., 2004) and TCS 46b preferentially blocking receptors containing NR1A/NR2B subunits (Gregory et al., 2000). PPDA was the strongest blocker of D-Asp currents observed in this study (~40%), with TCS 46b having minor but significant blocking effects. PPDA block implicates NR2C/D subunits in D-Asp receptors in BSC neurons. The slight block by TCS 46b could represent a small contribution of NR2B subunits to D-Asp whole cell currents, or

may have been due to non-specific blocking effects of other NR2 subunits by TCS 46b. In contrast to our results, Errico et al. (2010) did not observe block of D-Asp receptors implicating specific NMDAR subunits. Not greater than partial block of D-Asp currents by any L-GluR antagonist in our model further argues that D-Asp is an agonist at both L-Glu channels and unique, D-Asp channels.

It is interesting to note that the generalized NMDAR antagonist APV did not block Mg^{2+}-sensitive, putatively NMDAR-like currents evoked by L-Glu in *Aplysia* (Dale & Kandel, 1993) or in *Lymnaea* (Moroz et al., 1993). Despite this apparent lack of activity on molluscan NMDARs, APV has been used extensively in studies investigating synaptic transmission and plasticity associated with learning in *Aplysia* (Glanzman, 1994; Lin & Glanzman, 1994; Murphy & Glanzman, 1997; Schacher et al., 1997; Conrad et al., 1999; Murphy & Glanzman, 1999; Antonov et al., 2003; Ezzeddine & Glanzman, 2003). APV had unexpected mixed effects on D-Asp-induced currents in subsets of BSC neurons, blocking 10% of the current in most cells while potentiating D-Asp currents an average of 3-fold in a minority of cells. The slight reduction in current in the presence of APV in 75% of BSC neurons exposed to D-Asp supports the hypothesis that NMDAR-like channels are partial contributors to D-Asp whole cell currents. In contrast, the potentiating effect of APV on D-Asp currents in some cells may have been mediated via allosteric modulation of the receptor (Kenakin, 2004). Allosteric antagonists are those in which binding of an antagonist may appear as conventional block of the receptor, or alternatively, can enhance receptor activation by changing the rank order of agonist potency. The portion of D-Asp current in BSC cells not affected by NMDAR antagonists, as well as the potentiating effect of APV, CNQX and NBQX in some cells suggests a

novel D-Asp receptor may be involved in the whole cell D-Asp response. Although spermine and some endogenous neurosteroids potentiate NMDAR currents (Mony et al 2009), L-GluR current enhancement by L-GluR antagonists such as APV, CNQX or NBQX has not been observed before. No AMPA/kainate blocker used in this study inhibited D-Asp currents, and so the identity of the non-NMDAR D-Asp receptor as AMPA or kainate receptors can tentatively be ruled out. Therefore we hypothesize that the portion of current that was blocked by APV may represent NMDARs, while the portion potentiated may represent a non-L-Glu D-Asp receptor current affected by an unknown but diagnostic allosteric mechanism: enhancement by select L-GluR antagonists.

The observed L-GluR block by bath-applied D-Asp may be either competitive inhibition or desensitization. D-Asp inhibited L-Glu-evoked currents ~44%, yet L-Glu did not block D-Asp induced currents. D-Asp acting as a partial agonist of a putative NMDAR current in *Aplysia* culminated in apparent inhibition of these currents (Dale & Kandel, 1993) while D-Asp directly competing with L-Glu at AMPARs yet not inducing current was observed in rat hippocampal neurons (Gong et al., 2005). Based on the pharmacological results presented here, it is possible that bath-applied D-Asp blocked or desensitized AMPARs, and/or a subpopulation of NMDAR-like receptors that ordinarily contribute to the whole cell current induced by D-Asp. Interestingly, since D-Asp currents were blocked 40% by PPDA it is possible that bath D-Asp blocks the same NMDARs containing the NR2C or NR2D subunits as PPDA.

Despite the potential problems associated with pharmacological agents that block incompletely in Aplysia, we found support for our hypothesis that D-Asp activated a

mixed collection of receptors by the summary actions of many reagents. D-Asp may activate at least two different known ion currents in *Aplysia* BSC neurons: EAATs and NMDARs containing NR2C and/or NR2D subunits. The component of D-Asp current that is not NMDAR or EAAT D-Asp current may represent a unique receptor channel.

Given that D-Asp activates currents in cells in which L-Glu does not (Fieber et al., 2010; Chapter 2), I postulate that D-Asp activates a non-L-Glu receptor channel. While pharmacological isolation of D-Asp-induced currents with TBOA and PPDA may permit study of its specific physiological contribution, only molecular description of this receptor in cells expressing solely the unique D-Asp receptors will definitively identify it. To date, however, the NR1 subunits are the sole L-GluRs to have been cloned from *Aplysia* (Ha et al., 2006). The results of this chapter and Chapter 2 strongly support the findings of Errico et al (2010) that D-Asp is a neurotransmitter at dedicated receptors in multiple species.

Table 3.1. Effect of NMDAR glycine-site blocker HA-966 on D-Asp whole cell current amplitude.

HA-966 (100 μM)			
Conditions	**Voltage (mV)**	**% block (-) or potentiation (+)**	**n**
D-Asp	-30	-5±14%	7
D-Asp + Gly	-30	+39±66%	8
D-Asp	60	-9±27%	5
D-Asp + Gly	60	+19±24%	5

Table 3.2. Summary of effects of antagonists on D-Asp whole cell currents.

Antagonist	**Target Receptor**	**[antagonist]**	**% block (-) or potentiation (+)**	**n**
Kynurenate	Non-selective L-GluRs	1 mM	-23±24%*	9
APV	NMDARs	100 μM	+160±56%* -10±36%*	4 12
MK-801	NMDARs	500 nM	-13±36%	14
Memantine	NMDARs	100 μM	-6±9%	6
PPDA	NMDARs – selective for NR2C/NR2D	50 μM	-40±20%*	12
TCS 46b	NMDARs – selective for NR1A/NR2B	50 μM	-16±16%*	7
DNQX	Kainate Rs	100 μM	-22±37%	8
UBP302	Kainate Rs	50 μM	+10±23%	6
CNQX	AMPA/kainate Rs	100 μM	+30±18%*	8
NBQX	AMPA/kainate Rs	5 μM	+15±14%*	7
CTZ	AMPAR desensitization	200 μM	+3±20%	7
TBOA	EAATs	1 mM	-10±10%*	8
SITS	Cl^- channels	100 μM	-1±17%	7

