Inhibition of the *Aplysia* sensory neuron calcium current with dopamine and serotonin

Tyler W. Dunn and Wayne S. Sossin

Dept. Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada H3A 2B4
Abstract:
The inhibition of *Aplysia* pleural mechanosensory neuron synapses by dopamine and serotonin through activation of endogenous dopaminergic and expressed 5HT\textsubscript{1Apl(a)/b} receptors respectively, involves a reduction in action potential associated calcium influx. We show that the inhibition of synaptic efficacy is downstream of the readily releasable pool, suggesting that inhibition is at the level of calcium secretion coupling, likely a result of the changes in the calcium current. Indeed, the inhibitory responses directly reduce a Ca\textsubscript{V2} like calcium current in isolated sensory neurons. The inhibition of the calcium current is voltage-independent as it is not affected by a strong depolarizing prepulse, consistent with other invertebrate Ca\textsubscript{V2} calcium currents. Similar to voltage-independent inhibition of vertebrate nociceptors, inhibition was blocked with src tyrosine kinase inhibitors. The data suggest a conserved mechanism by which GPCR activation can inhibit the Ca\textsubscript{V2} calcium current in nociceptive neurons.

Introduction:
The pleural sensory neurons of the marine mollusc *Aplysia californica* function as body wall mechanosensory neurons with a nociceptive role (Walters et al. 2004; Walters et al. 1983a; b). Stimulation of the pleural sensory neurons excite defensive withdrawal motoneurons resulting in defensive behaviors (Walters et al. 1983a). Modulatory substances such as FMRFamide and dopamine reduce pleural sensory neuron excitability and synaptic efficacy to withdrawal motoneurons (Dunn et al. 2012; Edmonds et al. 1990; Guan et al. 2003). Such inhibition of pleural sensory neuron activity would serve an analgesic or hypoalgesic role, reducing the sensation of stimuli to the body wall and foot of the animal.

Previously we reported that dopamine had a strong inhibitory effect on synaptic efficacy at synapses between *Aplysia* pleural mechanosensory neurons and defensive withdrawal motor neurons in culture (Dunn et al. 2012). The inhibitory response also included a large reduction in the presynaptic calcium flux. However, it was not determined whether the reduction in the action potential associated calcium transient was through direct inhibition of the Ca\textsubscript{V2} like calcium current, the current responsible for triggering transmitter release, as was suggested previously for FMRFamide (Edmonds et al., 1990). Alternatively, the reduction in the calcium transient may be indirect, through changes in potassium currents that affect action potential duration, or from failure of the action potential to propagate to and depolarize all the presynaptic terminals.

Here we further examine the mechanisms of dopaminergic inhibition of synaptic efficacy at *Aplysia* sensory to motor neuron pairs in culture. We show that the action potential does conduct to distal neurites and that the readily-releasable pool (RRP) of synaptic vesicles is not significantly affected by the inhibitory dopaminergic response. An inward barium current that is insensitive to 10\textmu M nifedipine, and that has activation and inactivation characteristics consistent with a current mediated by a Ca\textsubscript{V2} calcium channel was isolated. Either dopamine or specific activation of over-expressed 5HT\textsubscript{1Apl(a)} receptors with 8-OH-DPAT, produces significant inhibition of this isolated Ca\textsubscript{V2} like barium current. A strong depolarizing prepulse has no effect on the inhibition of the
inward barium current, indicating that this type of inhibition is solely voltage-independent. Finally, we show that the inhibition of the Ca\textsubscript{v}2-like current is sensitive to a src kinase inhibitor, but not the inactive analog. Thus, the GPCR-mediated inhibition of Aplysia sensory neurons includes a significant reduction of the voltage-gated calcium current responsible for transmitter release and such inhibition is voltage-independent and therefore would not be relieved by sensory neuron firing. Similarly, voltage-independent inhibition of Ca\textsubscript{v}2 channels is important for analgesic mechanisms in vertebrates and the common sensitivity of this inhibition to tyrosine kinase inhibitors suggests that the mechanism of action is also conserved.

Methods:

Animals and cell culture

Aplysia californica were obtained from the University of Miami/National Resource for Aplysia mariculture facility (Miami, FL) at sizes from 50-120gr. 50-80gr animals were used for pleural sensory neurons, isolated from the ventro-caudal cluster of sensory neurons (Walters et al. 1983a). LFS motorneurons (siphon withdrawal motorneurons) (Hickie and Walters 1995), were identified based on anatomical position, morphology, and electrical properties (as in (Chitwood et al. 2001)). Sensory and motor neuron pairs were cultured for days in a 50-50 mixture of modified L-15 media and Aplysia haemolymph, supplemented with L-glutamine just prior to culture. Isolated sensory neurons used in figure 2 were cultured in the same media as synaptic pairs for 3 to 4 days, and neurons chosen with a large varicosity or terminal growth cone at least 200\textmu m from the soma of the neurons. Isolated sensory neurons used in figure 3-6 were cultured in a modified L-15 media with only 10% haemolymph to reduce neurite outgrowth. All cells were cultured in 50mm glass bottomed dishes precoated with poly-L-lysine. Changing of solutions during an experiment was achieved with a constant vacuum suction out to maintain bath volume and gravity flow into the recording dish. Unless otherwise noted, isolated sensory neurons for two-electrode voltage clamp experiments were plated at room temperature and left at room temperature until use within 24 hours. The exception to this was for 5HT\textsubscript{1Apl(a)} expression where instead of recording, the vector was injected at 24 hours and the plates were then put in an incubator at 18\textdegree C to reduce further neurite outgrowth and recording conducted after a further 24 hours allowing for vector expression and receptor plasma membrane localization. Dishes were allowed to acclimate to room temperature for at least one hour if taken from an incubator before recording.

