Inhibitory responses in Aplysia pleural sensory neurons act to block excitability, transmitter release and PKC Apl II activation.

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Abstract:

Expression of the 5HT1Apl(a) receptor in *Aplysia* pleural sensory neurons, inhibited 5HT-mediated translocation of the novel PKC Apl II in sensory neurons and prevented PKC-dependent synaptic facilitation at sensory to motor neuron synapses (Nagakura et al. 2010). We now demonstrate that the ability of inhibitory receptors to block PKC activation is a general feature of inhibitory receptors and is found after expression of the 5HT1Apl(b) receptor and with activation of endogenous dopamine and FMRFamide receptors in sensory neurons. Pleural sensory neurons are heterogeneous for their inhibitory response to endogenous transmitters with dopamine being the most prevalent, followed by FMRFamide, and only a small number of neurons with inhibitory responses to 5HT. The inhibitory response is dominant, reduces membrane excitability, synaptic efficacy and can reverse 5HT facilitation at both naïve and depressed synapses. Indeed, dopamine can reverse PKC translocation during the continued application of 5HT. Reversal of translocation can also be seen after translocation mediated by an analog of diacylglycerol, suggesting inhibition is not through blockade of diacylglycerol production. The effects of inhibition on PKC translocation can be rescued by phosphatidic acid (PA), consistent with the inhibitory response involving a reduction or block of production of this lipid. However, PA could not recover PKC-dependent synaptic facilitation due to an additional inhibitory effect on the non-L-type calcium flux linked to synaptic transmission. In summary, we find a novel mechanism downstream of inhibitory receptors linked to inhibition of PKC activation in *Aplysia* sensory neurons.
Keywords: *Aplysia*, membrane excitability, synapse, calcium, dopamine, serotonin, FMRFamide, PKC, phosphatidic acid, facilitation, depression, 5HT1
Introduction:

Sensory neurons involved in the defensive withdrawal reflexes of the marine mollusc *Aplysia californica* have long been studied as modulatory targets for serotonin. Serotonin release onto sensory to motor neuron synapses increases membrane excitability through modulation of a variety of currents (see Barbas et al. (2003) for review) and produces both short-term and long-term facilitation of transmitter release at naïve synapses through cAMP production followed by activation of PKA (Kandel 2001). Low frequency stimulation of *Aplysia* sensory neurons results in a depression of synaptic transmission at sensory to motor neuron synapses (Castellucci et al. 1970). 5HT-mediated facilitation following the low frequency depression is a major mechanistic component of behavioural dishabituation of the defensive withdrawal reflexes (Antonov et al. 1999; 2010). Activation of the novel, calcium-independent PKC in *Aplysia* Apl II, not PKA, is required for this form of facilitation (Ghirardi et al. 1992; Manseau et al. 2001).

While much of the initial work on serotonin was done on the siphon sensory neurons in the abdominal ganglia, the ventrocaudal (VC) cluster of mechanoafferent somata of the pleural ganglia are most commonly used for culturing *Aplysia* mechanosensory neurons as the cluster is quite distinct from the surrounding neurons allowing easy access for isolation in culture. These sensory neurons innervate the foot, body and parapodia of the animal and are involved in the head and tail-induced defensive withdrawal reflexes of the animal (Stopfer and Carew 1996; Walters et al. 1983a; b). 5HT is reported to have very similar effects in both abdominal and pleural sensory neurons (Wright and Kirschman 1995).
Previously we reported that overexpression of the 5HT$_{1\text{Apl(a)}}$ receptor in pleural sensory neurons resulted, not only in reversing the action of 5HT on membrane excitability, but also had a profound and unexpected inhibitory effect on PKC activation with 5HT, preventing the PKC-mediated reversal of synaptic depression (Nagakura et al. 2010). This receptor is known to be expressed only in a very small subset of pleural sensory neurons (Barbas et al. 2005), thus we wondered if any of the other known endogenous inhibitory responses on membrane excitability also had an inhibitory influence on PKC activation with 5HT. At Aplysia sensory neurons, the inhibitory effects of both dopamine and FMRFamide on membrane excitability have been previously noted (Abrams et al. 1984; Barbas et al. 2006). Here we demonstrate that the inhibitory responses produced by both dopamine and FMRFamide, acting through endogenous receptors, are also capable of strongly inhibiting synaptic efficacy, and the reversal of 5HT-dependent facilitation at both naïve and depressed synapses. Our results indicate that inhibition at depressed synapses occurs through at least two mechanisms; 1) a reduction in phosphatidic acid (PA), which is a requirement for the PKC Apl II activation at synapses (Farah et al. 2008), and 2) a reduction in the calcium flux that is responsible for triggering transmitter release.

**Materials and methods:**

**Animals and cell culture-** Mariculture reared Aplysia californica were purchased from The Nation Resource for Aplysia at the University of Miami. Pleural sensory neurons
were isolated from the ventrocaudal (VC) cluster of the pleural ganglia were isolated following either a 2 hour digestion at 37°C or an 18 hour digestion at room temperature with a predetermined concentration of protease in L-15 media. Cells were cultured on poly-L-lysine coated glass bottomed culture dishes filled with a culture media of 50% \textit{Aplysia} hemolymph and 50% L-15 media adjusted with salts to match \textit{Aplysia} hemolymph. Cells were cultured for 72-96hr at room temperature in a high humidity chamber. Experiments with synapses required pairing isolated pleural sensory neurons with isolated LFS siphon motor neurons from the abdominal ganglion. The identification for isolation of LFS motorneurons from abdominal ganglia was based on location and morphology and the identity of the LFS motor neurons was further confirmed electrophysiologically prior to assessing synaptic efficacy, through observing the occurrence of the “notch” potential following a hyperpolarizing pulse as described in Chitwood et al., (2001). In experiments with dopamine, a 1mM solution was prepared right before the start of the experiments and used for an hour or until discoloration indicative of oxidation. FMRFamide (Phe-Met-Arg-Phe-NH$_2$) was produced by Celtek Peptides (Celtek Bioscience LLC, Nashville, TN, USA), 1,2-dioctanoyl-sn-glycerol (DOG) and 1,2-dioctanoyl-sn-glycero-3-phosphate (DiC$_8$-PA) were purchased from Avanti polar lipids (Alabaster, AL, USA) and dissolved in DMSO. All other compounds used were purchased from Sigma-Aldrich. eGFP-5HT1$_{Apl(a)}$, eGFP-5HT1$_{Apl(b)}$, and eGFP-PKC Apl II sequences in pNEX3 vectors were microinjected into sensory neuron nuclei 24hr prior to experimentation as previously described in Farah and Sossin, (2011).
Electrophysiology - Prior to electrophysiological recordings, the culture media was replaced with a *Aplysia* recording saline containing in mM [NaCl 460, MgCl₂ 55, CaCl₂ 10, KCl 10, HEPES 10, pH 7.6]. Presynaptic sensory neuron and postsynaptic motor neuron membrane potentials were recorded and manipulated with intracellular sharp electrodes backfilled with 2M K-acetate, attached to an Axoclamp 900 (Molecular devices, Sunnyvale, CA, USA). Membrane potentials were held at -80mV with current injection. Depolarizing current pulses of 50ms were used to elicit action potentials in the sensory neuron and the resultant postsynaptic potential (PSP) recorded in the motor neuron. Electrodes were periodically rebalanced and input resistance measured with 500ms, 0.5nA hyperpolarizing pulses.