*significantly different from control via paired T-test at $p<0.05$

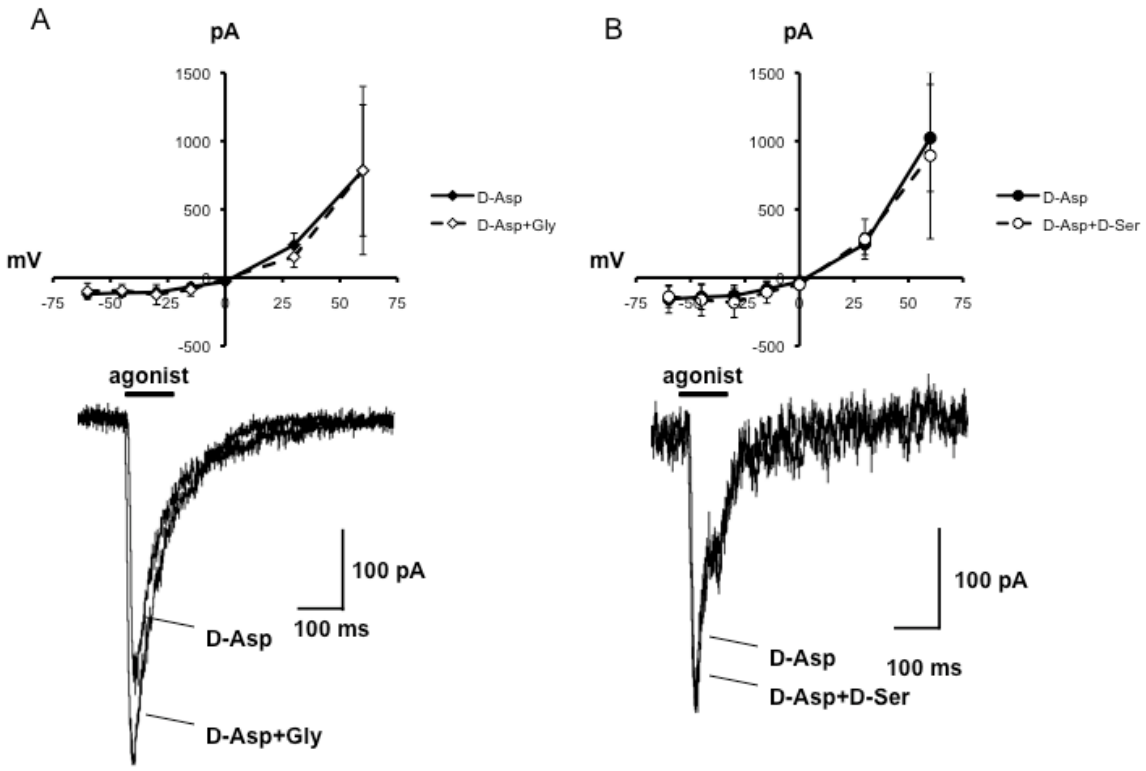

Fig. 3.1. D-Asp responses in BSC neurons in the presence of L-Glu co-agonists Gly and D-Ser.
A. Average current-voltage (I-V) relationship ±SD for D-Asp currents (1 mM; 100 ms) with and without Gly (added to the pressure ejection pipette, 1 mM; n=7).
B. Whole cell currents in D-Asp and in D-Asp + Gly in the same cell at -30 mV.
C. Average I-V relationship ±SD for D-Asp currents with and without added D-Ser (1 mM; n=7).
D. Whole cell currents in D-Asp and in D-Asp + D-Ser in the same cell at -30 mV.

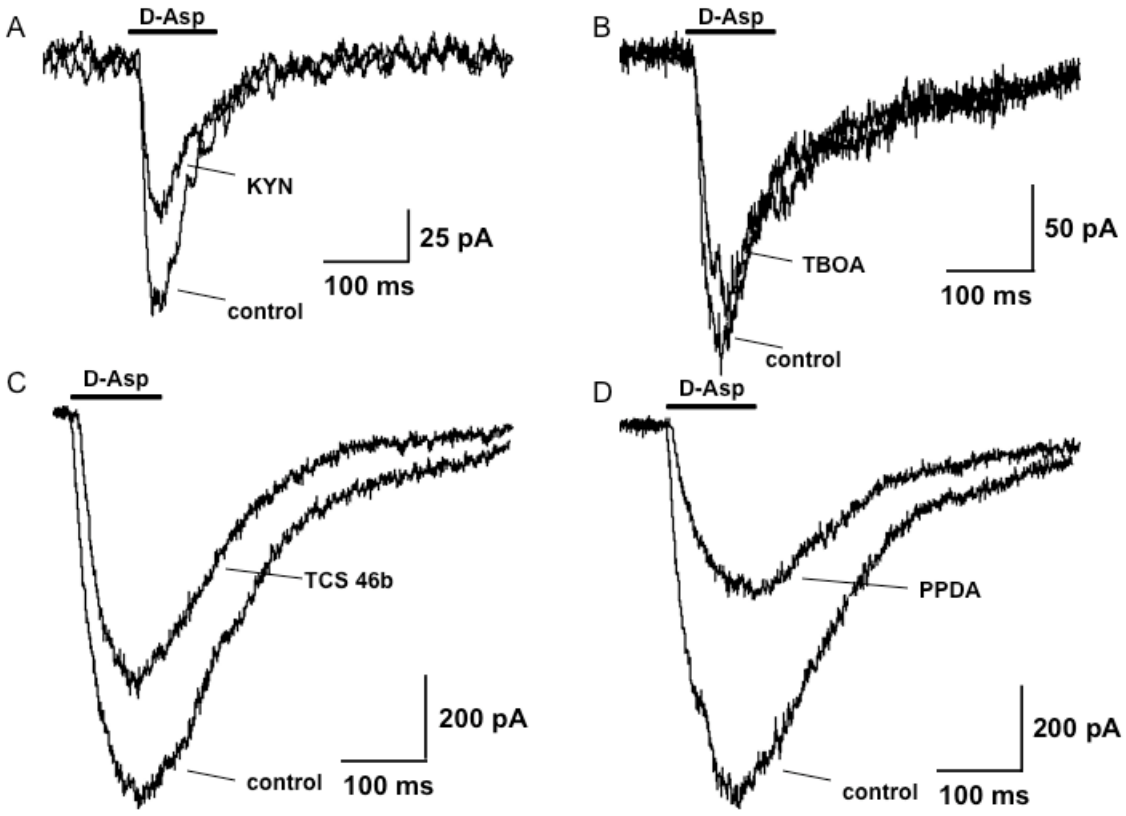

Fig. 3.2. Antagonists of D-Asp currents at -30 mV.
A. Whole cell currents in response to pressure application of D-Asp (1 mM) in ASW (control) and in ASW plus 1 mM kynurenate (KYN).
B. Whole cell currents in D-Asp in ASW (control) and in ASW plus 1 mM TBOA.
C. Whole cell currents in D-Asp in ASW (control) and in ASW plus 50 μM TCS 46b.
D. Whole cell currents in D-Asp in ASW (control) and in ASW plus 50 μM PPDA.