Sharp electrode recordings

Sharp electrode recordings were made using glass capillary pipettes pulled on a Sutter P89 (Sutter Instruments Inc., Novato, CA) to 15-20M\textOmega when backfilled with 2M K\textsubscript{2}SO\textsubscript{4}. Electrodes were bridge-balanced before cell impalement and rebalanced regularly following impalement with positive pulses in sensory neurons and negative pulses in motorneurons. Prior to recording the culture media was replaced with artificial sea water (ASW) of the following composition in (mM): NaCl (460), MgCl\textsubscript{2} (55), CaCl\textsubscript{2} (10), KCl (10), D-Glucose (10), HEPES (10), pH 7.6. The presynaptic electrode was used to hyperpolarize the sensory neuron and generate action potentials with 50ms depolarizing
current pulses. The postsynaptic electrode was used to hyperpolarize the membrane potential of the motorneuron to -80mV and to measure post synaptic potentials (PSP) and spontaneous, miniature PSP. Membrane voltages and command currents were controlled and recorded with an Axoclamp 900 amplifier, Digidata 1440 digitizer, and pClamp software (Molecular Devices, Sunnyvale, CA). Changes in synaptic strength were examined by measuring the PSP rise-rate as the amplitude change over 1ms after the initial non-linear change in amplitude using pClamp software. The initial PSP rise-rate was used to measure changes in synaptic efficacy with postsynaptic voltage recordings (Fig. 1Ai). With small PSP amplitudes (<20mV), PSP amplitude and the initial PSP rise-rate change proportionally (see Fig. 1Aiii for correlation between PSP amplitude and PSP initial rise rate). However, as PSP amplitude increases, non-linear summation of the underlying quantal currents and the activation of voltage-dependent currents produce errors in the measurement of changes in synaptic efficacy from PSP amplitude. Fig. 1Aii displays a variety of PSPs (>20mV) that all activate voltage-dependent currents that obscure changes in transmitter release from being estimated from the measurement of peak PSP amplitude. Thus, the initial PSP rise-rate provides a more accurate measurement of changes in transmitter release and was used here. Unitary transmitter release events observed as spontaneous mPSPs and sucrose evoked unitary PSPs were analyzed with MiniAnalysis (Synaptosoft, Fort Lee, NJ) or pClamp, where events where individually examined and measured only if the temporal characteristics were similar to the action potential evoked PSP and of an amplitude >5 times the standard deviation of the noise band for the particular recording.

**Loose-patch recording of action potential occurrence in distal neurites**

Fire-polished patch pipettes at 1MΩ tip resistances (backfilled with ASW) were created using a Sutter P89. Using an airtight microelectrode holder, suction was applied through a side port with a syringe. The loose-patch pipette was connected to an Axoclamp 2D in voltage clamp mode. While firing action potentials at 0.1-0.3 Hz in the soma with a sharp electrode, suction was applied to the pipette until the occurrence of the action potential was visible at the neurite, suction was continued until the action potential amplitude in the neurite was at least five-fold greater than the standard deviation of the noise band (though the signal to noise ratio was often many times greater than this).

**Two-electrode voltage clamp**

Two-electrode voltage clamp experiments were conducted on isolated sensory neurons cultured for 24hr unless otherwise stated, by impaling the sensory neuron with two sharp electrodes (10-15MO) attached to an Axoclamp 900 (Molecular Devices, Sunnyvale, CA). Cells were preferentially taken from 50-80gr animals to limit neuron soma size. Five to ten minutes prior to the experiment the culture media was replaced with a TEA (Tetraethylammonium) based saline of the following composition (in mM): TEA Cl2 (460), 4-AP (4-aminopyridine) (10), MgCl2 (45), CaCl2 or BaCl2 (20), HEPES (25) Glucose (10), nifedipine (0.1), pH 7.6 with CsOH. Nifedipine, being light sensitive, was added just prior to use and was dissolved in DMSO to final concentration of 0.1%. Following successful impalement with both electrodes, two-electrode voltage clamp was initiated, clamping the cell membrane at -80mV unless stated otherwise. All raw data was attained from averaging the traces of two successive runs of the same command.
voltage protocol with ClampEx software into a single trial. In experiments with
dopamine or 8-OH-DPAT at least 3-4 trials (one minute per trial) were acquired prior to
addition to insure current stability, with experiments removed if either inward or outward
currents are unstable during this period.

Vector nuclear microinjection and fluorescence imaging
5HT_1Apl(a)-eGFP in pNEX3 vectors were prepared and injected as described (Nagakura et
al. 2010). eGFP was imaged 24hr later with an Axioobserver D1 with standard Zeiss
GFP filter set (ex:470/40 and em:525/50) (Carl Zeiss Canada) and a Expo HBO arc (X-
cite, Mississauga, CAN).

Data analysis
Electrophysiological data was analyzed with pClamp software (Molecular Devices,
Sunnyvale, CA) and statistical analyses with GraphPad Prism (GraphPad software, La
Jolla, CA). All statistical tests were paired t-tests unless otherwise stated. Data are
presented at means, and error bars are standard errors of the mean.