Fluo-4 imaging - Sensory neurons were loaded with micropipettes backfilled with 1mL of 6mM membrane impermeant fluo-4 using with -1nA current pulses. The fluo-4 load was monitored optically and 15-30min were allowed between loading and imaging/recording. Fluo-4 fluorescence was imaged with an ex.470/40nm-515/50nm filter set through a 1.3NA 40x objective using a Photometrics (Tucson, AZ, USA) QuantEM: 512SC EM CCD camera. Rapid image acquisition was achieved using the Fast Acquisition Solution from Zeiss (Carl Zeiss Inc.), involving hardware and software modifications that allow less time delay between subsequent frames. Exposure times were set to 20ms, and regions of interest chosen to reduce frame size so that at least 20 frames per second were acquired. Fluorescence intensity was transformed into $\Delta F/F_0$ following background subtraction using the average intensity of the ten frames preceding each action potential as the value for $F_0$. Peak fluorescence change values (occurring on
the first or second frame following the action potential) for three successive action
potential induced-fluorescence transients were averaged before and after addition of
dopamine for comparison.

Data analysis. All PSP amplitudes are normalized as a percentage of the first PSP
observed at the synapse (PSP#1). At synapses with PSPs large enough to activate
voltage-dependent currents in the motor neuron, PSP rise-rate was measured over 1ms
and normalized to PSP#1. eGFP-PKC Apl II cytosol to membrane translocation was
measured and analyzed as described in Farah et al., (2008). For the dopamine and PA
groups in Fig.4CD, cells were treated first with DOG (10 μg/ml) for 5min OR with DOG
(10 μg/ml) and PA (25 μg/ml) for 5min. Since translocation in the presence of DOG and
PA was not different from translocation in the presence of DOG alone, the two groups
were pooled for data analysis. All statistical analysis was preformed using t-tests and all
values are means +/- standard error of the mean.
Results:

Modulation of pleural sensory neuron membrane excitability.

Previously we reported that over-expression of the 5HT1A\textsubscript{Ap}(a) receptor in sensory neurons resulted in a reduction in excitability with 5HT, requiring more current to reach action potential threshold and the inability in many cases to fire more than one action potential (Nagakura et al. 2010; Fig. 1AiiBiiCD). This response with 5HT is opposite to the primary endogenous response (Fig. 1AiBiCD). Since the 5HT1A\textsubscript{Ap}(a) receptor had been previously characterised as negatively coupling to adenylate cyclase (AC) (Angers et al. 1998), we questioned whether the similar 5HT1A\textsubscript{Ap}(b) receptor, also observed to negatively couple to AC (Barbas et al. 2002) had similar effects, or whether this was a unique property of the 5HT1A\textsubscript{Ap}(a) receptor. The 5HT1A\textsubscript{Ap}(b) receptor is not known to be expressed in pleural sensory neurons (Barbas et al. 2005). Therefore we expressed this receptor with an eGFP tag (eGFP-5HT1A\textsubscript{Ap}(b)), similar to the tag previously used with the 5HT1A\textsubscript{Ap}(a) receptor, for visualization of expression levels in pleural sensory neurons.

Similar to what was observed with 5HT1A\textsubscript{Ap}(a) receptor expression, expression of eGFP-5HT1A\textsubscript{Ap}(b) greatly reduced membrane excitability with addition of 5HT, increasing the amount of current required to reach threshold and reducing the number of action potentials per unit of depolarizing current (Fig. 1AiiiBiiiCD). Using a 500ms hyperpolarizing pulse, membrane input resistance was also observed to decrease with both eGFP-5HT1A\textsubscript{Ap}(a) and eGFP-5HT1A\textsubscript{Ap}(b) receptor activation (Fig. 1E). As reported with eGFP-5HT1A\textsubscript{Ap}(a) expression in pleural sensory neurons paired with motor neurons in culture (Nagakura et al. 2010), eGFP-5HT1A\textsubscript{Ap}(b) expression in the sensory neuron blocked
the facilitation of postsynaptic potential (PSP) amplitude with 5HT following synaptic
depression with low frequency stimulation (Fig. 1F). Thus, the inhibitory responses
appear to be a general feature of these receptors, and we therefore searched for an
endogenous transmitter that could also produce this response.

Inhibition of pleural sensory neuron excitability has been reported with the
neuromodulatory peptide FMRFamide (Critz et al. 1991), and also with dopamine
(Barbas et al. 2006). We found that both of these modulatory substances had a
significant inhibitory effect on sensory neuron excitability, the inhibition with
FMRFamide was generally observed to be less than that of dopamine (Figure 1), also
apparent when an individual sensory neuron responded to both FMRFamide and
dopamine. A decrease in input resistance was also observed with dopamine and with
FMRFamide, as with 5HT when eGFP-5HT_{1APl(a)} or eGFP-5HT_{1APl(b)} were expressed
(Fig. 1E). The increase in the S-K^+ current in *Aplysia* sensory neurons with FMRFamide
leads to hyperpolarization of the resting membrane potential (Belardetti et al. 1987). As
the sensory neurons were held at -80mV with negative current injection, the effect of
FMRFamide on the resting membrane potential manifest as a reduction in the amplitude
of the holding current (reduction to 66±6% of before FMRFamide, n=7). A similar
reduction in the holding current was also observed with dopamine, consistent with the
two substances acting on similar conductances (holding current reduced to 63±6% of
before dopamine, n=11).