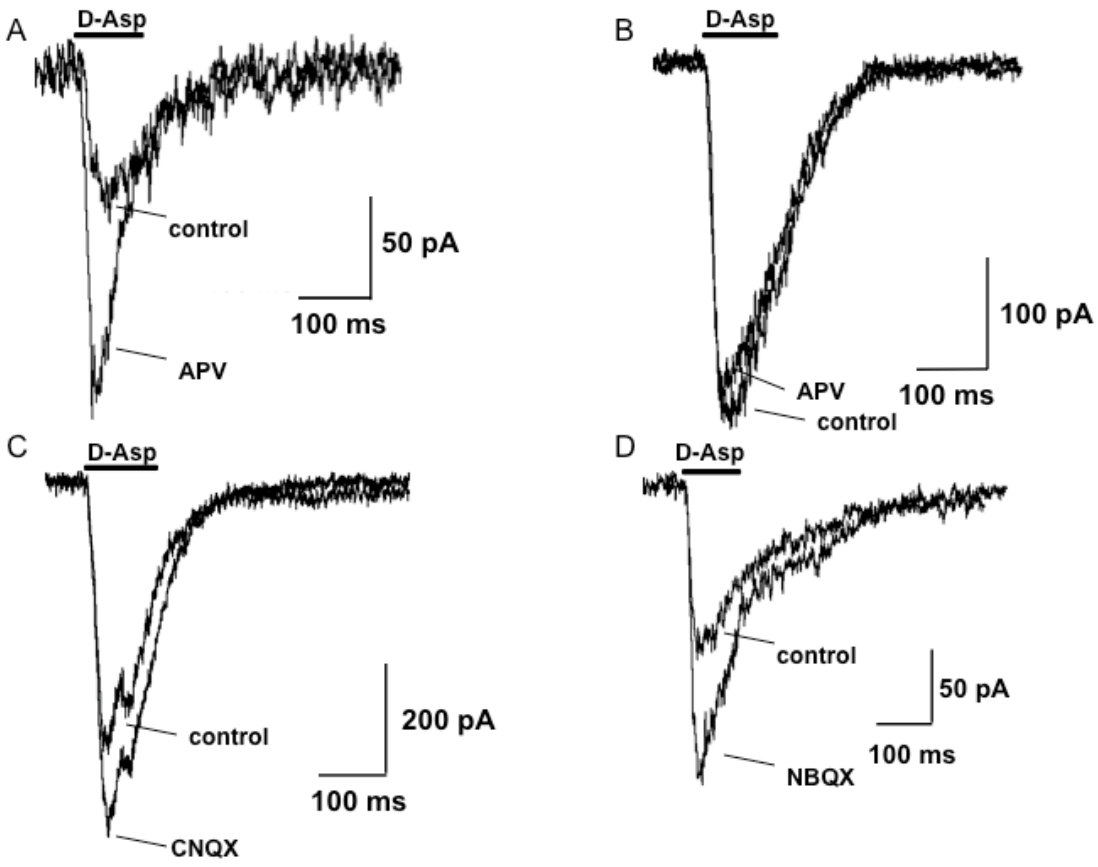

Fig. 3.3. Antagonists of D-Asp currents at -30 mV.
A-B. Currents in D-Asp (1 mM) in ASW (control) and in ASW with 100 μM APV.
C. Currents in D-Asp in ASW (control) and in ASW plus 100 μM CNQX.
D. Currents in D-Asp in ASW (control) and in ASW plus 5 μM NBQX.

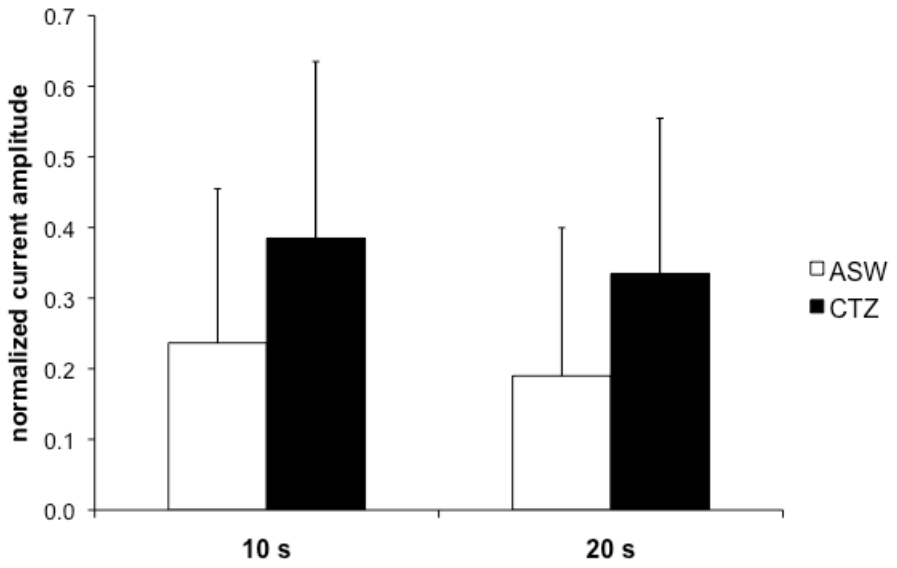

Fig. 3.4. Effects of CTZ on desensitization of D-Asp-induced currents. Control D-Asp-induced currents were elicited in ASW or ASW plus CTZ (200 μM), and then again at 10 and 20 s and plotted as a fraction of control current amplitude.

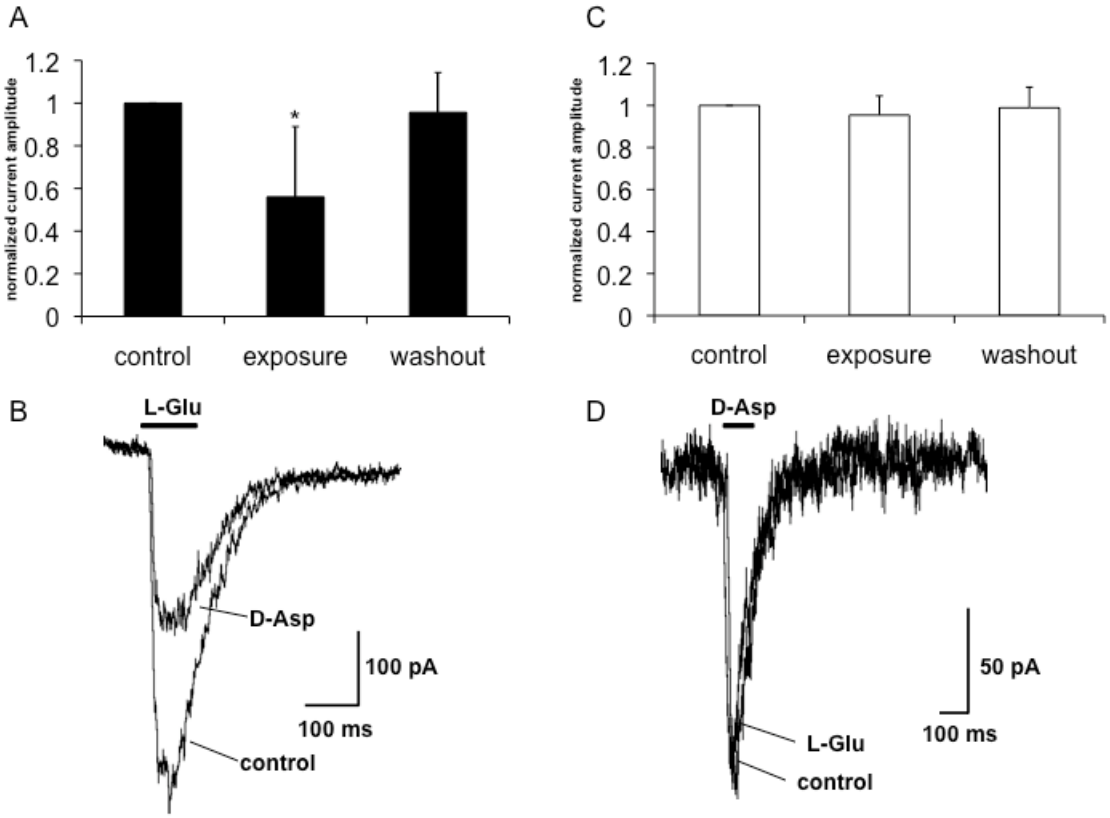

Fig. 3.5. Effects of bath-applied D-Asp and L-Glu on agonist-evoked currents.
A. Summary of effects of 0.5 mM bath-applied D-Asp ("exposure") on L-Glu-activated currents (1 mM). *denotes significant difference from control and washout at $p<0.05$; paired T-test, n=24.
B. Example L-Glu-evoked currents in ASW (control) and in ASW plus D-Asp .
C. Summary of effects of 0.5 mM bath-applied L-Glu on D-Asp-activated currents (1 mM; n=6).
D. Example D-Asp-evoked currents in ASW (control) and in ASW plus L-Glu .

Chapter 4:

Alternate agonist comparison and 5-HT-induced plasticity of D-Asp currents

4.1 Summary

The non-L-Glu receptor component of D-Asp currents in *Aplysia* 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. ACh and 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 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 three agonists. Charge area comparisons with L-Glu, however, supported previous results suggesting some overlapping receptor sites with D-Asp. Ten minute exposure to 5-HT induced facilitation of D-Asp-evoked responses in BSC neurons. This effect was mimicked by phorbol ester and blocked by bisindolylmaleimide (Bis), suggesting that PKC was involved. D-Asp induced excitation is subject to 5-HT induced

modulation that is similar to that involving AMPARs, but this modulation occurs at unique receptors for D-Asp.