Results:
The inhibitory dopaminergic reduction in synaptic efficacy in pleural sensory neurons
does not include a reduction in the RRP
Synaptic efficacy is strongly inhibited by dopamine in a majority of pleural sensory to
motor neuron synapses in culture. The reduction in synaptic efficacy was accompanied
by a large reduction in the action potential associated calcium influx into the presynaptic
contacts with postsynaptic motorneurons (Dunn et al. 2012). This calcium flux is
responsible for triggering evoked transmitter release from the readily releasable pool
(RRP) (Edmonds et al. 1990). While these results are consistent with the inhibitory
action of dopamine being at the calcium current, they did not rule out a role for dopamine
in regulating the RRP. To test this directly we measured the RRP at pleural sensory
neuron to abdominal LFS motorneuron synapses reconstructed in culture before and after
addition of dopamine. The RRP was measured following two successive action potential
evoked postsynaptic potentials (PSP) evoked before and after 2 min in 1μM dopamine to
assess the degree of inhibition on synaptic strength (Fig. 1B). At Aplysia synapses in
culture, 1M sucrose results in a ‘burst’ of unitary events or minis as the RRP empties
over approximately 45 seconds, much slower than many other preparations, but
conveniently allowing for a count of unitary events rather than a charge measurement
from release of the entire RRP ((Rosenmund and Stevens 1996; Zhao and Klein 2002),
Fig. 1C). The distribution of amplitudes observed with sucrose release is similar to that
of the spontaneous minis (mPSP) observed at a particular synapse (Fig. 1D), confirming
that the sucrose events are largely unitary release events allowing for an estimate of the
RRP with a simple count of the mPSPs. Application of 1M sucrose within 30sec of the
second PSP allowed for estimation of the size of the RRP during the inhibitory
dopaminergic response, followed immediately by a third action potential evoked PSP
(PSP3) to confirm depletion of the RRP. To avoid errors in the estimate of synaptic
strength using PSP amplitude at strong synaptic connections, the initial PSP rise-rate was
used here instead of amplitude (see Methods). As described previously (Dunn et al. 2012), the inhibitory dopaminergic response occurs at approximately 60% of cultured pleural sensory neurons, thus the synaptic pairs can be put into two groups, a group showing a reduction in membrane excitability and PSP amplitude, and a group without this inhibitory response (Fig. 1E). In the group showing a strong inhibitory response to dopamine, the PSP rise-rate significantly reduced by over 95% (average amplitudes reduce from 19.1±5.9mV to 0.8±0.4mV) often to synaptic failure, whereas sensory neurons insensitive to dopamine show only a mild reduction in PSP rise-rate, a result of homosynaptic depression (Fig. 1E, difference between PSP1 and PSP2; (Armitage and Siegelbaum 1998)). Following the 45s exposure to 1M sucrose, PSP amplitude was greatly reduced, a result of RRP depletion (Fig. 1E, PSP3). Comparison between the two groups of synaptic connections (Inh. DA or No Resp.) found a modest, but not significant, decrease in the unitary event count with the 1M sucrose estimate of the RRP (Fig. 1F). Thus, the dopaminergic reduction in PSP amplitude at pleural sensory neuron synapses cannot be entirely accounted for by a reduction in the size of the RRP.

The inhibitory dopaminergic reduction in membrane excitability does not prevent action potential propagation to distal sensory neuron varicosities

One explanation for the dopamine-induced reduction in presynaptic calcium influx after an action potential is a lack of action potential propagation to the presynaptic terminals (Dunn et al. 2012). Indeed, the large reduction in membrane excitability induced by dopamine suggests this explanation. To examine this question directly we measured action potential propagation at isolated sensory neurons following three to four days in culture to allow extensive neurite outgrowth. Action potentials were evoked and measured in the soma with a sharp electrode before and after dopamine application. At the same time a loose-patch electrode was used to observe the occurrence of an action potential at the end of a neurite as displayed in figure 2A. In all of the trials, the distance between the extracellular electrode and the soma were at least 200μm. When dopamine reduced excitability resulting in action potential failure in the soma, no action potential was observed in the neurite as would be expected. When enough depolarizing current was injected into the sensory neuron soma to overcome the dopaminergic inhibition, producing an action potential, the action potential was again observed in the neurite at amplitudes consistent with what was observed before addition of dopamine (Fig. 2B-D). In this experiment every action potential in all the preparations examined with dopamine, somatic action potentials were observed to propagate to the neurite examined with a loose-patch electrode. Thus, the reduction in calcium influx at presynaptic terminals with dopamine is not the result of dopamine preventing action potential propagation to distal neurites.

Isolation of an Aplysia CaV2-like calcium current in cultured pleural sensory neurons

The data thus far suggest that the effect of dopamine on synaptic efficacy is a result of reduced presynaptic calcium influx through CaV2-like voltage-dependent calcium channels that conduct the calcium current responsible for transmitter release. Next we sought to isolate and directly measure this calcium current in sensory neurons and then determine if this current is reduced by dopamine. To measure calcium currents in pleural
sensory neurons we used two-electrode voltage clamp. Sensory neurons were cultured in reduced hemolymph (reduced to 10% from 50%) and only cultured for 24hr to reduce neurite outgrowth so as to achieve an acceptable space clamp over the neuron. The sodium of the extracellular solution was replaced with TEA (460mM) and 4-AP (10mM) to block potassium currents and limit sodium influx through voltage-gated sodium channels. The calcium/barium to magnesium ratio was increased to 20mM calcium/barium, 45mM magnesium (from 10:55 respectively) to enhance the inward current. Replacing barium with calcium reduces the peak inward current and shifts the current/voltage (IV) curve consistent with other calcium currents (Fig. 3ABC; Spafford et al. 2003). Barium also strongly reduces the contaminating residual K+ current as noted elsewhere (Armstrong et al. 1982), further aiding in isolation of the inward current. Consistent with a Cav2 current, substitution of calcium for barium reduces the peak current but does not change the inactivation tau of the current as would have been expected for a Cav1 current (Fig. 3C). Addition of 100μM cadmium completely inhibits the inward current, allowing measurement of the residual K+ current that can be used for subtraction (Fig. 3AB). To remove any potential Cav3 or T-type calcium currents, sensory neurons were held at -50mV, a potential that should inactivate any T-type influx (Senatore and Spafford 2010). Comparison of current/voltage (IV) relationships of the inward currents with SNs held at -80mV or -50mV reveals no differences in current kinetics, indicating that T-type currents are likely not present in pleural sensory neurons (Fig. 3D). To measure the contribution of the Cav1 or L-type calcium currents, 10μM nifedipine (Brezina et al. 1994; Edmonds et al. 1990; Laurienti and Blankenship 1996; Ludwar et al. 2009) was applied to reveal a modest contribution (~10%) of the Cav1 current to the overall inward current under these conditions (Fig. 3E). The amount of peak current reduced with nifedipine was equal to the amount of current reduced when the current is measured at the end of a 200ms step pulse, consistent with the removal of a slowly-inactivating component such as a Cav1 current (Fig. 3E). To ensure that this concentration of nifedipine is sufficient to completely block the Aplysia Cav1-like current, we took advantage of the observation that cultured sensory neurons that lack residual axons and any neurite outgrowth have only a small, slowly inactivating, inward barium current (Cav1-like). Under these conditions, the inward current is completely blocked by 10μM nifedipine (Fig. 3F). Furthermore, the slow inactivation rate (>200ms) of the nifedipine-sensitive current seen in the subtracted current traces (Fig. 3F) is also characteristic of a Cav1 or L-type calcium current.

