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**Inhibition of human recombinant T-type calcium channels by N-arachidonoyl serotonin**

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Inhibition of human recombinant T-type calcium channels by N-arachidonoyl serotonin

Running Title: NA-5HT inhibits T-type calcium channels

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Summary

**Background and Purpose:** *N*-arachidonyl serotonin (NA-5HT) has anti-nociceptive effects reported to be mediated by inhibitory actions at the transient receptor potential vanilloid receptor 1 (TRPV1) and fatty acid amide hydrolase (FAAH). Anandamide and *N*-arachidonoyl dopamine (NADA), endocannabinoids which activate TRPV1 or are metabolised by FAAH, also inhibit T-type calcium channels ($I_{Ca}$). T-type $I_{Ca}$ are expressed by many excitable cells, including neurons involved in pain detection and processing. We sought to determine whether NA-5HT also modulates T-type $I_{Ca}$.

**Experimental Approach:** Human recombinant T-type $I_{Ca}$ ($Ca_{V}3$ channels) expressed in HEK 293 cells were examined using standard whole cell voltage clamp electrophysiology techniques.

**Key Results:** NA-5HT completely inhibited $Ca_{V}3$ channels with a rank order of potency ($pEC_{50}$) of $Ca_{V}3.1$ (7.4) $>$ $Ca_{V}3.3$ (6.8) $\geq$ $Ca_{V}3.2$ (6.6). The effects of NA-5HT were voltage-dependent and it produced significant hyperpolarizing shifts in $Ca_{V}3$ steady state inactivation relationships. NA-5HT selectively affected $Ca_{V}3.3$ channel kinetics.

**Conclusions and Implications:** NA-5HT increases the steady state inactivation of $Ca_{V}3$ channels, reducing the number of channels available to open during depolarization. These effects occur at NA-5HT concentrations at or below those at which NA-5HT affects TRPV1 receptors and FAAH. NA-5HT is one of the most
potent inhibitors of T-type $I_{Ca}$ described to date, and it is likely to exert some of its biological effects, including anti-nociception, via inhibition of these channels.

Key Words:
T-type calcium channel; NA-5HT; anandamide; nociception; acyl amino acid; arachidonoyl amino acid
### Abbreviations

<table>
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<th>Abbreviation</th>
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<tr>
<td>Anandamide</td>
<td>$N$-arachidonoyl ethanolamide</td>
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<tr>
<td>FAAH</td>
<td>fatty acid amide hydrolase</td>
</tr>
<tr>
<td>GIRK</td>
<td>G protein-gated inwardly rectifying K channels</td>
</tr>
<tr>
<td>$I_{Ca}$</td>
<td>voltage gated calcium channel current</td>
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<tr>
<td>NAAN</td>
<td>$N$-acyl amino acid/neurotransmitters</td>
</tr>
<tr>
<td>NA-5HT</td>
<td>$N$-arachidonoyl serotonin</td>
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<tr>
<td>NA-DA</td>
<td>$N$-arachidonoyl dopamine</td>
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<tr>
<td>NA-Gly</td>
<td>$N$-arachidonoyl glycine</td>
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<tr>
<td>TRPV1</td>
<td>transient receptor potential vanilloid receptor 1</td>
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Introduction

Fatty acids conjugated with amino acids or neurotransmitters (N-acyl amino acid/neurotransmitters, NAAN) are a class of endogenous compounds with a widespread tissue distribution and potential to modulate many aspects of cellular function (reviewed in Connor et al., 2010). Several NAAN have been shown to have profound effects on nociception and inflammatory processes following local or systemic administration or incubation with tissue in vitro (Burstein et al., 2000; Huang et al., 2001, 2002, Succar et al., 2007; Vuong et al., 2008, Barbara et al, 2009). These effects are likely to be mediated via a range of molecular targets including cannabinoid CB1 receptors (Bisogno et al., 2000), transient receptor potential vanilloid 1 (TRPV1) receptors (Huang et al., 2002), glycine transporters and receptors (Wiles et al., 2006; Yang et al., 2008) and T-type calcium channels (Ca\textsubscript{v}3, Barbara et al., 2009, Ross et al., 2009). It is an open question whether tissue levels of any of these NAAN are high enough to effect these proteins (Marinelli et al., 2007) but NAAN are becoming valuable pharmacological tools as both structurally novel ligands for existing modulatory sites and probes for new sites potentially amenable to pharmacological manipulation (Edington et al., 2009; Yevenes and Zeilhofer, 2011).