4.2 Background

D-Asp activates multiple receptor sites in the *Aplysia* nervous system (Chapters 2 and 3). While some of these receptors overlap with the well-characterized neurotransmitter L-Glu, including NMDARs and EAATs (Chapter 3), D-Asp also activates channels independently of L-Glu (Errico et al., 2010; Fieber et al., 2010; Chapter 2). D-Asp currents in *Aplysia* are pharmacologically distinctive from L-Glu, with many NMDAR antagonists and all non-NMDAR blockers that are known to be effective against L-Glu receptors in *Aplysia* ineffective at inhibiting these currents, such as the AMPAR-specific DNQX and CNQX (Chitwood et al., 2001; Trudeau and Castelluci 1993; Chapter 3).

Molluscs use a variety of small molecules for excitatory synaptic transmission. 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. 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-HT_3 receptors. Receptors for both ACh (Kehoe, 1972; Nierop et al., 2005; Nierop et al., 2006) and 5-HT_3 (Walcourt-Ambakederemo & Winlon, 1995; Green et al., 1996; Clemens & Katz, 2001) have

been identified in the molluscan nervous system. The experiments in this chapter were designed to study the ability of D-Asp to activate these receptors.

5-HT-induced modulation at the synapse is one of the fundamental processes associated with learning and memory formation in both *Aplysia* and mammalian systems (reviewed in Glanzman, 2008). At the postsynaptic membrane, sustained exposure to 5-HT induces 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 upregulation of AMPAR expression (Li et al., 2005; Villareal et al., 2007). 5-HT appears to produce facilitation via activation of PKC (Villareal et al., 2009). As D-Asp receptors may be related to ionotropic L-GluRs, I tested D-Asp current modulation by 5-HT. The results presented in this chapter suggest a role for D-Asp specific receptors in synaptic plasticity in *Aplysia*.

4.3 Materials and Methods

Cell culture/Electrophysiology

Preparation of primary cell cultures of *Aplysia* BSC cells and whole cell voltage clamp measurements were made as described in Chapter 2 Materials and Methods section. 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, with <25 ms turnover time, at a concentration of 1 mM, and whole cell current figures are presented at a holding potential of -30 mV.

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.

Solutions

Unless otherwise noted, reagents were from Sigma-Aldrich (St. Louis, MO). ECS normally consisted of ASW containing (mM) 417 NaCl, 10 KCl, 10 $CaCl_2$ (2 H_2O), 55 $MgCl_2$ (6 H_2O), 15 HEPES-NaOH, pH 7.6. Control ICS contained (mM) 458 KCl, 2.9 $CaCl_2$ (2 H_2O), 2.5 $MgCl_2$ (6 H_2O), 5 Na_2ATP, 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 μM 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) were made in water. Stocks of Bis (500 μM; 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.).

Statistical tests

Data are presented as mean±SD. Significance for pharmacology, agonist co-application, and potentiation experiments were assessed using Student's paired t tests. Analyses were performed using Data Desk software (version 6.2; Data Description, Inc., Ithaca, NY). Differences at $p<0.05$ were accepted as significant.

4.4 Results

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. 4.1A). 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. 4.1B). 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. 4.1C). Due to unavailability of kainate at the time of these experiments, this compound was not tested. Pharmacological evidence, however, suggests D-Asp does not activate kainate receptors (Chapter 3).

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. 4.2), therefore known antagonists of ACh and 5-HT receptor currents were tested on D-Asp currents (Fig. 4.3 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 (Chapter 2), 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. 4.3A; 1 mM; mean decrease 25±29%; $p<0.05$, Student's paired t test; n=9) and the nicotinic blocker d-TC (Fig. 4.3B; 1 mM; mean decrease 58±28%; $p<0.05$, Student's paired t test; n=5), while D-Asp induced currents were unaffected. Conversely, PPDA (50 μM) significantly reduced D-Asp currents (Fig. 4.3C; mean decrease 46±15%; $p<0.05$, Student's paired t test; n=6), while ACh currents were unaffected.

5-HT activated currents were significantly reduced in the 5-HT_3 receptor antagonist granisetron (Fig. 4.4A; 100 μM; mean decrease 79±28%; $p<0.01$, Student's paired t test; n=5) and the $5HT_1/5HT_2$ receptor antagonist MES (Fig. 4.4B; 1mM; mean decrease 82±27%; $p<0.01$, Student's paired t 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. 4.4C; 44±20%; $p<0.05$, Student's paired t test; n=5).

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. 4.5A; 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. 4.5B; 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 slightly greater than that obtained by application of D-Asp and L-Glu together (Fig. 4.5C; mean difference of 30±23%; $p<0.05$, Student's paired t test; n=5), indicating some receptors associated with channels are activated by both D-Asp and L-Glu.

5-HT facilitation

5-HT approximating physiological levels (20 μM) was bath applied to BSC neurons for durations of 80 s or 10 min before D-Asp induced currents were elicited 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. 4.6A). Ten min application, however, significantly increased D-Asp current amplitude (Fig. 4.6B; mean increase 44±60%; $p<0.05$, Student's paired t test; n=8). This potentiation was sustained and remained after

10 min washout of 5-HT (Fig. 4.7A; mean increase 40±50%; $p<0.05$, Student's paired t 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. 4.6C), 10 min of PMA significantly increased current amplitude (Fig. 4.6D; mean increase 65±57; $p<0.05$, Student's paired t test; n=6). The potentiating effect of PMA on D-Asp current amplitude remained after 10 min of washout (Fig. 4.7B; mean increase +51±59; $p<0.05$; Student's paired t test; n=6), similarly to the 10 min bath application of 5-HT.

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. 4.8A and B, respectively). Eighty second application of 5-HT + Bis also had no effect on D-Asp current amplitude (Fig. 4.8C). While D-Asp currents appeared to increase after 10 min bath applied 5-HT + Bis, there was no significant difference in current amplitude, suggesting that Bis blocked the potentiating effect of 5-HT (Fig. 4.8D). There also was no significant difference when comparing the relative current amplitude increase of cells exposed to 5-HT for 10 min to cells exposed to 5-HT + Bis for 10 min (compare 2nd bars in Fig. 4.8B and D; two sample t test; n=8 and n=5, respectively).

4.5 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 that is permeable to Na^+ and K^+ (Chapter 2). While D-Asp currents show some similarities with L-Glu currents, D-Asp activates currents in many cells not responding to L-Glu, indicating independent target receptors. D-Asp shows some pharmacological similarities with L-Glu (Chapter 3). D-Asp currents are partially blocked by the L-Glu antagonists kynurenate, APV, PPDA, TCS 46b, and the EAAT blocker TBOA. Thus, in addition to activating non-L-Glu currents, D-Asp currents are partially characterized by activation of EAAT currents and L-Glu channels, or channels showing pharmacological similarity to L-Glu receptor channels. The present study extends 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 clearly has actions in *Aplysia* ganglia independent of NMDARs (Chapters 2 and 3). 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 some aspect of dissociating the cells for this study caused them to be NMDAR-deficient.

The lack of an AMPA-induced current, while surprising given the presumably ubiquitous nature of AMPARs (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 & Castellucci, 1993). It is possible that the eponymous ligand used to characterize AMPARs in vertebrates lacks activity in Aplysia, due to differences in the binding site of the receptors, yet the receptors may be AMPA subtype-like. So, while pharmacological evidence suggests that D-Asp does not activate AMPARs (Chapter 3), the failure of BSC cells to respond to AMPA may reflect that it is not the diagnostic agonist for these receptors in *Aplysia*, at least in BSC neurons, 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 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.

ACh and 5-HT activated excitatory currents in cells also responding to D-Asp. Currents activated by ACh and 5-HT had I-V relationships similar to D-Asp currents, but their distinct pharmacological profiles indicate that D-Asp did not activate nicotinic or serotonergic receptor channels.