*Heterogeneity of pleural sensory neurons to dopamine and FMRFamide.*
The reduction in pleural sensory neuron excitability with dopamine only occurs in some of the pleural sensory neurons (Fig. 2A). We found that the inhibitory response to dopamine was maximal at 500nM, well below the reported activation of 5HT1Apl(a) with dopamine which has a minimal activation of the 5HT1Apl(a) at concentrations above 10μM (Angers et al. 1998). To ensure that the inhibitory response to dopamine is not a result of dopamine acting on endogenous 5HT1Apl(a) receptors, dopamine up to 20μM was applied to sensory neurons over-expressing eGFP-5HT1Apl(a). While 5HT led to extensive inhibition of excitability in all cells expressing eGFP-5HT1Apl(a), dopamine only led to inhibition of excitation in some of the sensory neurons indicating that 5HT1Apl(a) receptor does not mediate the inhibitory dopamine response (Fig. 2B). Expression of the eGFP-5HT1Apl(a) receptor in sensory neurons allowed us to confirm that this receptor is activated by nanomolar concentrations of the selective agonist 8-OH-DPAT as previously reported (Angers et al. 1998). Activation of the eGFP-5HT1Apl(a) receptor with 500nM 8-OH-DPAT results in a large reduction in excitability in all neurons expressing the receptor (Fig. 2C) similar to what was observed with 5HT on eGFP-5HT1Apl(a) expressing neurons (see Fig. 1). Conversely, 8-OH-DPAT on pleural sensory neurons not expressing the eGFP-5HT1Apl(a) only rarely shows a decrease in excitability, which is likely the result of activation of an endogenous 5HT1Apl(a) response (circled in Fig. 2C). The reduction in excitability with 8-OH-DPAT occurs in all cells expressing eGFP-5HT1Apl(a), confirming the efficacy of the agonist and indicating that dopamine is not activating a potential endogenous 5HT1Apl(a) response, but rather acting through another receptor.
Examination of many neurons isolated from the same pleural pedal ganglia revealed that the inhibitory dopamine response varied from 10% to 90% of the sensory neurons examined, with on average of the eleven animals examined (99 sensory neurons in total) 58.2±0.1% showed a reduction in excitation with dopamine (Fig. 2D). Next, greater numbers of neurons were examined with 500nM 8-OH-DPAT to estimate the percentage of pleural sensory neurons with an endogenous 5HT1A<sub>pl(a)</sub> response. In the animals used in this experiment the percentage of pleural sensory neurons with the inhibitory dopamine response was similar to the previous estimates at 50%, while the percentage of neurons with an endogenous 5HT1A<sub>pl(a)</sub> response (sensitive to 500nM 8-OH-DPAT) was much lower at 5-7% (Fig. 2E). To estimate the percentage of pleural sensory neurons responsive to FMRFamide with a reduction in excitability, sensory neurons isolated from the same ganglion were examined first for FMRFamide sensitivity with 20μM FMRFamide and then for dopamine sensitivity with 1μM dopamine. Approximately half of the examined pleural sensory neurons were inhibited by FMRFamide (41.2±5.9%), slightly less than the number of sensory neurons responsive to dopamine (59.4±13.1% in this experiment). Many pleural sensory neurons respond to both FMRFamide and dopamine, though the extent of inhibition is generally weaker with FMRFamide (as in Fig.1). These data indicate that pleural sensory neurons are heterogeneous with respect to their sensitivity to modulatory substances, with dopamine producing the most prominent inhibitory effect on membrane excitability.

Dopamine and FMRFamide regulate the recovery from synaptic depression.
Following the generation of forty action potentials at low frequency stimulation (0.05Hz) of the sensory neuron, PSP amplitude decreases to approximately 20% of the initial amplitude, and addition of 5HT to the depressed synapse leads to facilitation. Both eGFP-5HT\textsubscript{1Apl(a)}, and eGFP-5HT\textsubscript{1Apl(b)} expression in sensory neurons inhibited the PKC-mediated reversal of synaptic depression with 5HT (Nagakura et al. 2010; Figure 1). Furthermore, dopamine also blocked the reversal of depression with 5HT (data not shown), thus we wondered if the 5HT reversal of depression could be inhibited or reversed once already initiated. After only four 5HT facilitated PSPs, dopamine was added in combination with 5HT. As observed in the above experiments, not all pleural sensory neurons (now paired with LFS motor neurons) responded to dopamine with a decrease in membrane excitability. Therefore, synaptic pairs could be grouped according to the change in sensory neuron excitability with dopamine (Fig. 3A). Addition of dopamine had no effect on 5HT facilitation at depressed synapses if the sensory neuron did not also show a decrease in membrane excitability (Fig. 3A-D). However, if membrane excitability of the sensory neuron was inhibited with 500nM dopamine, then rapid synaptic depression also occurred (Fig. 3A-D). Thus dopamine can reverse 5HT-mediated facilitation at depressed synapses once already initiated (Fig. 3). With the similar effects of 5HT\textsubscript{1Apl(a)}, 5HT\textsubscript{1Apl(b)}, and dopamine on membrane excitability and 5HT facilitation at depressed synapses, we wondered whether FMRFamide which also decreases membrane excitability (Fig. 1) and depresses synaptic transmission at naïve synapses (Guan et al. 2003), could reverse 5HT facilitation at depressed synapses. Again, since not all pleural sensory neurons respond to FMRFamide, individual synaptic pairs could be separated for analysis of the effect of
FMRFamide on sensory neuron membrane excitability. Five of the sensory neurons showed no change in excitability with FMRFamide (95.1±3.4% of current required before FMRFamide) while the other five synaptic pairs showed a large reduction in excitability requiring 230.2±30.2% more current to reach action potential threshold after FMRFamide (represented in Fig. 3inset). And similar to what was observed with dopamine, 5HT facilitation at depressed synapses was reversed at synaptic pairs with FMRFamide-sensitive sensory neurons only (Fig. 3E). Since the inhibitory responses produced by both dopamine and FMRFamide are variable between pleural sensory neurons, the correlation between the change in membrane excitability with the change in synaptic efficacy was examined (Fig. 3F). A significant correlation was found between the change in the amount of current required to reach action potential threshold (change in membrane excitability) and the reduction in PSP amplitude during 5HT-mediated facilitation at depressed synapses with dopamine and FMRFamide (number of cells?? P<0.0001). Therefore, similar to activation of 5HT1_Apl(a), 5HT1_Apl(b), and the endogenous inhibitory dopamine response, the inhibitory FMRFamide response is also capable of reversing the 5HT facilitation at depressed synapses.

Dopamine reverses PKC Apl II translocation with 5HT and DOG

5HT-mediated facilitation at naïve synapses requires cAMP whereas 5HT facilitation at depressed synapses requires the calcium-independent PKC, Apl II (Ghirardi et al. 1992; Manseau et al. 2001). We previously reported that eGFP-5HT1_Apl(a) over-expression inhibited 5HT-dependent translocation of the novel PKC Apl II (Nagakura et al. 2010), suggesting the inhibitory effect of 5HT1_Apl(a) is upstream of PKC activation.
Since, dopamine could reverse the PKC-mediated reversal of depression, we wondered if dopamine could reverse the 5HT-mediated eGFP-PKC Apl II translocation from the cytosol to the plasma membrane. Cultured pleural sensory neurons expressing eGFP-PKC Apl II show a redistribution of eGFP-PKC Apl II from the cytosol to the plasma membrane with application of 5HT (the eGFP membrane to cytosol ratio after 5HT in the control group was 1.87±0.10 and 2.51±0.18 in group yet to receive dopamine) (Fig. 4A). Subsequent application of dopamine in the continued presence of 5HT, resulted in a significant reduction in eGFP-PKC Apl II translocation compared to neurons not treated with dopamine (P<0.001, Fig. 4). Translocation of PKC Apl II downstream of 5HT requires production of both DAG by phospholipase C (PLC) and phosphatidic acid (PA) by phospholipase D (PLD) (Farah et al. 2008). To test if dopamine was acting upstream of PLC, we examined whether dopamine could still reverse translocation when a cell permeable, exogenous DAG analog, DOG was used. Activation and translocation of eGFP-PKC Apl II with DOG could also be reversed with addition of dopamine, although this was slower than the dopamine reversal of 5HT-dependent translocation (Fig. 4CD). These results suggest that dopamine was not acting on membrane DAG levels. To test if dopamine was acting on membrane PA levels, we examined whether dopamine could reverse translocation in the presence of a cell-permeable analog of PA, DiC8-PA. Unlike with DOG alone, the dopamine-induced reversal of eGFP-PKC Apl II translocation was inhibited by the combination of DOG and DiC8-PA (Fig. 4CD). This suggests that dopamine is inhibiting or inactivating PKC Apl II activation at least in part through a reduction in plasma membrane PA levels.
Since adding DiC8-PA rescued the inhibition of eGFP-PKC Apl II translocation, we attempted to prevent the dopamine-mediated inhibition of 5HT facilitation at depressed synapses with pre-application of 25μg/ml of DiC8-PA. Though this concentration was sufficient to prevent the inhibitory effect of dopamine on eGFP-PKC Apl II translocation with DOG, it failed to alter the inhibitory action of dopamine on the 5HT-mediated reversal of depression (Fig. 4EF). This suggests that dopamine has additional actions on inhibiting transmitter release independently of PA production.