Ca\textsubscript{v}3 channels are expressed in many neurons involved in sensory processing, and knockout of Ca\textsubscript{v}3.1 or Ca\textsubscript{v}3.2 in mice has revealed deficits in the detection or processing of noxious stimuli sensory neurons in several brain regions (Kim et al., 2003; Bourinet et al., 2005; Park et al., 2010). In contrast to the situation with high voltage activated calcium channels (HVA $I_{\text{Ca}}$), there are few selective inhibitors of Ca\textsubscript{v}3 channels available, which makes defining their function by means other than
gene knockout difficult. Previous work has shown that NA-DA, NA-Gly and NA-GABA inhibit CaV3 channels in a manner similar to that of the prototypic endocannabinoid anandamide, although the fine details of channel modulation differ, indicating subtle differences in the interactions of these molecules with the putative binding site on the channels (Chemin et al., 2001, 2007; Barbara et al., 2009; Ross et al., 2009). Interestingly, NA-DA and NA-Gly do not inhibit HVA ICa (Guo et al., 2008). Together, these data suggest that the study of NAAN effects on CaV3 ICa may provide insight into the design of more specific modulators of these channels.

NA-5HT was one of the earliest NAAN synthesized (Bisogno et al., 1998), but it was not until quite recently that it was found to occur in vivo (Verhoeckx et al., 2011). NA-5HT is a very poor CB1 receptor ligand, a relatively weak inhibitor of the anandamide degrading enzyme fatty acid amide hydrolase (FAAH), but a potent TRPV1 antagonist (Bisogno et al., 1998; Fowler et al., 2003, Maione et al., 2007). These properties have been exploited to explore the potential contribution of the endocannabinoid system to the modulation of responses to nociception and inflammation in brain (Suplita et al., 2005; de Novellis et al., 2008; 2011), spinal cord (Suplita et al., 2006) and the periphery (D'Argenio et al., 2006; Costa et al., 2010). While some of these studies show that the effects of NA-5HT are consistent with an increase of cannabinoid receptor-mediated effects of endocannabinoids secondary to inhibition of FAAH or via inhibition of TRPV1 activation, there are other actions of NA-5HT that are not easily ascribed to either of these mechanisms (D'Argenio et al., 2006; Di Marzo et al., 2008). Further, cannabinoid receptor- and TRPV1-independent effects of NA-5HT on cancer cell growth (Jacobsson et al., 2001; Bifulco et al., 2004) has been reported. In light of these studies and our previous findings
with NA-DA, we examined the actions of NA-5HT on T-type calcium channels. We find that NA-5HT is a potent inhibitor of Ca\textsubscript{V}3 channel activity, particularly that of Ca\textsubscript{V}3.1. These effects of NA-5HT on T-type channels are likely to contribute to the previously described effects of the compound in brain and cell lines and make NA-5HT the most potent endogenous modulator of T-type calcium channels identified to date.
Materials & Methods:

Cell culture

HEK 293 cells stably transfected with plasmids containing cDNA for the human Ca\textsubscript{v}3.1, Ca\textsubscript{v}3.2 or Ca\textsubscript{v}3.3 (Cribbs et al., 1998; 2000; Gomora et al., 2002, Ross et al., 2008) were cultivated in Dulbecco’s modified Eagle’s Medium supplemented with 100U penicillin, 100 µg streptomycin, 10% fetal bovine serum and 1 mg ml\textsuperscript{-1} G418 (Invitrogen or Invivogen, Australia). For some experiments we used HEK 293 cells in which the same channels had been integrated into a FLP site under the control of a tetracycline repressor. In these experiments channel expression was induced by overnight incubation with tetracycline (1 µg ml\textsuperscript{-1} Sigma Australia). AtT-20 cells stably expressing human 5-HT\textsubscript{1B} receptors were grown as outlined above, except that the G418 concentration was 500 µg/ml (Heblinski & Connor, 2012).