Change in inward barium current with morphology suggests the channel is in growth cones

The isolation of a Cav2-like current without consideration of neuron outgrowth resulted in currents of widely varying inactivation rates that were clearly related to cell morphology. Pleural sensory neurons with a single large growth cone at the end of a short axon (as in Fig. 4A left image) produced the largest peak currents with the fastest inactivation rates. Occasionally a cultured neuron would have no outgrowth. However with such an ideal voltage-clamp morphology, no inward current is observed suggesting that the Cav2-like channel is not expressed at the plasma membrane of the pleural sensory neuron somata (see Fig. 3F with nifedipine).
The largest inward currents were observed in cells with low neurite outgrowth typical of 24hr cultures. Examining current activation and inactivation kinetics in cells cultured for 24, 48, or 72hr shows a clear trend towards a reduction in the peak current and a slowing of the inactivation rate of the barium current (Fig. 4ABC). These changes are indicative of channels conducting the isolated inward barium current being located in distal neurites. As the terminals grow outside of the space clamp of the two-electrode voltage-clamp, the measured current is reduced and slowed as a result of errors in membrane potential.

With a well-isolated inward current (with barium and nifedipine, 24hr culture/low outgrowth) a 10s prepulse was used to measure the steady-state inactivation kinetics to further characterize the isolated inward current (Fig. 4D). The steady-state inactivation curve of the isolated inward current is similar to that reported for the *Lymnaea CaV2* current (Spafford et al. 2006).

All subsequent experiments were preformed in the TEA, 4-AP, 20mM Ba\(^{2+}\), and 10\(\mu\)M nifedipine saline with sensory neurons cultured for 24hrs with no to low outgrowth (as is Fig. 4A left panel) so as to isolate and maximize the inward barium current, while removing the contribution of CaV1 channels as described in the previous two figures.

**Inhibition of the isolated inward barium current with dopamine or activation of over-expressed 5HT\(_{1A}\) receptor**

To examine whether the inhibitory dopaminergic response includes modulation of the isolated barium current, voltage steps to -10, 0, +10, +20mV were used to measure the peak amplitude of the inward barium current. A final voltage step in the IV curve was to +60mV, which is at or near the reversal potential for calcium currents (Fig. 3, (Spafford et al. 2006)) and was thus used to measure the change in the residual potassium currents concurrent with the change in the peak inward current which includes the contaminating residual K\(^+\) currents. The amplitude of the residual K\(^+\) current is more than three fold larger at +60mV than at +10mV which is the voltage step used for measurement of the peak inward current. Thus, if there is no significant increase in the amplitude of the current measured at +60mV, then we can be assured that there will not be a contribution of changing residual K\(^+\) currents to the measured inward barium current at +10mV.

Application of 1\(\mu\)M dopamine significantly reduces the isolated inward barium current, with no concurrent change in the residual K\(^+\) current measured at +60mV. In the experiment represented in figure 5, within one minute of dopamine application the peak inward current amplitude was reduced to on average 41±11% of the amplitude before dopamine (Fig. 5C). The amplitude of the residual K\(^+\) currents were unaffected at this time point by dopamine, indicating the reduction of the peak inward current was not the result of changes in the residual K\(^+\) current. As reported with the inhibitory changes in membrane excitability and synaptic strength, dopamine only reduced the isolated barium current in some sensory neurons. In about half of the sensory neurons, no reduction was observed in the inward current with dopamine.

The molecular identity of the receptor(s) mediating the inhibitory dopaminergic response is unknown. Similar inhibition (reduction in membrane excitability and synaptic efficacy) is observed with activation of a known 5HT receptor, the *Aplysia*
5HT$_{1A}$(a) receptor, that can be exogenously expressed (Angers et al. 1998; Dunn et al. 2012). The 5HT$_{1A}$(a) receptor is only endogenously expressed in a very small subset of pleural sensory neurons (Barbas et al. 2005; Nagakura et al., 2010; Dunn et al. 2012). Therefore we over-expressed the receptor with an eGFP tag (5HT$_{1A}$(a)-eGFP) in cultured sensory neurons with nuclear microinjection of pNEX3 vectors containing the tagged receptor (Nagakura et al. 2010). With over-expression, the receptor is visible through the eGFP tag in neurites 24hr after injection (Fig. 5D). The isolation of the inward barium current was as described above, except an additional 24hr of culture was allowed for receptor expression. Using the 5HT$_{1A}$(a) agonist 8-OH-DPAT, we examined whether the inhibitory 5HT response includes inhibition of the inward barium current similar to the inhibitory dopaminergic response. One minute after application of 10μM 8-OH-DPAT the inward current was examined with step pulses from -10mV to +20mV and the residual K current with a step pulse to +60mV in sensory neurons over-expressing 5HT$_{1A}$(a)-eGFP (Fig. 5E). There was a significant reduction in the isolated barium current in cells over-expressing 5HT$_{1A}$(a)-eGFP, with no change in the residual K$^+$ current measured at +60mV (Fig. 5F). Thus, similar to the inhibitory dopaminergic response, the inhibitory serotonergic response through activation of 5HT$_{1A}$(a) includes a reduction of the isolated inward barium current that is mediated by CaV2-like channels. While the effect of 8-OH-DPAT is qualitatively larger than the effect of dopamine, it is not clear if this is due to the exogenous expression leading to a larger amount of receptor than the endogenous dopamine receptor, or additional signalling pathways activated by the 5HT$_{1A}$(a) receptor compared to the inhibitory dopamine receptor.