Facilitation and depression at naïve synapses.

In the typical pleural sensory neuron, 5HT not only increases excitability (as in Fig 1i), and increases transmitter release at depressed synapses, but also increases transmitter release at naïve synapses (no stimulation history since isolation and culture) through an independent mechanism (Ghirardi et al. 1992). In contrast, FMRFamide is reported to depress PSP amplitude at naïve synapses (Edmonds et al. 1990; Guan et al. 2003; Montarolo et al. 1988), whereas only a few published papers mention the short-term inhibitory effect of dopamine on synaptic efficacy (Abrams et al. 1984; Montarolo et al. 1988), none present any form of data as to the extent or mechanism of inhibition, and no reports involve the pleural sensory neurons. To examine the effect of dopamine on naïve synapses, PSP amplitude was first measured with a single action potential and then either 5HT or dopamine was added to modulate PSP amplitude. Following two minutes, PSP amplitude was measured again with a second action potential in the sensory neuron and the amplitude of PSP#2 expressed as a percentage of PSP#1. While 5HT produced facilitation, dopamine produced a dramatic depression in PSP amplitude.
However this occurred only if membrane excitability of the sensory neuron was also inhibited with dopamine (Inh. DA resp., had on avg. a 209±38% change in the amount of current required to reach threshold, Fig. 5AB). In a second experiment, following measurement of PSP#1 and addition of 5HT, facilitation was measured with PSP#2 after five minutes in 5HT. Then dopamine was added and a third PSP measured following an additional two minutes with now dopamine and 5HT. When dopamine did not reduce excitability of the sensory neuron (No DA resp, in this group the amount of current required to fire an action potential with a 50ms pulse was 88±7% of what was required before dopamine), PSP amplitude remained facilitated with dopamine for a third PSP (Fig. 5C). Conversely, if dopamine produced a reduction in membrane excitability (Inh. DA resp.; in this group the amount of current required to fire an action potential in dopamine was on average 690±216% of the amount required before dopamine) PSP amplitude was greatly depressed (Fig. 5C). Thus, dopamine results in depression that can over-come or reverse 5HT facilitation, much like that observed at depressed synapses. These data show that the pleural sensory neuron-specific inhibitory dopamine response includes a dramatic inhibitory effect on synaptic efficacy, independent of prior stimulation history. Similarly, eGFP-5HT\textsubscript{1Apl(a)} expression alters the response of synapses to 5HT from facilitation to depression (eGFP-5HT\textsubscript{1Apl(a)} expression at two synaptic pairs resulted in a block of PSP amplitude with 5HT, avg. initial PSP amplitude was 9.6mV).

The inhibitory dopaminergic response includes a reduction in the non-L-type calcium flux
The mechanism of FMRFamide mediated inhibition of synaptic transmission remains in contention. A previous paper (Edmonds et al. 1990), reported a reduction in the non-L-type calcium current with FMRFamide, and since this current is responsible for transmitter release we wondered whether dopamine also affected this calcium flux. The L-type calcium current in *Aplysia* is not involved in transmitter release and can be selectively blocked with dihydropyridines (Braha et al. 1993). To selectively examine the relative calcium flux through the channels responsible for transmitter release, we iontophorectically preloaded the sensory neurons with membrane impermeant fluo-4, and blocked the L-type flux with 5\(\mu\)M nifedipine. With a sharp electrode in the sensory neuron to evoke single action potentials, fluo-4 imaging was conducted such that >20Hz frame rates were achieved of regions of interest at axon ends or at the contacts between the sensory neuron axon/neurite and that of the LFS motor neuron (Fig. 6A). These subcellular regions were chosen as previous observations of calcium flux modulation with 5HT observed the changes to be localized to these regions (Eliot et al. 1993; Leal and Klein 2009). A single action potential in the sensory neuron produced a fluorescence transient that was greater in some regions and these were selected for measuring intensity change (Fig. 6B). Three successive action potentials generated by brief 50ms depolarizing pulses, produced individual fluo-4 fluorescent transients of similar amplitude (Fig. 6CD). As in previous experiments, sensory neurons were grouped according to the change in membrane excitability with dopamine for analysis. In sensory neurons where dopamine had no effect on membrane excitability, the fluo-4 fluorescence transient was unaffected when three more action potentials were generated following the addition of dopamine (Fig. 6C). However when dopamine reduced excitability, the fluo-
4 transients were also greatly reduced (Fig. 6D). Therefore, one of the actions of

dopamine is to inhibit the calcium flux responsible for transmitter release.

Discussion:

The excitatory and facilitatory properties of 5HT at *Aplysia* sensory to motor

neuron synapses are usually the focus of examination, as these mechanisms underlie the

sensitisation of the behavioural defensive withdrawal reflexes with noxious stimuli, a

very simple and highly accessible behaviour for neurophysiological examination

( Antonov et al. 1999). Though examined less frequently, the inhibitory responses are no

less dramatic in affecting both membrane excitability and transmitter release.

Inhibitory (D$_2$-like) dopaminergic reduction of transmitter releasing CaV2 calcium

currents has been well documented, observed in a variety of preparations indicating a

conserved mechanism ( Kline et al. 2009; Missale et al. 1998; Ramanathan et al. 2008;

Salgado et al. 2005; Wikstrom et al. 1999; Zhang et al. 2004). In *Aplysia*, stimulation of

a connective to the abdominal ganglia is known to reduce the calcium current in the

abdominal neuron L10 and PSP amplitude in follower cells, suggesting that release of a

substance reduces transmitter release by reducing calcium flux in this neuron ( Shapiro et

al. 1980). In abdominal LE sensory neurons, inhibition of excitability with both

FMRFamide and dopamine was described by Abrams et al., (1984) and though they state

that both transmitters also reduce synaptic transmission, no data is presented. Montarolo

et al., (1988) also state that dopamine results in short-term depression of synaptic efficacy

but again present no data to describe the extent of the inhibition. Here we describe the
extent of the inhibitory response and relative frequency of the different inhibitory
responses produced by different neuromodulators in pleural sensory neurons.