Electrophysiology

Calcium channel currents in HEK293 cells were recorded in the whole-cell configuration of the patch-clamp method (Hamill et al., 1981) at room temperature (Ross et al., 2008). Dishes were perfused with HEPES buffered saline (HBS) containing (in mM): 140 NaCl, 2.5 KCl, 2.5 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 HEPES, 10 glucose (pH to 7.3, osmolarity = 330 ± 5 mosmol). For recording Ca\textsubscript{v}3.1 and 3.2 currents, cells were bathed in an external solution containing (in mM): 140 tetraethylammonium chloride, 2.5 CsCl, 10 HEPES, 10 glucose, 1 MgCl\textsubscript{2}, 5 CaCl\textsubscript{2} (pH to 7.3, osmolarity = 330 ± 5 mosmol). For recording Ca\textsubscript{v}3.3 currents, 5 mM CaCl\textsubscript{2} was replaced by 5 mM BaCl\textsubscript{2} in the external solution (see Ross et al., 2008) unless otherwise stated. Recordings were made with fire-polished borosilicate glass pipettes with resistance ranging from 2-3 MΩ. For recording Ca\textsubscript{v}3.1 and 3.2 currents,
the internal solution contained (in mM): 130 CsCl, 10 HEPES, 2 CaCl₂, 10 EGTA, 5 MgATP (pH to 7.3, osmolarity = 285 ± 5 mosmol). For recording of Caᵥ3.3 currents, 10 mM EGTA was replaced by 10 mM BAPTA, and the concentration of MgATP was reduced to 1 mM. The different solutions used to record Caᵥ3.3 were necessary to minimize current rundown (Ross et al., 2008), although the reason(s) underlying this rundown in our cells are not established. Recordings were made with a HEKA EPC 10 amplifier with Patchmaster acquisition software (HEKA Elektronik, Germany), and an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, California) using AxoGraph X software (http://axographx.com/). Data was sampled at 5-20 kHz, filtered at 2 kHz, and recorded on hard disk for later analysis. Series resistance ranged from 3 to 10 MΩ, and was compensated by at least 80% in all experiments. Leak subtraction using a P over 4 protocol (with 10 mV test steps) was used for a few of the experiments where cells were being stepped to a single potential, but it was not employed for experiments where more complex waveforms were applied to the cells (e.g. inactivation). Uncompensated leak in any experiment did not exceed -30 pA at –86 or –106 mV, and cells with a leak current of greater than –30 pA were discarded. Cells were exposed to drugs via flow pipes positioned approximately 200 µm from the cell. Concentration-response curves were generated by fitting data to a sigmoidal dose response function in GraphPad Prism 4. Steady-state activation curves for Caᵥ3.1 and 3.2 were generated from current-voltage relationships, while steady-state inactivation curves for each channel were generated by measuring the peak current from a 50 ms step to –26 mV following a series of 5 s steps ranging from potentials of –126 mV to –46 mV. Reported potentials are corrected for a junction potential of –6 mV. Activation curves were generated by fitting data to a Boltzmann sigmoidal function $Y=1/(1+e^{-(V_{0.5}-Vm)/Slope})$. Inactivation curves were
generated by fitting data to a Boltzmann sigmoidal function $Y=1 - \frac{1}{1+e^{(V_{0.5}-V_{m})/\text{Slope}}}$.

Ca$_V$3.3 has unusual permeation properties and a relatively positive activation potential, which makes determining activation curves from chord conductances unreliable (Frazier et al., 2001). Thus, we obtained activation curves for Ca$_V$3.3 channels by measuring the amplitude of tail currents at -90 mV following steps to positive potentials. The amplitude of the tail current is a direct measure of the number of open channels. To reduce the possible confounding effects of interactions in the pore of the channels between permeant monovalent and divalent cations, these experiments were carried out in solutions with only permeant monovalent ions. External solution for these experiments comprised (mM): NaCl 140, MgCl$_2$ 5, HEPES 10, TEACl 10; pH to 7.3, osmolarity = 330 ± 5 mosmol.

K currents in AtT-20 cells were recorded using an external solution comprising (mM) KCl 130, NaCl 35, CaCl$_2$ 1.5, HEPES 10, glucose 10, pH 7.3. The intracellular solution comprised (mM) KCl 130, NaCl 5, EGTA 10, CaCl$_2$ 2, HEPES 20, MgATP 5, NaGTP 0.2, pH 7.3. In some experiments GBP$_B$S or GTP$_Y$S (both 1.2 mM) were substituted for NaGTP. AtT-20 cells were voltage clamped at -60 mV and continuous recordings of current made. In these conditions opening of K channels produces an inward current.