Voltage-independent modulation of the isolated inward barium current in pleural sensory neurons is sensitive to the src kinase inhibitor PP2

Heterotrimeric G-protein G$\beta\gamma$ subunits are known to inhibit CaV2 channels through direct interaction with the pore forming alpha subunit (Tedford and Zamponi 2006; Zamponi and Currie). This type of inhibition is referred to as voltage-dependent inhibition as it can be relieved with a strong depolarizing prepulse, however, this type of modulation has only been observed with vertebrate CaV2 channels (Huang et al. 2010). Following isolation of the inward barium current in Aplysia sensory neurons a strong prepulse to +120mV 10ms before a test pulse to +10mV, was delivered to assess the amount of voltage-dependent inhibition (Fig. 6A). The strong depolarizing prepulse had no significant influence on the inhibition of the inward barium current with dopamine (Fig. 6AB). The reduction of the inward current with dopamine was equivalent with or without a strong prepulse, indicating the inhibition with dopamine is only voltage-independent. Similarly, a strong positive prepulse failed to relieve the inhibition of the isolated barium current with activation of over-expressed 5HT$_{1A}$(a)-eGFP, 8-OH-DPAT reduced the peak inward current following a prepulse (pulse 2, reduced to 23.8±5.0% of before agonist) similar to the reduction of the peak inward current without a prepulse (pulse 1, reduced to 34.8±6.5% of before agonist, data from 3 neurons). Rapid voltage-independent inhibition of CaV2 channels with GPCR activation at vertebrate DRG nociceptors is known to require src kinase phosphorylation of the C-terminal of the channels $\alpha_1$ subunit (Raingo et al. 2007). As the Aplysia pleural sensory neurons likely serve a nociceptive function for the animal (Walters et al. 2004; Walters et...
al. 1983a; b), and are rapidly inhibited by activation of a variety of inhibitory GPCRs, we pretreated sensory neurons with either the c-src kinase inhibitor PP2 or the control analog PP3. To avoid ambiguities between a block in the inhibitory response and a lack of an endogenous response as might be the case with the endogenous dopaminergic response, we over-expressed the 5HT1Apl(a)-eGFP receptor in pleural sensory neurons as in figure 5. Using the specific agonist 8-OH-DPAT to activate 5HT1Apl(a)-eGFP receptor we can inhibit the isolated inward barium current in all of the eGFP positive cells. The 5HT1Apl(a) agonist 8-OH-DPAT significantly reduced the peak inward current with pretreatment of the control analog PP3 (Fig. 7AC). However, pretreatment with the c-src kinase inhibitor PP2 blocked the reduction of the inward barium current at one minute following application of 8-OH-DPAT (Fig. 7BC). The residual potassium currents measured with the +60mV step pulse were not affected by the agonist with either PP2 or PP3 pretreatment.

Discussion:

In a previous report we described inhibition of *Aplysia* cultured pleural sensory neurons with dopamine that included a reduction in membrane excitability and PSP amplitude, along with an inhibition of the novel PKC Apl II (Dunn et al. 2012). Here we show that the effect on PSP amplitude cannot be entirely explained by a decrease in the RRP (Figure 1), nor the lack of propagation of the action potential to the nerve terminal (Fig. 2). Consistent with the decrease in PSP amplitude being due to a direct effect on the calcium influx through the CaV2 channels that mediate transmitter release (Edmunds et al., 1990), we observed a significant reduction in the isolated CaV2-like calcium current (Fig. 5-7), with both dopamine and activation of the over-expressed 5HT1Apl(a) receptor. Furthermore, the lack of any significant change in the residual potassium currents remaining after inhibition with TEA, 4-AP, and barium at the time of measurement indicates that the observed reduction in the inward current is not an indirect result of an increase in the residual potassium conductance. Thus, the effect of dopamine on transmitter release (Fig. 1) is likely a result of reduced calcium influx through CaV2 channels, which are coded for by a single gene in most invertebrate animals. Inhibition of this calcium current has been observed in other preparations; with the neuron VD4 in the related mollusc *Lymnaea*, the current is rapidly inhibited by 5HT (McCampbell et al. 2008), and dopamine is known to inhibit CaV2.2 channels in rat visceral afferents (Kline et al. 2009), when both D2 and CaV2.2 are coexpressed in NG108-15 cells (Brown and Seabrook 1995), and at rat ventral mesencephalon neurons (Cardozo and Bean 1995).