We show the relative frequency of a variety of short-term inhibitory responses
observed at *Aplysia* sensory neurons isolated from the VC cluster of the pleural ganglion.
The strongest and most prominent inhibitory response observed was produced with
dopamine in a majority of sensory neurons examined (Fig. 2&3). The inhibitory effect of
dopamine on membrane excitability was very strong, increasing the amount of current
required to reach threshold while also reducing input resistance and the resting membrane
potential. Thus, in the presence of dopamine this subset of pleural sensory neurons is
unlikely to generate more than one action potential per stimulus, regardless of the
stimulus intensity. In addition, dopamine also had a profound inhibitory effect on
synaptic efficacy (Fig. 3&5), further reducing the likelihood of a stimulus to the
mechanosensory neuron exciting the motor neuron.

At both naïve and depressed synapses, the inhibitory action of dopamine was
dominant to excitatory effects of 5HT, able to reverse the 5HT-mediated facilitation and
recovery from depression, the former requiring PKA and the latter a form of synaptic
plasticity requiring the activation of PKC (Ghirardi et al. 1992; Liu et al. 2004; Manseau
et al. 2001). Therefore, as the typical response of the pleural sensory neuron to 5HT is an
increase in excitability and synaptic efficacy, dopamine produces the opposite response
with a reduction in excitability and synaptic efficacy. While FMRFamide also produces a
similar reduction in excitability and synaptic efficacy, the dopaminergic inhibitory
response is stronger (Fig. 1) and more prevalent (Fig. 2) in pleural sensory neurons. The
dominance of the inhibitory response of FMRFamide over 5HT had been reported
previously (Critz et al. 1991), here we extend this observation to depressed synapses and
include the $5HT_{1A\text{pl(a)}}$ and $5HT_{1A\text{pl(b)}}$ responses and dopamine as producing a similar
mechanism of inhibition.

Heterogeneity of the inhibitory responses in pleural sensory neurons

The VC cluster pleural sensory neurons innervate the tail, foot, head, body, and
the posterior parapodia of the animal acting as primary mechanoreceptors (Walters et al.
2004; Walters et al. 1983a). Some of these sensory neurons innervate the tail, so that
when the tail is stimulated, a defensive withdrawal of the tail occurs through direct
excitation of the tail motor neurons in the pedal ganglion. The noxious stimulus to the
tail, results in serotonin release in local regions of a variety of ganglia including the
pleural ganglia (Marinesco and Carew 2002; Marinesco et al. 2004a; Marinesco et al.
2006; Marinesco et al. 2004b), resulting in increased excitability and facilitation of
transmitter release from the tail sensory neurons (Ghirardi et al. 1992). Conversely, a
noxious stimulus to the head (which leads to a defensive withdrawal of the head), inhibits
the siphon withdrawal reflex through an inhibitory action of 5HT at interneuron to
motorneuron synapses (Marinesco et al. 2006). A small subpopulation of VC pleural
neurons express the inhibitory 5HT$_{1A\text{pl(a)}}$ receptor (Barbas et al. 2005), from the data
presented in Fig. 2 and in Nagakura et al., (2010), these pleural sensory neurons would be
inhibited with 5HT, a response also reported in RF abdominal sensory neurons
(Storozhuk and Castellucci 1999), and some cerebral sensory neurons (Rosen et al.
1989). In support of these observations, in situ hybridizations indicate $5HT_{1A\text{pl(a)}}$ or
$5HT_{1A\text{pl(b)}}$ receptor expression in a variety of abdominal sensory neurons and
approximately 20% of the cerebral sensory neurons (Barbas et al. 2005). Thus, like the abdominal and cerebral ganglia, the pleural ganglia contain a small subset of mechanosensory neurons that are inhibited by 5HT.

The heterogenous response of pleural sensory neurons to dopamine reported here was also observed between the different sensory neuron clusters of the abdominal ganglia, with the RF sensory neurons showing less of an inhibitory change in membrane excitability with dopamine than sensory neurons in the other clusters (ie LE, rLE, RE) (Dubuc and Castellucci 1991). Furthermore, our observation that about half of the pleural sensory neurons show no response to FMRFamide is supported by the observations of Buttner and Siegelbaum, (2003), however, they suggest this may be due to a requirement for an initial excitation for inhibition to counter. Our data contradicts this possibility as FMRFamide insensitive sensory neurons were incapable of counteracting 5HT-mediated facilitation following depression (Fig. 3E). We also observed that some pleural sensory neurons showed a response to dopamine but not to FMRFamide, and vice versa, further indicating that the lack of response was more likely due to a lack of the specific receptor rather than the state of the neuron. The responses we observed to FMRFamide and dopamine were largely heterogeneous indicating that like the cerebral and abdominal sensory neurons, the pleural ganglion VC cluster of mechanosensory neurons are a heterogeneous population of neurons in their specific sensitivity to neuromodulatory substances. Thus the inhibitory changes to pleural sensory neuron excitability and synaptic efficacy will differ depending on the specific neuron examined.

Physiological relevance
The strong inhibition of a majority of the pleural sensory neurons with dopamine predicts that dopamine release in the pleural ganglia would strongly inhibit body-induced defensive withdrawal reflexes. The appetitive behaviours of *Aplysia* are regulated by dopamine, such that dopamine in the hemocoel or from increased dopaminergic neuronal release induces feeding behaviours (Kabotyanski et al. 2000). Indeed, there is evidence that dopamine release mediates the reinforcement of both operant and associative conditioning to food in *Aplysia* (Baxter and Byrne 2006; Lorenzetti et al. 2008). Though habituation of the siphon and gill withdrawal reflexes are reported to be inhibited with perfusion of the gill with dopamine (Ruben and Lukowiak 1983), a variety of defensive responses of the animal that would involve pleural sensory neurons including siphon withdrawal, locomotion after salt stimulation, and inking responses were all observed to be highly attenuated a half hour after feeding (Advokat 1980). The cerebral-pedal regulator neurons are activated with food-induced arousal and act to reduce defensive reflexes including suppression of the head withdrawal reflex through inhibitory action in the pleural/pedal ganglia (Teyke et al. 1990). Dopamine has been observed in the neuropile of pedal ganglia (McCaman et al. 1973), the location of the pleural sensory to tail motor neuron synapses (Wainwright et al. 2002), whether release is modulated by appetitive behaviour has not been assessed. State-dependent changes in the defensive responses may represent the physiological relevance of the inhibitory responses observed in the pleural sensory neurons. Alternatively, the inhibitory responses may represent endogenous hypoalgesic mechanisms serving to prevent activation of withdrawal reflexes in favour of other defensive behaviors. A noxious stimulus to the tail of the animal alters subsequent defensive withdrawal of the siphon in favour of siphon flaring for directed
inking towards the tail (Illich et al. 1994). Furthermore, extrinsic inhibition of pleural sensory neuron action potential discharge has been observed previously, indicating that under some noxious stimulation patterns inhibition of sensory neuron activity occurs (Clatworthy and Walters 1993).