Whereas previous studies in *Aplysia* neurons have observed a mixed response to ACh, consisting of multiple inhibitory and excitatory components (Kehoe, 1972), only excitatory currents were observed in BSC cells in response to ACh. The currents likely represent activation of the molluscan homolog of vertebrate nAChR (Nierop et al., 2005). Pharmacologically, the ACh currents were similar to those underlying excitatory post-synaptic potentials induced by ACh and blocked by C6 and d-TC (Kehoe, 1972). The absence of block of D-Asp currents by these antagonists confirms that D-Asp does not activate nAChRs in BSC neurons.

The activation of an ionotropic 5-HT receptor in *Aplysia* BSC cells is consistent with previous studies examining 5-HT_3 responses in molluscs (Walcourt-Ambakederemo & Winlon, 1995; Green et al., 1996; Clemens & Katz, 2001). Unlike previous studies, however, in which a biphasic or dual receptor-induced current was observed in response to 5-HT, 5-HT currents in BSC neurons superficially appeared to be due to activation of a single ionotropic receptor type and were blocked by the 5-HT_3 receptor-specific antagonist granisetron. Thus these receptor channels likely represent a homologue of the vertebrate 5-HT_3

receptor. The observation that the 5-HT_1/5-HT_2 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 subtracted 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. The pharmacological data presented in Chapter 3 and here corroborate this. 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. These data support previous conclusions about the ionotropic target of D-Asp (Chapter 3), in which EAATs and NMDARs, two targets also activated by L-Glu, are partial contributors to D-Asp currents. 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 & Kandel, 1993; Gong et al., 2005; Chapter 3). Due to the poor efficacy of L-Glu receptor blockers in Aplysia it is not possible to distinguish between these possibilities in voltage clamp studies, however, binding studies with intact membranes or in 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 washout further implicates involvement of second messengers, as does its mimic by the phorbol ester PMA, a direct activator of PKC, and block by the PKC inhibitor Bis. 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 may be similar.

Whereas the L-Glu current facilitation by 5-HT at sensorimotor synapses was blocked by the antagonist DNQX but not APV, implicating an increase in the number of AMPARs at the post synaptic membrane (Chitwood et al., 2001), pharmacological evidence suggests that D-Asp does not activate AMPARs (Chapter 3). D-Asp receptors may substitute for AMPAR in this role in BSC cells.

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 (Walter 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 & Byrne, 2006). Thus, this system should provide an ideal model for the investigation of modulation of D-Asp responses related to learning.

The results in the current and previous chapters confirm that D-Asp activates a unique receptor in the *Aplysia* central nervous system and suggests D-Asp induced

excitation may be subject to synaptic modulation by known mechanisms. The identification of non-L-Glu channels activated by D-Asp will require molecular characterization. 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.

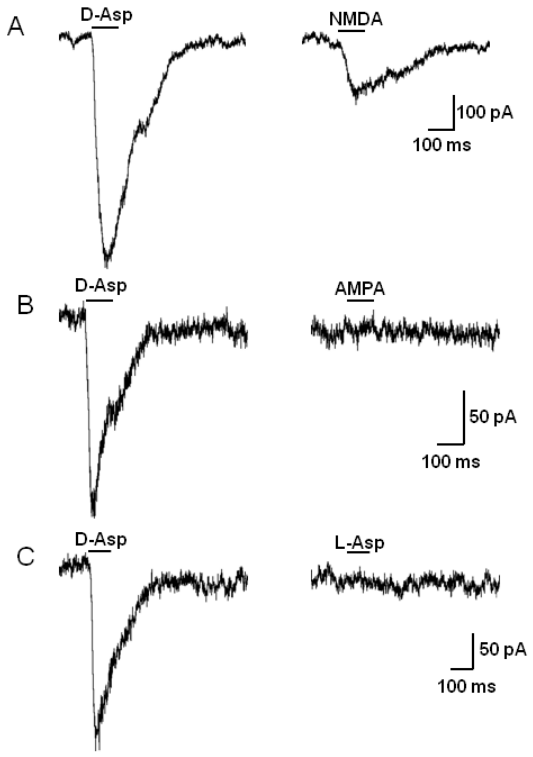

Fig. 4.1. BSC whole cell current responses induced by D-Asp, specific L-Glu receptor agonists, and L-Asp (1 mM; 100 ms).
A. Whole cell currents in a single cell induced by D-Asp (left) and NMDA (right).
B. Whole cell currents in a single cell induced by D-Asp (left) and AMPA (right).
C. Whole cell currents in a single cell induced by D-Asp (left) and L-Asp (right).

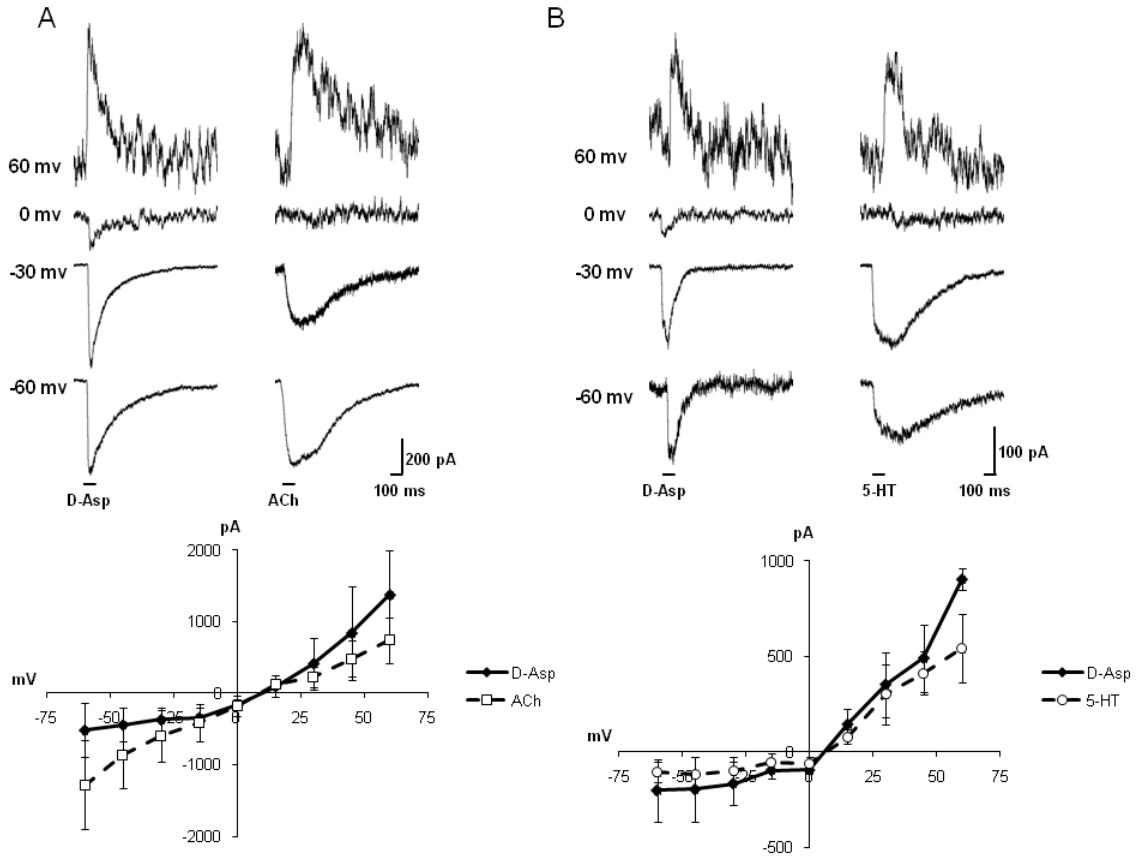

Fig. 4.2. Currents and current-voltage (I-V) relationships of D-Asp currents and currents evoked by ACh and 5-HT.
A. top - D-Asp (left) and ACh (right) whole cell currents in a single cell at different holding
potentials. bottom - average I-V ±SD of currents evoked by D-Asp and ACh (both 1 mM; 100 ms; 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 ±SD of currents evoked by D-Asp and 5-HT (both 1 mM; 100 ms; n=5).