The isolated inward barium current is likely mediated by CaV2 channels

The isolated CaV2-like calcium current cannot be confirmed as arising solely from the channel coded by the CaV2 gene until this channel is cloned in *Aplysia*. However, the following points suggest the isolated inward current described here is predominantly mediated by CaV2 channels: 1) The current has voltage-dependent kinetics typical of a high-voltage activated calcium channel, that is doubled with barium substituted as the charge carrier (Fig. 3ABC), and the current is also inhibited by micromolar cadmium (Fig. 3; (Spafford et al. 2003)). 2) The current is insensitive to 10μM nifedipine, which would completely inhibit a CaV1 current (Fig. 3EF; (Spafford et al. 2006)). 3) The
activation kinetics are unaffected by the membrane holding potentials between -80 and -50mV suggesting no contaminating CaV3 current (Fig. 3D; (Senatore and Spafford 2010)) and the inactivation rate is maintained in barium, uncharacteristic of a CaV1 current (Fig. 3C). These points suggest the inward current isolated in figures 3&4 is mainly mediated by CaV2 channels. The apparent change in this isolated CaV2-like calcium current with cell morphology suggests the channels measured in isolated sensory neurons are in distal neurites rather than evenly distributed in the neurites (Fig. 4). This is consistent with the observation in neurons from the related mollusc Lymnaea and from the neuroendocrine bag cells of Aplysia where CaV2 channels are observed in growth cones (Spafford et al. 2004; Zhang et al. 2008). Our previously reported observations with the calcium indicator Fluo-4, included 10μM nifedipine so we could be certain of no significant contamination from CaV1 channels, however we had no way of knowing whether a CaV3 current might be participating in the change in excitability and thus could result in the observed change in calcium influx (Dunn et al. 2012). The results presented in figure 3 suggest that there is no CaV3 calcium current in pleural sensory neurons, so it is likely that the calcium transient observed to be modulated in some neurites and varicosities is likely mediated by CaV2 channels. Leal and Klein (Leal and Klein 2009) also reported modulation of the nifedipine-insensitive calcium transient in neurites of Aplysia pleural sensory neurons with Fluo-4, however it is interesting to note that the current was only observed to be increased with 5HT when the sensory neuron was paired with a synaptic partner in culture. Inhibition of the CaV2-like current with dopamine (Fig. 5C), expression and activation of the inhibitory 5HT1Apl(a) receptor (Fig. 5F), or with FMRFamide (Edmonds et al. 1990) were all observed in isolated sensory neurons. This suggests different physiological requirements for the mechanisms underlying excitatory and inhibitory GPCR-mediated modulation of the CaV2-like current in Aplysia pleural sensory neurons.

A significant reduction in the CaV2 calcium current would be expected to result in a significant reduction in transmitter release. With the calcium cooperativity of transmitter release (Schneggenburger and Neher 2005), such a reduction in calcium influx would have a dramatic impact on transmitter release, consistent with what is observed with dopamine at pleural sensory neuron synapses (Fig. 1).

Voltage-dependent and Voltage-independent inhibition

At vertebrate CaV2 channels, G-protein beta/gamma subunits can directly interact with and inhibit the channel, an interaction that can be relieved with strong membrane depolarization and thus is referred to as voltage-dependent (VD) inhibition. Such inhibition is physiologically distinct in that strong depolarization of the neuron as with bursting would also relieve this inhibition (Brody et al. 1997). Expression of another Molluscan CaV2 channel, the Lymnaea CaV2 in HEK293 cells, provides evidence that invertebrate CaV2 channels do not show voltage-dependent inhibition (Huang et al. 2010). Even with vertebrate CaV2 channels, VD-inhibition only accounts for a portion of the G-protein mediated inhibition of the channel. The remaining type of inhibition, referred to as voltage-independent (VI) inhibition, appears to account for all of the inhibition at invertebrate CaV2 channels. There are a variety of different VI-inhibition mechanisms including changes in channel density, local lipid microenvironment (specifically PIP2 and metabolites), and through changes in phosphorylation (Zamponi...
Any of these possibilities could contribute to the inhibition of the channel based on studies in *Aplysia*. Alterations in Cav2 surface expression in *Aplysia* bags cells is controlled by PKC phosphorylation of the channel (White et al. 1998; Zhang et al. 2008), and there is evidence that inhibition with FMRFamide and dopamine includes changes in lipid metabolism in *Aplysia* sensory neurons (Dunn et al. 2012; Guan et al. 2003; Piomelli et al. 1987). The high conservation of the src kinase phosphoregulation site on the C-terminus of Cav2 channels along with the observed sensitivity of the inhibition to the src kinase inhibitor PP2 (Fig. 7) strongly suggests a conserved mechanism involving channel phosphorylation.

**Nociception and Analgesia**

As stated above, the pleural sensory neurons serve as mechanoreceptors with a nociceptive role, and thus inhibition with dopamine (or with 5HT in the small subset of sensory neurons that express endogenous 5HT1A, (Barbas et al. 2005; Dunn et al. 2012)) would be expected to have a hypoalgesic or analgesic effect on behaviour. Interestingly, modulation of nociception in vertebrates appears to function through a conserved general mechanism, namely a reduction in membrane excitability and reduced transmitter release through reduced calcium influx (Cregg et al. 2010). A mechanism of opioid action at primary afferent DRG synapses is through G-protein inhibition of the Cav2 calcium channel (Wiley et al. 1997).

Src kinase inhibition of the Cav2 current was first noted at chick spinal neurons where voltage-independent inhibition of the Cav2.2 calcium current with GABA required tyrosine kinase activity (Diverse-Pierluissi et al. 1997). Later this src kinase-dependent inhibition was attributed to phosphorylation of a single reside in the C-terminal of the α1-subunit of the channel. Interestingly, mammalian Cav2.2 channels have evolved a splice variant that is insensitive to this src kinase modulation by lacking the key tyrosine residue (Castiglioni et al. 2006; Raingo et al. 2007). This splice form is the predominant isoform expressed in the mammalian CNS with the exception of the DRG nociceptors, where the src kinase sensitive isoform is upregulated and accounts for a significant portion of the analgesic actions of morphine at the level of the spinal cord (Andrade et al. 2010). Further experimentation will be required to elucidate this apparently highly conserved mechanism of the src kinase-mediated VI-inhibition of the voltage gated calcium channels through the action of inhibitory heterotrimeric G-protein coupled receptors in *Aplysia* pleural sensory neurons.