The pleural sensory neuron inhibitory 5HT response from endogenous 5HT1Apl(a) only occurs in a very small subset of pleural sensory neurons (Fig. 2; Barbas et al. 2005). The majority of pleural sensory neurons respond in an excitatory manner with 5HT so the physiological function of the inhibitory response in this small subset of neurons is unclear but may reflect a more complicated or alternate function, as suggested for the RF abdominal sensory neurons that are also inhibited by 5HT (Storozhuk and Castellucci 1999). On the other hand, the inhibitory dopamine and FMRFamide responses likely function to modulate the body-induced defensive withdrawal reflexes in a state-dependent manner, as suggested above.

Reversing PKC activation and inhibition of the non-L-type calcium flux

Facilitation with 5HT following synaptic depression requires activation of calcium-independent PKC, Apl II (Manseau et al. 2001). A fluorescent protein tagged PKC Apl II, eGFP-PKC Apl II translocates from the cytosol to the plasma membrane when activated with 5HT (Farah et al. 2008; Zhao et al. 2006). Recently we reported that 5HT-mediated translocation of eGFP-PKC Apl II is inhibited with over-expression of the 5HT1Apl(a) receptor (Nagakura et al. 2010). Here we extend the list of receptors capable of inhibiting PKC activation to the very similar 5HT1Apl(b) receptor, but also to the receptors producing the endogenous inhibitory responses to dopamine and FMRFamide.
Application of dopamine following the reversal of depression with 5HT demonstrates that the dopamine inhibitory response is able to reverse or over-power the 5HT/PKC-mediated facilitation (Figure 3). eGFP-PKC Apl II membrane translocation with 5HT is reversed with dopamine suggesting that the effect on facilitation following depression is a reversal of the facilitation through inactivation of the novel PKC Apl II (Figure 4).

Translocation of eGFP-PKC Apl II with 5HT involves a synergistic interaction of the two membrane metabolites DAG and PA to localize the active kinase at the plasma membrane (Farah et al. 2008). PKC Apl II translocation with DOG is not inhibited with dopamine if PA is included, indicating that the impact of dopamine on PKC Apl II translocation is likely through reducing plasma membrane PA levels. However, the same addition of PA was unable to prevent the inhibitory effect of dopamine on synaptic efficacy at both naïve and depressed synapses. Similar to what has been previously observed with FMRFamide, dopamine strongly reduces the non-L-type calcium flux that is responsible for synaptic transmission. The mechanism of the calcium current inhibition was not examined here but may be due to direct action on the channel or indirectly through a failure of the action potential to sufficiently depolarize the presynaptic terminals. While in many cases the inhibitory dopaminergic response is insufficient to fully block synaptic transmission, indicating that action potential propagation to at least some presynaptic terminals is maintained (as in the traces displayed in figure 3), the large reduction in synaptic efficacy with dopamine is likely a consequence of the large reduction in presynaptic calcium flux. We do not think the deficit of PKC Apl II translocation is due to the decrease in the calcium current, since PKC Apl II is not sensitive to calcium (Sossin et al. 1996) and since exogenous PA can
rescue the translocation without rescuing transmitter release. Thus, the inhibitory response is complicated, acting on membrane PA levels either through PLD or one of the lipid metabolic proteins, as well as acting on at least one more separate mechanism to reduce membrane excitability and calcium entry.

Dissociation of inhibitory responses from inhibition of adenylate cyclase

Both the 5HT1\textsubscript{Ap(a)} and 5HT1\textsubscript{Ap(b)} receptors are known to negatively couple to AC when expressed in cell lines (Angers et al. 1998; Barbas et al. 2002), as expected for G\textsubscript{\alpha_i} - coupled receptors (Taussig et al. 1993). This suggests that reducing cellular cAMP may result in the observed inhibitory response in these neurons. However, over-expression of an isoform of the cAMP phosphodiesterase apPDE4 that inhibits facilitation at naïve synapses and long-term facilitation, both of which require cAMP increases, did not alter 5HT facilitation at depressed synapses (Jang et al. 2011). Overexpression of the phosphodiesterase should reduce any basal cAMP levels and therefore reduce the modulatory range for any potential signalling through a reduction of cAMP levels. This is also consistent with the findings of Lee et al, (2009) where removal of the 5HT receptor coupled to AC removed facilitation at naïve synapses, but did not affect 5HT facilitation at depressed synapses and Chang et al., (2003) where expression of an exogenous AC-coupled octopamine receptor allowed for octopamine facilitation at naïve, not depressed synapses.

The work of Jang et al., (2011) also suggests that the inhibitory mechanism acting on synaptic strength and excitability in pleural sensory neurons is also unlikely to be signalled through reducing cAMP levels as they report no decreases in initial synaptic
strength or in membrane excitability with apPDE4 (only the 5HT-mediated increases are impaired). Bernier et al., (1982) observed inhibitory changes in membrane excitability with both 5HT and dopamine in abdominal neuron R15 concurrent with an increase in cAMP, also in support of an inhibitory mechanism that is not mediated through a down-regulation in cAMP. Thus the evidence is many of the actions noted here are independent of the inhibition of AC.

There is no direct evidence for G\(\alpha_i\) inhibition of PKC activation, though inhibition of PLC-\(\epsilon\) was noted with G\(\alpha_i\) activation by mAChR expressed in HEK293 cells (vom Dorp et al. 2004). There is precedence for G\(\alpha_i\) receptors coupling to Src\(\rightarrow\)RAS pathway (Ma and Huang 2002) and to RAP\(\rightarrow\)Erk pathway (Weissman et al. 2004) independent of cAMP modulation. Interestingly, neurite outgrowth in vertebrate neurons can also be regulated by 5HT1 and inhibitory D\(_2\) dopamine receptors through a non-canonical G\(\alpha_i/o\) coupling to RAP1GAP (reviewed in Ma'ayan et al. (2009)). However, the relevance of these pathways for inhibiting excitability and blocking PKC Apl II translocation is not clear. In sensory neurons, the activated G-proteins and subsequent second messenger pathways that produce the common inhibitory responses described here remain unknown and the mechanisms of these inhibitory responses appear to be complex involving a variety of targets.

The molecular identity of the inhibitory dopamine receptor (or the inhibitory FMRFamide receptor) receptor is not clear. A dopamine receptor positively coupled to AC has been cloned from *Aplysia*, but this receptor is not expressed in sensory neurons (Barbas et al, 2006). Bioinformatic screens of the *Aplysia* ESTs and genomic sequences resulted in identification of a second dopamine receptor orthologous to other invertebrate
dopamine receptors (DinvApt) (Nagakura et al. 2010), but this receptor is also postulated to
be positively coupled to adenylate cyclase (Han et al. 1996). No D2-like receptor was
identified in the available *Aplysia* genomic sequences, but D2-like receptors are found in
the mollusk *Lottia* (Nagakura et al, 2010) and D2-like responses are seen in the mollusk,
*Lymnaea* (Dobson et al. 2006; Werkman et al. 1987). Since the *Aplysia* genome is still
incomplete, it is most likely that *Aplysia* also has an orthologue of a D2-like receptor, and
this receptor most-likely underlies the inhibitory responses observed.
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Figure Descriptions:

Figure 1. Reducing sensory neuron excitability with 5HT, DA, and FMRFamide.