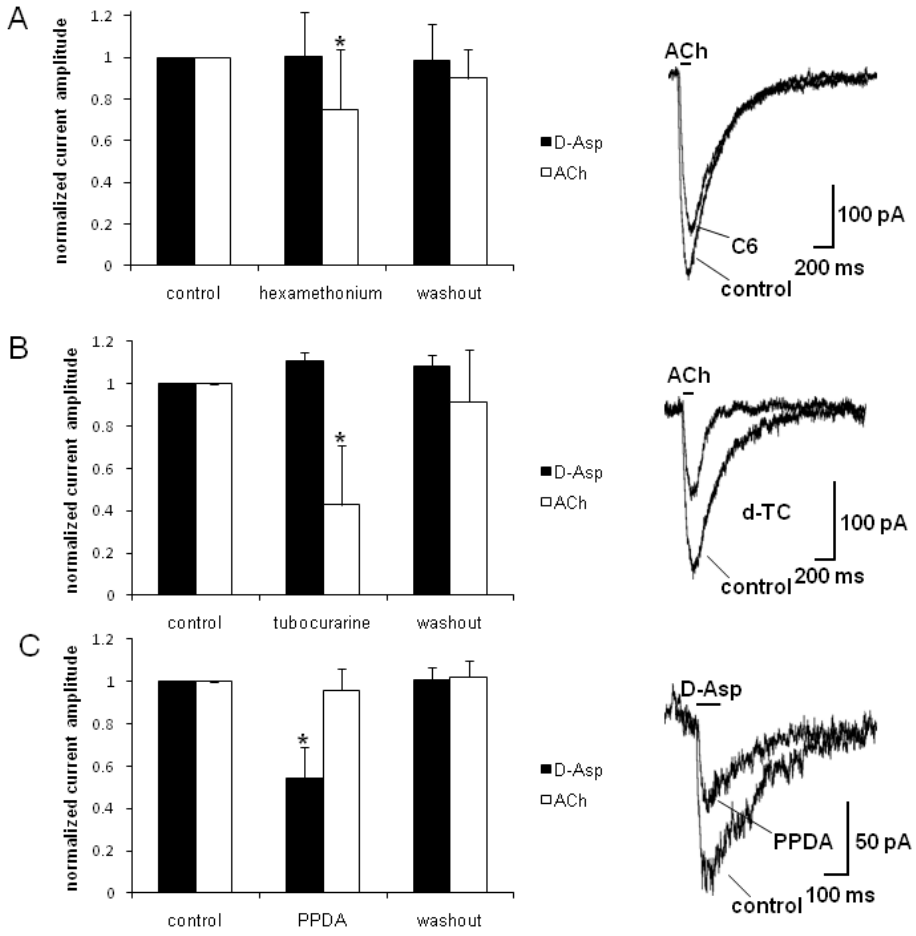

Fig. 4.3. Pharmacological differentiation of D-Asp and ACh currents.
A. Summary of effects of bath-applied C6 (1 mM) on D-Asp- and ACh-activated currents (left; n=9) and example control ACh induced currents and ACh currents in bath-applied C6 (right). *denotes significant difference between control/washout and application of blocker at $p<0.05$; paired T-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 μM) 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).

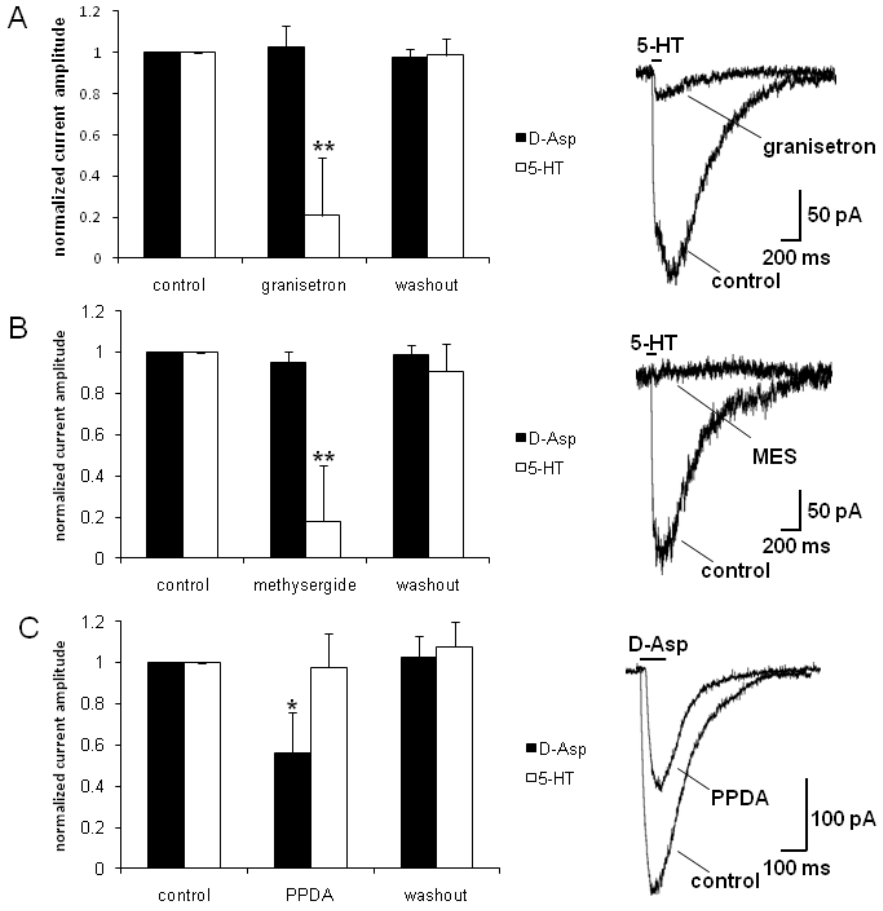

Fig. 4.4. Pharmacological differentiation of D-Asp and 5-HT currents.
A. Summary of effects of bath-applied granisetron (100 μM) on D-Asp- and 5-HT activated currents (left; n=5) and example control 5-HT induced currents and 5-HT currents in bath-applied granisetron (right). ** denotes significant difference between control/washout and application of blocker at $p<0.01$; paired T-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 μM) 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 $p<0.05$; paired T-test.

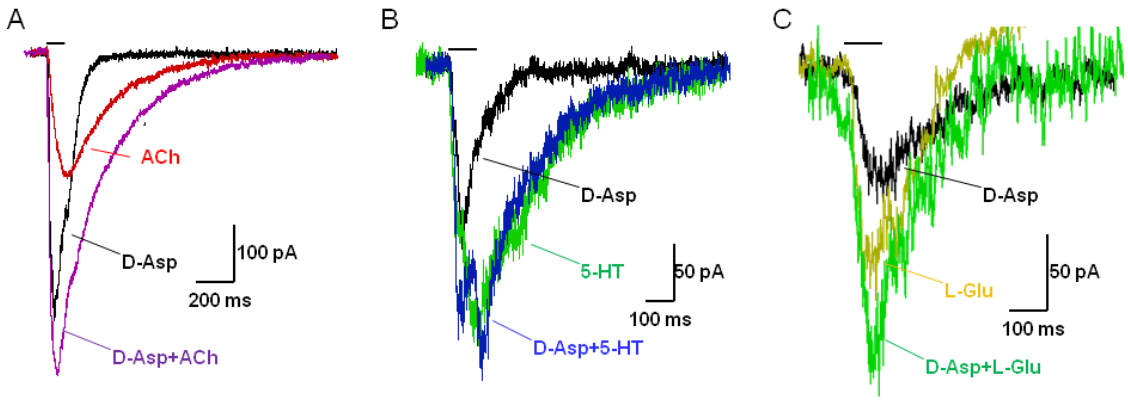

Fig. 4.5. Currents in response to individual and combined agonists.
A. Currents in response to pressure application (1 mM; 100 ms) of D-Asp (black), ACh (red), and D-Asp + ACh (purple). Mean difference +3±28%.
B. Currents in response to pressure application (1 mM; 100 ms) of D-Asp (black), 5-HT (green), and D-Asp + 5-HT (blue). Mean difference -5±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±23%; $p \leq 0.05$, paired T test (n=5).