**Figure Descriptions:**

Figure 1. The large reduction in PSP amplitude with dopamine is not accompanied by a similar reduction in the ready releasable pool (RRP). A) i) PSP with cursor positions separated by 1ms (a-b) measuring the initial PSP rise-rate following the initial non-linear change in membrane potential. The arrow points to potentials from the activation of voltage-dependent currents in the motor neuron that prevent using PSP amplitude to
estimate changes in transmitter release. Scale bar is 15mV/2ms. ii) Traces of PSPs
>20mV evoked at different synapses in this experiment, all of which activate voltage-
dependent currents (scale bar is 20mV/5ms). iii) Representative correlation between
initial PSP rise-rate (mV/ms) and PSP peak amplitude (mV) from a single synaptic
connection is strong (Pearson r =0.9801) when PSP amplitude is low (<20mV). B)
Experimental chronology for assessing PSP amplitude before and after dopamine, the
RRP assessment with 1M sucrose after dopamine, and a final PSP after one minute in
sucrose to assure the effective depletion of the RRP. C) 1M sucrose produces a burst of
unitary events over a period of about 45 seconds the sum of which is used to estimate the
RRP (upper trace). The lower trace is an expansion of a small section of the upper trace
to highlight individual events marked with *. * are events >5*SD of RMS noise band.
Scale bars are 3/1.5mV and 4/0.12s. D) The unitary events produced by sucrose are
similar in amplitude to the spontaneous “minis” produced prior to sucrose. Frequency
histogram of mini amplitudes and sucrose evoked unitary events at a single synaptic
connection with mPSPs recorded for 10min, grouped in 0.5mV bins (29 minis and 98
sucrose events). Inset, is the same data as relative cumulative frequency distributions of
mPSP amplitudes and sucrose event amplitudes showing no significant difference when
compared with a Kolmogorov–Smirnov test, (P>0.05). E) Comparison of the change in
synaptic strength as measured with the change in PSP rise-rate at synaptic connections
with sensory neurons lacking an inhibitory dopaminergic response (No Resp) and
synaptic connections that show a strong reduction in PSP rise-rate, often to PSP failure
(Inh. DA). Synaptic connections without an inhibitory response to DA are easily
distinguished as they lack the strong reduction in sensory neuron membrane excitability
observed in the Inh. DA group. F) mPSP count over the 45s exposure to 1M sucrose,
with synapses separated according to dopamine sensitivity as in E are not significantly
different, P>0.05. Data from E and F from n=10 synaptic connections with No Resp and
n=8 synaptic connections showing the Inh. DA response, data compared with Mann-
Whitney U tests, **P<0.01.

Figure 2. Action potentials that are evoked in the soma conduct to terminals during
inhibition with dopamine. A) Photomicrograph of a representative single isolated pleural
sensory neuron with a sharp intracellular electrode in the soma and an extracellular
electrode on the distal neurite at least 200μm from the soma. Scale bar is 150μm. B)
Representative traces where action potentials generated in the soma conduct to the distal
neurite (i, before inhibition with DA). Addition of dopamine reduces excitability so that
action potentials are not generated (ii and iii). iv, when enough depolarizing current is
delivered to the soma, an action potential is generated in the soma and observed in the
distal neurite with the extracellular electrode. C) Action potentials delivered at 1Hz
rapidly fail when the sensory neuron shows the inhibitory response to dopamine added at
action potential 1. When the action potential fails in the soma it fails in the distal neurite.
When enough depolarizing current is delivered to the soma the action potential recurs and
is observed both in the soma and the distal neurite. The amplitude of the action potential
measured in the soma is reduced with DA, likely a result of error due to the large amount
of current simultaneously passing through the electrode. The amplitude of the action
potential measured in the distal neurite is a similar amplitude. D) There is no significant
change in the amplitude of the action potential measured at the neurite before and after
the inhibitory dopaminergic response (P>0.05, n=4, comparing data before and after with
a paired t-test).

Figure 3. Isolation of the CaV2-like calcium current with a TEA based saline with 4-AP
and two electrode voltage clamp. (The experiments presented in this figure used 10μM
nifedipine to block DHP-sensitive, CaV1-like calcium channels, with the exception of
panels E&F where the impact of 10μM nifedipine on the isolated inward barium current
is examined). A) Using a TEA based saline with 4-AP and barium results in isolation of
an inactivating inward current (left traces) that is reduced in amplitude with calcium
(middle traces). Addition of 100μM Cd2+ completely blocks the inward current but has
no effect on the residual K currents (right traces). Scale bars are 2nA and 100ms. B) IV
curve of the peak inward current measured with extracellular solutions containing 20mM
Ca2+ or 20mM Ba2+ as the charge carrier. 100μM cadmium completely blocks the inward
current and reveals the residual potassium currents. Data from five sensory neurons. C)
Comparison of the average peak and inactivation tau for the inward current with barium
and with calcium in the recording solution (n=5, P<0.05 comparing groups with a paired
t-test). D) Holding the membrane potential at -80mV versus -50mV has no effect on the
voltage-dependence kinetics of the inward current indicating that no high-voltage
activated calcium currents are in the pleural sensory neurons (n=6 sensory neurons). E)
At typical cultured sensory neurons with a residual axon and low to no neurite outgrowth
(as pictured in inset) the inward current is only slightly reduced with 10μM nifedipine.
The reduction in the current is similar at the peak as at the end of the pulse consistent
with a slowly inactivating CaV1-like barium current (n=6 sensory neurons). F) At
cultured sensory neurons with no residual axon and no neurite outgrowth (spherical, as
pictured in inset) only a small inward barium current is visible without nifedipine (left
traces, scale bars are 2nA/ 80ms). Addition of 10μM nifedipine inhibits the inward
current, leaving only the residual potassium currents (right traces). The subtracted
currents (lower traces) reveal a slowly inactivating inward barium current. The IV curve
before and after nifedipine, normalized to the peak inward current without nifedipine,
suggests effective blocking of the inward current in cells with this morphology. (n=5
sensory neurons).