A) Representative traces of voltage recordings made in isolated sensory neurons to a 500ms depolarizing current pulse before and after addition of neuromodulatory substances. i) Change in excitability as the relationship between the amplitude of the depolarizing current pulse and the number of action potentials evoked in sensory neurons expressing eGFP before and after 10μM 5HT. ii) Change in excitability at sensory neurons over-expressing the *Aplysia* eGFP-5HT$_{1Apl(a)}$ before and after addition of 10μM 5HT. iii) Change in excitability of sensory neurons over-expressing the eGFP-5HT$_{1Apl(b)}$ with 10μM 5HT. iv) Change in sensory neuron excitability with 500nM dopamine and v) 10μM FMRFamide. B) The typical relationship between the number of action potentials and the amplitude of the depolarizing current pulse with 5HT on sensory neurons over-expressing either eGFP (i), eGFP-5HT$_{1Apl(a)}$ (ii), or eGFP-5HT$_{1Apl(b)}$ (iii), and the endogenous responses to 500nM dopamine (iv) and 10μM FMRFamide (v). C) Change in the amplitude of depolarizing current required to reach action potential threshold with a 50ms pulse with 5HT on sensory neurons over-expressing GFP, eGFP-5HT$_{1Apl(a)}$ or eGFP-5HT$_{1Apl(b)}$, or with dopamine DA, or with FMRFamide (as a percentage of the amount of current required before addition of the respective neuromodulatory substance). 5HT on eGFP-5HT$_{1Apl(a)}$ or eGFP-5HT$_{1Apl(b)}$ expression or dopamine or FMRFamide significantly increased the amount of current required to reach threshold (* is P<0.0001, compared to eGFP, N are 13,5,5,13,11 neurons). D) Change in the slope of the depolarizing current to action potential number under the same condition in C). E) Change in input resistance under the same conditions as in A-D, with input resistance
measured with a -0.5nA, 500msec pulse with membrane potential at -80mV. Comparison of eGFP (with 5HT) group to other groups with a one-way analysis of variance, and Tukey’s post-hoc test where *P<0.05, **P<0.01, and ***P<0.001. F) Over-expression of the eGFP-5HT₁Ap(b) prevents 5HT-mediated facilitation following low-frequency depression. PSP amplitude normalized to PSP#1, and every ten successive PSPs averaged to reduce scatter. 5HT was added after (not including) PSP#40, so the final point at PSP#50 represents the average of all PSPs after 5HT addition. PSP amplitude in LFS motorneurons, synapsed with pleural sensory neurons over-expressing eGFP show significant facilitation with 5HT (* is P<0.05 comparing eGFP in 5HT to eGFP-5HT₁Ap(b) in 5HT, n is five in each group), unlike PSP amplitude at synapses with sensory neurons over-expressing eGFP-5HT₁Ap(b).

Figure 2. Pharmacological differentiation of the 5HT₁Ap(a) and DA responses in pleural sensory neurons and relative numbers of neurons with endogenous 5HT₁Ap(a), dopaminergic, and FMRFamideergic inhibitory responses. A) Dopamine in the nanomolar range leads to an inhibition in excitability in some cells, while other sensory neurons show no response or an excitatory response. Examining fifteen sensory neurons, seven require more that 150% of the current required to fire a single action potential (Inh. DA resp.). The other eight neurons show little or no change in membrane excitability with dopamine (No DA resp., highlighted with black box). B) A similar pattern of dopamine responses is observed in sensory neurons with (triangles) or without (squares) over-expression of eGFP-5HT₁Ap(a), with some cells responding to DA with a large reduction
in excitability now requiring more depolarizing current to reach threshold. Sensory neurons in both groups tend to show a reduction in excitability; however some neurons in both groups show no change in excitability (highlighted with the black box). (P>0.05, comparing mean change in excitability with DA between eGFP to eGFP-5HT$_{1Ap}(a)$ expression groups). Unlike with dopamine, when sensory neurons express eGFP-5HT$_{1Ap}(a)$, 5HT leads to a reduction in excitability in all neurons examined (circles). C) 5HT$_{1Ap}(a)$ sensitivity to the agonist 8-OH-DPAT allows for specific activation of this receptor when it is expressed in sensory neurons. Pleural sensory neurons over-expressing the 5HT$_{1Ap}(a)$ receptor (eGFP-5HT$_{1Ap}(a)$) all respond to 500nM 8-OH-DPAT with a reduction in membrane excitability (triangles). All but one of the sensory neurons without eGFP-5HT$_{1Ap}(a)$ expression (No Exp.) show no change in membrane excitability (P<0.05, comparing No Exp. to eGFP-5HT$_{1Ap}(a)$). The single response in the no expression group (highlighted with dashed circle), likely represents activation of an endogenous 5HT$_{1Ap}(a)$ response. D) Percentage of cultured pleural sensory neurons showing an inhibitory response to dopamine. Each bar is the percentage of sensory neurons examined from a single animal with the inhibitory dopamine response, with the number of neurons examined under the bar. This variation represents either sampling errors or animal-to-animal differences, the average from all experiments is highlighted with dashed line, E) Percentage of cultured sensory neurons with an inhibitory response to 8-OH-DPAT or dopamine. Each set of bars represents data from a single animal. The x-axis is the number of sensory neurons examined. F) Percentage of sensory neurons responding to FMRFamide and/or DA. All sensory neurons in each pair of bars were isolated from the same ganglia with the number of sensory neurons on the x-axis. G)
Representative plot of the depolarizing current pulse amplitude for a 500ms pulse versus the number of action potentials generated, before and after modulation of an endogenous 5HT1Apl(a) response in a pleural sensory neuron. The endogenous 5HT1Apl(a) response (activated with 500nM 8-OH-DPAT) results in a change in the amount of current required to reach threshold (x-intercept) and the number of action potentials/nA.