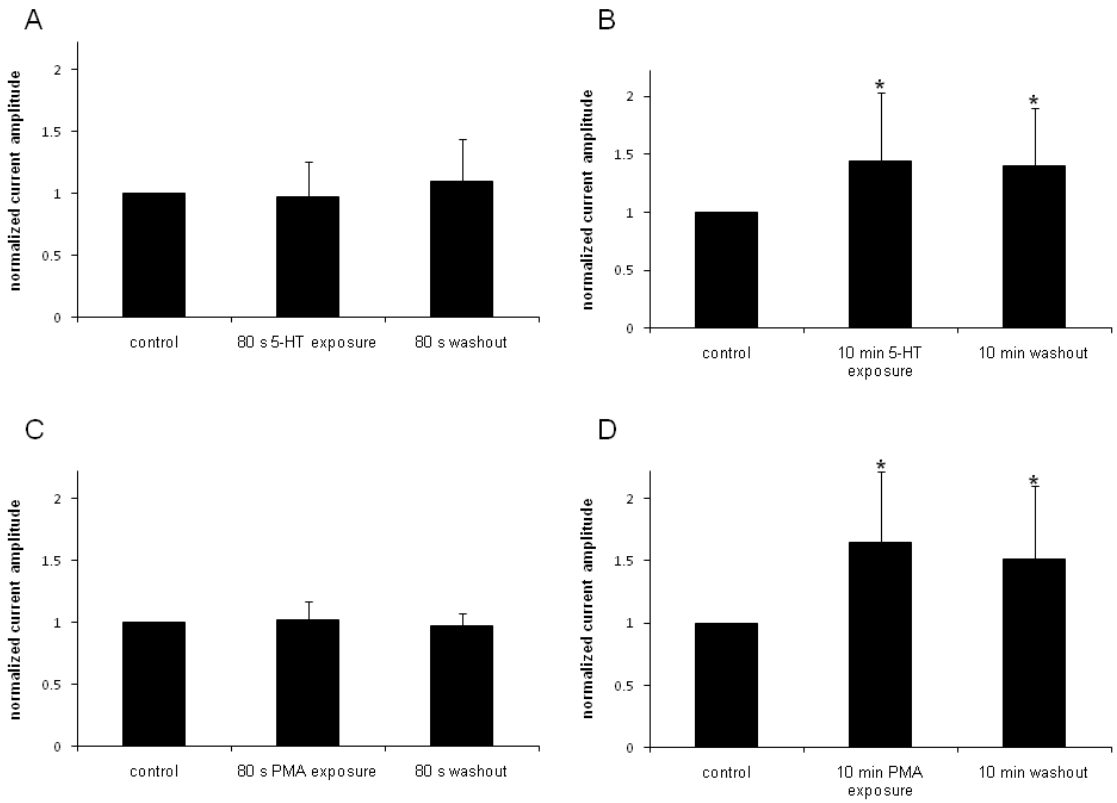

Fig. 4.6. Effects of exposure to bath-applied 5-HT and PMA on D-Asp-activated currents.
A. Effects of 80 s bath application of 5-HT on D-Asp currents (n=11).
B. Effects of 10 min bath applied 5-HT (20 μM) on D-Asp currents. * denotes significant different between control and 10 min application/10 min washout of 5-HT at $p \leq 0.05$, paired T 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).

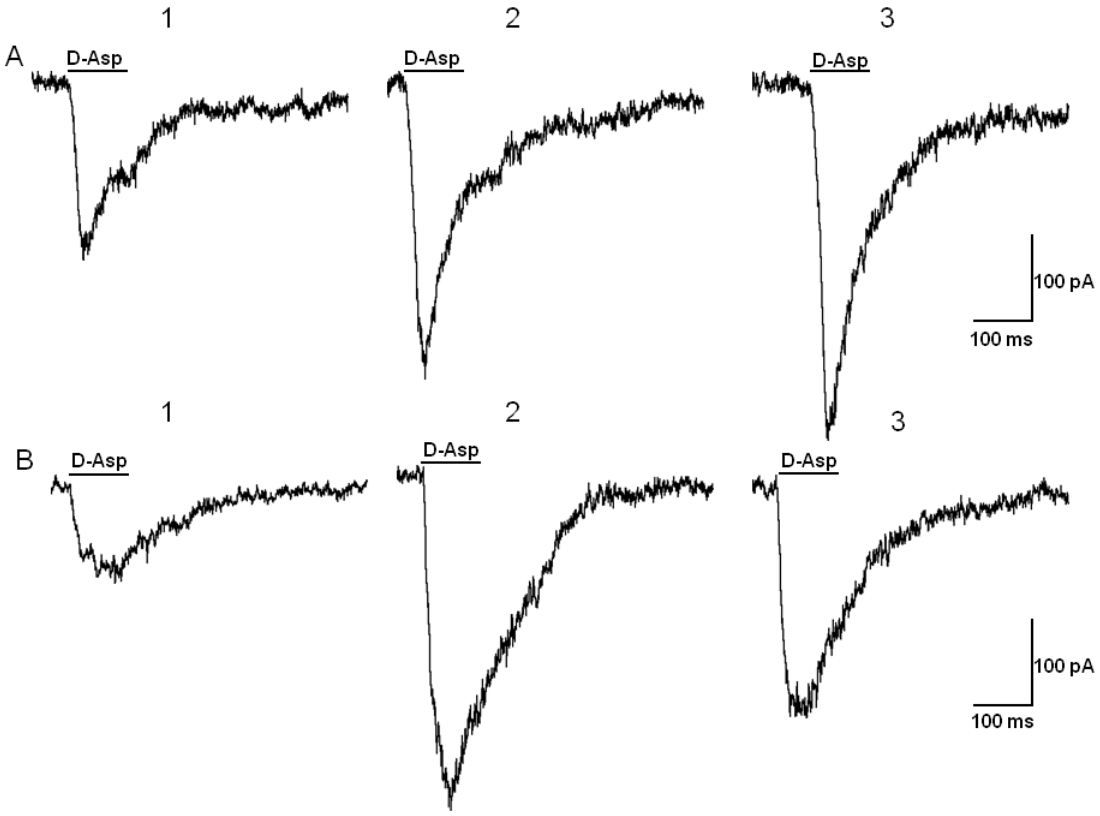

Fig. 4.7. Response of D-Asp currents to bath application of 5-HT and PMA.
A. D-Asp-activated currents: 1- before application of 5-HT. 2- after 10 min bath application of 5-HT (20 μM). 3- 10 min after washout of 5-HT.
B. D-Asp-activated currents: 1- before application of PMA. 2- after 10 min bath application of PMA (0.5 nM). 3-10 min after washout of PMA.