Figure 4. Kinetic changes in the isolated inward barium current with changes in cell
morphology. A) Photomicrographs of representative pleural sensory neurons cultured for
24, 48, or 72hr before recording. The amount of neurite outgrowth tends to increase with
duration of the culture period. B) Representative traces of peak inward barium currents
from sensory neurons of different culture periods. Increasing the culture duration from
24h to 48h to 72h reduces the peak amplitude and increases the inactivation rate of the
inward barium current. Scale bars are 2nA and 30ms. C) The average peak inward
barium current significantly reduces when neurons are cultured for periods longer than
24hr (P<0.01 comparing 24hr to both 48hr and 72hr with a one-way ANOVA with
Tukey’s multiple comparison post test). D) Steady-state inactivation rate for the isolated
inward barium current (with 10μM Nifedipine). Holding at -80mV with a prepulse to
various membrane potentials for 10s followed immediately by a test pulse to +20mV (n=5, avg. peak inward current was 4.3nA).

Figure 5. Reduction in the isolated CaV2-like calcium current with dopamine or activation of exogenously expressed 5HT1Apl(a) cannot be accounted for by an increase in the residual K+ currents. A) Representative traces of currents from voltage steps to -10mV, 0, +10, +20, and +60mV, before and one minute after 10μM dopamine along with a representative IV curve. DA reduces the inward currents without changing the current at +60mV, where a typical high-voltage activated calcium current will reverse. Scale bars are 2nA/40ms. B) IV curve of isolated ICaV2 from six sensory neurons demonstrating the initial inhibitory dopaminergic response. C) Dopamine significantly reduces the inward current at +10mV (P<0.001) measured near or at the peak inward current, but does not affect the amplitude of the current measured at +60mV (P>0.05) near or at the calcium reversal potential (all currents normalized to the peak inward current before dopamine, comparisons made with a two-way repeated measures ANOVA and Bonferroni post tests, n=6). There is no significant difference (P>0.05) in the time to peak (8.4±0.1 and 8.2±0.2ms) or inactivation tau (44.7±1.5 and 41.1±0.8ms) before and after dopamine. About half of the sensory neurons are not inhibited with DA, in this group the average peak current with DA at 1min. was 101.3±5.6% of control (n=5). These non-responding sensory neurons were removed from the analysis in Fig. 5BC for clarity. D) Images of a pleural sensory neuron in phase contrast (left panel) and epifluorescence image with GFP filters (right panel) of 5HT1Apl(a)-eGFP over-expression. Lower panel is 5HT1Apl(a)-eGFP over-expressed in the membrane of a varicosity. Scale bars are 50μm and 30μm. E) IV curves of peak inward current and at the calcium reversal potential before and after 8-OH-DPAT in sensory neurons over-expressing 5HT1Apl(a)-eGFP. The change in current with application of 8-OH-DPAT on sensory neurons over-expressing 5HT1Apl(a)-eGFP was measured 1 minute after 8-OH-DPAT application. F) Change in inward current measured at +10mV and at +60mV with 8-OH-DPAT in sensory neurons over-expressing 5HT1Apl(a)-eGFP. The data is normalized to the amplitude of the peak inward current. The inward current at +10mV (at or near the peak) is significantly reduced with 8-OH-DPAT (P<0.001) however the current measured at +60mV (at or near the calcium reversal potential) remains unaffected (P>0.05). Comparisons made with a two-way repeated measures ANOVA and Bonferroni post tests, n=8. There was no significant change in the time to peak (8.1±0.1 and 8.2±0.1ms) or the inactivation tau (54.0±1.3 and 50.8±2.4ms) at +10mV before and after 8-OH-DPAT (P>0.05, compared with paired t-tests).

Figure 6. The inhibition of the isolated CaV2-like calcium current with dopamine is voltage-independent. Only sensory neurons showing inhibition of the inward current with dopamine were used for this analysis. A) Top trace- two-electrode voltage-clamp stimulus command with a test pulse to +10mV (100ms), a strong prepulse to +120mV (50ms), followed closely (10ms) by a test pulse back to +10mV (100ms). The broken line represents a 5s gap between pulse 1 and the prepulse. Middle traces- are representative current traces before (in grey) and after dopamine (in black). Scale bars are 2nA and
20ms. Lower traces are same traces in middle panel normalized to the amplitude of the first pulse for each trace showing that the inhibition is not relieved by the strong depolarizing prepulse. B) Averaged data of peak inward current with dopamine inhibition as a percentage of the amplitude before dopamine. Both pulse 1 and pulse 2 are equally inhibited by dopamine as represented with the ratio of the inhibition with (P2) and without (P1) a strong prepulse.

Figure 7. Src kinase inhibitor PP2 prevents the inhibition of the CaV2-like current with 5HT1Ap(a) activation. A&B) IV curves before and one minute after application of 10μM 8-OH-DPAT to SNs expressing 5HT1Ap(a)-eGFP following a 10 minute pretreatment with either 25μM of the c-src inhibitor PP2 (B) or the inactive analog PP3 (A). Pretreatment with PP2 prevents the reduction in the inward current observed at one minute following 8-OH-DPAT observed with PP3 or without either drug. Neither drug, nor the 8-OH-DPAT application affected the residual K+ current measured with a +60mV pulse. C) The inhibition of the peak inward current with 8-OH-DPAT in cells pretreated with PP3 is significant, but not with PP2 pretreatment, comparing before to one minute after 8-OH-DPAT application with a paired t-test, ****P<0.0001, n=8 for PP2 and n=9 for PP3.

References:


Huang X, Senatore A, Dawson TF, Quan Q, and Spafford JD. G-proteins modulate invertebrate synaptic calcium channel (LCa(v)2) differently from the classical voltage-dependent regulation of mammalian Ca(v)2.1 and Ca(v)2.2 channels. *J Exp Biol* 213: 2094-2103, 2010.


Zamponi GW, and Currie KP. Regulation of Ca(V)2 calcium channels by G protein coupled receptors. *Biochim Biophys Acta* 2012.