Figure 3. Dopamine reverses 5HT-mediated facilitation following depression at synapses with sensory neurons displaying the inhibitory changes in membrane excitability with dopamine. A) Change in excitability in pleural sensory neurons synapsed with LFS motoneurons with addition of dopamine at PSP#46 on. Only two of the nine sensory neurons showed no change with dopamine (highlighted with box), the other seven neurons showed a strong reduction in excitability, observed as a many fold increase in the amount of current required to reach action potential threshold. B) Time-course of PSP amplitude change with low frequency stimulation of the sensory neuron. Addition of 5HT occurs before PSP#41 and 5HT+DA before PSP#46. Synapses were separated into two groups dependent on the change in membrane excitability in the sensory neurons with DA addition. C) Representative voltage traces from AB) of sensory neurons (top traces) synapsed with motor neurons (bottom traces). The depressed PSP (lower left trace) is facilitated with the addition of 5HT (lower middle trace), however this facilitation can be reversed with addition of dopamine (lower right trace), only when the presynaptic sensory neuron shows the inhibitory reduction in excitability. D) While 5HT significantly facilitates PSP amplitude (5HT PSP41-46) at depressed synapses (before
PSP36-40) (P<0.05), DA depresses PSP amplitude even in the presence of 5HT only when presynaptic sensory neuron membrane excitability was significantly inhibited with DA (Inh DA resp). N is nine sensory-motor neuron pairs where seven show the inh. DA resp. *P<0.05. E) In another experiment similar to B-E, except FMRFamide was added at PSP#46. Synaptic pairs were separated based on whether or not there was a reduction in membrane excitability with FMRFamide (inset, cells in box show no change in excitability with FMRFamide are No FMRFresp group). Similar to what was observed with dopamine, FMRFamide significantly reduced the 5HT-mediated reversal of depression (n=5 sensory-motor neuron pairs in each group, *P<0.05). F) At each synaptic pair the correlation between the current required to reach action potential threshold in the sensory neuron and the change in PSP amplitude with application of both dopamine and FMRFamide during 5HT facilitation at depressed synapses. The measured values are normalized to percentages so the log_{10} of the change in current required to reach threshold and the log_{10} of the change in PSP amplitude with either dopamine (circles) or FMRFamide (triangles) were plotted. The data were best fit with a straight line of slope -1.25, having a strong correlation (Pearson r=-0.8197, P<0.0001, n=18).

Figure 4. Dopamine inhibits PKC Apl II translocation in isolated sensory neurons with 5HT or DOG, but not DOG and PA. A) Representative confocal fluorescence images of *Aplysia* sensory neurons expressing eGFP-PKC Apl II before (Pre 5HT), 5 min following first treatment with 5HT 10 µM (Post 5HT #1) and 1-5 min following second treatment with 5HT 10 µM (Post 5HT #2) in the absence (control group) or presence of dopamine 1
μM or 10 μM (dopamine group). B) The translocation ratio normalized to Post 5HT #1 is shown for the conditions cited in A. Error bars represent standard errors of the means; n = 7 for the control group and n = 16 for the dopamine group. For the dopamine group, the translocation ratio Post 5HT #2 was significantly lower than Post 5HT #1 (***, P < 0.001 by two-tailed paired Student’s t tests). No difference was observed between the translocation ratios Post 5HT #1 and Post 5HT #2 for the control group. C) Sensory cells expressing eGFP-PKC Apl II were treated first with DOG (10 μg/ml) for 5min (DOG #1) and then for treatment #2 with: 1) DOG (10 μg/ml) for 15min (control group), 2) DOG (10 μg/ml) and dopamine 1 μM (dopamine group) or 3) DOG (10 μg/ml), dopamine 1 μM and PA (25 μg/ml) [dopamine and PA group]. D) The translocation ratio post 5, 10 and 15min treatment #2 was normalized to 5min DOG #1 for the different conditions cited in A. Dopamine inhibits DOG-dependent translocation of PKC Apl II (***, P < 0.001 and **, P < 0.01 in the dopamine group by Repeated Measures Analysis of Variance) and PA blocks the effect of dopamine (dopamine and PA group). Error bars represent standard errors of the means; n = 11 neurons for the control group, 16 for the dopamine group and 7 for the dopamine and PA group. E) 25 μg/mL PA was unable to rescue the inhibition of the 5HT-mediated reversal of synaptic depression. Representative traces of a low frequency stimulation depressed PSP (PSP39), subsequent facilitation with 5HT (PSP41), and depressed with dopamine (PSP47). Scale bars are 20mV/20ms. F) Summary of attempted rescue of dopamine depression following 5HT facilitation at depressed synapses with 25μg/mL PA. Low frequency stimulation depresses synaptic transmission (PSP36-40, is the average of the last five PSPs before 5HT addition). 5HT facilitates PSP amplitude at depressed synapses (PSP41-45, is average of first five PSPs.
Addition of dopamine overcomes the 5HT facilitation and depresses the synapse further than that observed with low frequency stimulation (PSP 46-50, is the average of the first five PSPs after addition of dopamine). * P<0.05 comparing dopamine depression to both low frequency depression (PSP36-40) and 5HT facilitation (PSP41-45).

Figure 5. Facilitation and depression at naïve synapses with 5HT and dopamine (DA). A) (Upper traces) Representative traces for B) of PSP#1 and PSP#2 following two minutes in 5HT (top left traces) or DA with the sensory neuron showing an inhibition of excitability with dopamine (top middle traces), or DA with the sensory neuron not showing an inhibitory change in membrane excitability with dopamine. (Lower Traces) Representative traces for C) of PSP#1 and PSP#2 following five minutes in 5HT and PSP#3 following an additional two minutes in 5HT and dopamine. Left traces show no inhibitory change in membrane excitability, whereas the right traces are of PSPs from neurons showing the inhibitory DA response (Inh. DA resp.) where dopamine produces a large reduction in membrane excitability. B) 5HT produces facilitation after 2 minutes, whereas dopamine depresses PSP amplitude only in sensory neurons that also show a reduction in membrane excitability (Inh. DA resp.). C) 5HT for 5 minutes produces greater facilitation, and dopamine for two minutes greatly reduces facilitation producing depression only in sensory neurons that showed the reduction in excitability. Sensory neurons not responsive to dopamine with a reduction in excitability (No DA Resp.) still show facilitation from 5HT after two minutes in dopamine.
Figure 6. Single action potential fluo-4 fluorescence transients in the presence of nifedipine are greatly reduced with dopamine only in pleural sensory neurons that have a concurrent reduction in excitability. A) Phase contrast image on pleural sensory neuron axon at point of contact with motor neuron, the yellow box is the region to be used for fast (>20fps) fluo-4 fluorescence imaging. B) Fluo-4 fluorescence intensity in pseudo color before (left panel) 50ms after (middle panel) a single action potential. The subtraction of the two images (right panel) reveals regions that show a stronger fluorescence transient that will be used as regions of interest. CD) Time-course of fluo-4 fluorescence before and after dopamine expressed as a DF/Fi where Fi is the average fluorescence intensity in the ten frames preceding each action potential. The fluo-4 DF/F for three sequential single action potentials evoked at 0.1Hz were averaged before and after addition of dopamine. As in previous figures, sensory neurons were grouped according to whether or not the neuron showed the reduction in membrane excitability with dopamine (D, Inh DA resp) or not (C, No DA resp). The neuron represented in C required a similar amount of current to reach threshold following dopamine addition, whereas the neuron in D required 275% more current to generate an action potential with dopamine addition. E) Dopamine significantly reduces the peak single action potential fluo-4 fluorescence transient only in sensory neurons that also show a reduction in membrane excitability. *P<0.05, n is 7 sensory neurons (No DA resp) and 5 neurons (Inh DA resp).