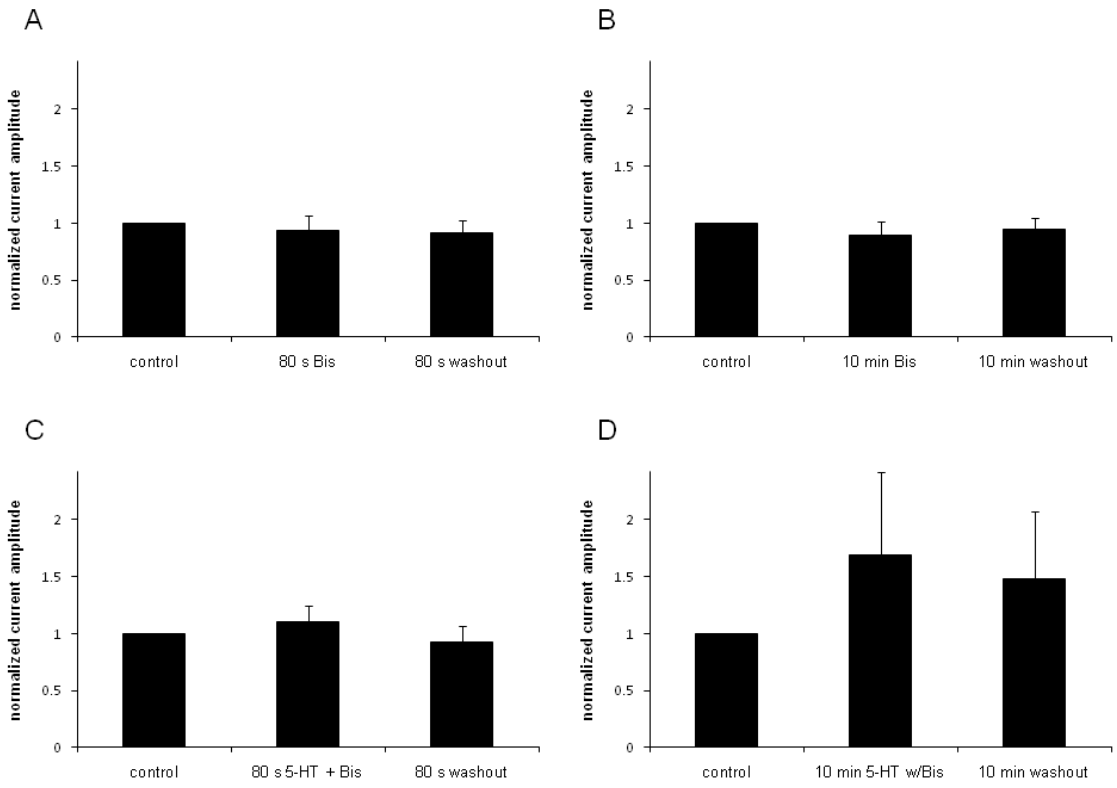

Fig. 4.8. Effects of exposure to Bis or Bis + 5-HT on D-Asp-activated currents.
A. Summary of effects of 80 s bath application of Bis (500 nM) on D-Asp currents (n=6).
B. Summary of effects of 10 min bath application of Bis on D-Asp currents (n=5).
C. Summary of effects of 80 s application of Bis+5-HT (20 μM) 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).

Chapter 5:

Conclusions

D-Asp appears to act as an excitatory neurotransmitter in *Aplysia*, activating a non-specific cation current. Channels activated by D-Asp are permeable to Na^+ and K^+. In addition to activating excitatory currents, D-Asp apparently acts as an endogenous antagonist of L-Glu currents in *Aplysia* neurons.

D-Asp whole cell currents share many characteristics with those induced by L-Glu, such as similar time courses, current-voltage relationships, and current densities. Receptor channels activated by these two agonists appear to be distinct in many instances, however, with over half of cells responding to one agonist, but not the other. Furthermore, D-Asp-activated currents activate and inactivate significantly faster and undergo a prolonged desensitization, requiring ~40 s to recover to full amplitude during repetitive applications of D-Asp, whereas L-Glu-activated currents recover from desensitization within a few seconds.

Pharmacological evidence suggests that D-Asp may activate multiple receptor sites in *Aplysia* BSC cells. D-Asp-induced currents are partially blocked by the EAAT antagonist TBOA, suggesting that currents associated with these transporters contribute to D-Asp whole cells currents. Several lines of evidence suggest that part of the D-Asp whole cell current is due to by activation of NMDAR channels. D-Asp-induced currents share several features of NMDAR currents: 1) they undergo voltage-dependent Mg^{2+} block, 2) they are partially blocked by the NMDAR channel blockers PPDA, TCS 46b,

kynurenate, and in some cells by APV, and 3) they potentiat in the presence of Gly at –30 mV. Experiments in which both D-Asp and L-GLu are applied and in which the charge crossing the membrane in response to individual versus simultaneous application of D-Asp and L-Glu are compared confirms that the two agonists may activate overlapping receptors. None of the non-NMDAR channel antagonists blocked D-Asp currents, moreover AMPA did not activate currents in BSC cells, suggesting that AMPA and kainate receptors are not mediators of D-Asp-induced excitation.

Additional experiments with receptor specific agonists of ionotropic L-Glu receptors support the pharmacological data. NMDA activates currents in only some (33% of cells examined) neurons responding to D-Asp. These currents are much smaller in amplitude compared to D-Asp-induced currents in the same cells. As mentioned, AMPA did not activate currents in any of the cells tested, while kainate could not be tested due to the rarity of this chemical reagent in the 21st century. Notably, L-Asp did not elicit currents in cells responding to D-Asp, attesting to stereospecificity of the actions of D-Asp at the receptors studied in this dissertation.

D-Asp does not activate nACh or 5-HT_3 receptor channels. Antagonists of ACh- and 5-HT-induced excitatory currents in *Aplysia* BSC cells have no effect on D-Asp-induced currents, while the D-Asp antagonist PPDA has no effect on ACh- or 5-HT currents. Experiments examining the amount of charge crossing the membrane in response to application of D-Asp with or without ACh or 5-HT further support independent receptor actions of these agonists.

Finally, D-Asp currents are modulated by activation of second messenger systems. Ten minute exposure to 5-HT potentiated D-Asp whole cell current responses.

This effect was mimicked by bath application of phorbol ester, and blocked by bisindolylmaleimide, suggesting that it occurs via activation of PKC.

D-Asp activated channels are non specific for monovalent cations, while lacking significant Ca^{2+} or Mg^{2+} permeation. Ca^{2+} permeability is a diagnostic feature of NMDARs. In this respect D-Asp receptors are similar to non-NMDARs; however, they share many pharmacological features with NMDAR channels, such as voltage-dependent Mg^{2+} block, glycine-induced current potentiation at -30 mV, and partial block by NMDAR antagonists. This research supports a partial contribution of NMDARs to D-Asp activated currents, and it may be the NMDAR current fraction, rather than the L-Glu receptor- or the unique D-Asp receptor fractions that exhibits these characteristics. Alternatively, NMDAR subunits may contribute to functional D-Asp receptors

While these studies provide a foundation for deciphering the physiological role of D-Asp, there is still much work to be done in characterizing the actions of this endogenous compound. These experiments confirm activation of two targets overlapping with L-Glu – NMDARs and EAATs. The identity of the putative non-L-Glu D-AspR, however, remains elusive. Identification of this receptor channel awaits the use of molecular techniques outside the scope of this study, because thus far only the NR1 subunits of NMDA-like receptors have been cloned from *Aplysia* (Ha et al., 2006).

Finally, the importance of 5-HT-induced facilitation of D-Asp responses remains to be investigated. Both the identity of the receptor channels being modulated, whether they are a novel D-Asp receptor channel or the NMDAR fraction of D-Asp whole cell current, as well as the mechanism of modulation, are unclear. Additionally, it is unknown if this form of synaptic plasticity may relate to learning in *Aplysia*. Use of reduced

preparations and simple forms of learning as with previous studies involving L-Glu should help clarify this. Experiments, for example, utilizing the sensory-motor connections involved in simple forms of learning such as habituation of the gill or tail withdrawal reflex could be used to investigate the potential for modulation of D-Asp responses. As it has numerous times in the past, *Aplysia* provides a relevant model system to learn about the link between an excitatory response, in this case the novel D-Asp induced response, and behavior in intact animals. Such studies will find relevance in the numerous neurophysiological systems in which free D-Asp is present.

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