**NA-5HT synthesis**

NA-5HT, \(5Z,8Z,11Z,14Z-N-(2-(5-hydroxy-1H-indol-2-yl)ethyl)icoso-5,8,11,14-tetraenamide\), was synthesized as follows: To a stirred solution of arachidonic acid
(54 µl, 0.16 mmol) in anhydrous acetonitrile (1 ml) at 0 °C and under atmosphere of argon was added triethylamine (32 µl, 0.23 mmol, 1.4 equiv) followed by isobutylchloroformate (26 µl, 0.197 mmol, 1.2 equiv). The reaction mixture was stirred at 4 °C for 40 minutes during which time a white precipitate formed. The solvent was evaporated under a stream of argon and the residue obtained was treated with a solution of serotonin hydrochloride (38 mg, 0.197 mmol, 1.2 equiv) and triethylamine (25 µl, 0.181 mmol, 1.1 equiv) in anhydrous N,N-dimethylformamide (1.0 ml). The resultant pale yellow solution was stirred at 4 °C for 18 hours after which time the reaction mixture was diluted with water and extracted with ethyl acetate. The organic extracts were combined, washed sequentially with water and brine, dried over anhydrous sodium sulfate and concentrated in vacuo. The crude residue obtained was purified by flash chromatography on silica gel, eluting with hexane-ethyl acetate (1:1), to afford (5Z,8Z,11Z,14Z)-N-(2-(5-hydroxy-1H-indol-2-yl)ethyl)icosa-5,8,11,14-tetraenamide (71 mg, 93%) as a colourless gum.

$^1$H and $^{13}$C NMR spectra were acquired at 300 ± 1 K using either a Bruker DP X400 (400 MHz). $^1$H chemical shifts are reported relative to residual non-deuterated solvent resonance or tetramethylsilane. Low-resolution mass spectra were recorded on a Finnigan LCQ mass spectrometer. High-resolution mass spectra were recorded by the Mass Spectrometry Unit of the School of Chemistry, University of New South Wales. Structural information was as follows: $^1$H NMR (400.2 MHz, CDCl$_3$) δ 7.21 (1H, d, $J$ = 8.8 Hz), 7.00 (1H, dd, $J$ = 17.8, 2.2 Hz), 6.80 (1H, dd, $J$ = 8.8, 2.4 Hz), 5.58-5.30 (10H, br m), 3.57 (2H, dd, $J$ = 12.8, 6.8 Hz), 2.89 (2H, t, $J$ = 6.8 Hz), 2.84-2.76 (6H, m), 2.13 (2H, t, $J$ = 7.6 Hz), 2.10-2.02 (4H, m), 1.72-1.64 (4H, m), 1.39-1.23 (6H, m), 0.88 (3H, t, $J$ = 6.8 Hz); $^{13}$C NMR (100.6 MHz, CDCl$_3$) δ 173.1, 149.9, 131.6, 130.6, 129.2, 128.7, 128.6, 128.2, 128.1, 127.9, 127.5, 123.0, 112.4,
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112.2, 111.9, 130.3, 39.7, 36.2, 31.5, 29.3, 26.7, 25.6, 25.5, 22.6, 14.1;

**MS** (+ESI) m/z 485 ([M + Na]+, 28%), 947 ([2M + Na]+, 100); **HRMS** (+ESI) calculated for C$_{30}$H$_{42}$N$_2$O$_2$ ([M + Na]$_+$) 485.31385, found 485.31381.

**Pharmacological agents:** NA-5HT was obtained from either Alexis Biochemicals (Lausen, Switzerland), Biomol (Plymouth Meeting, PA, USA), Cayman Chemical (Ann Arbor, MI, USA) or synthesized by us. Results were similar with drugs purchased from all sources. Oleoyl- and palmitoyl-5HT were from Cayman Chemical. Arachidonic acid for synthesis was from Biomol. All other drugs and chemicals were from Sigma Australia.

**Drug vehicle (ethanol):** Drugs were kept in concentrated stock solutions in ethanol and stored at -30°C. Daily dilutions from these stocks were made; the final ethanol concentration in all solutions was 0.1%. Ethanol at this concentration does not significantly affect the properties of the Ca$_v$3 channels (Ross et al., 2009).

**Statistics:**
Statistical significance for comparing the V$_{0.5}$ values of activation and inactivation was determined using an unpaired Student’s t-test comparing values of V$_{0.5}$ calculated for individual experiments. In order to compare the changes in the time constants of inactivation and deactivation, a two-way ANOVA test was used with a Bonferroni post-test to compare values at different potentials.

Drug and molecular target nomenclature conforms to the BJP Guide to Receptors and Channels (Alexander et al., 2011).
NA-5HT inhibited each of the human CaV3 subtypes (Figure 1). The inhibitory effects of NA-5HT on CaV3 channels developed relatively slowly and did not readily reverse on washing. However, a substantial reversal of NA-5HT (300 nM) inhibition of CaV3.1 could be achieved by inclusion of BSA (1 %) in the wash buffer (Figure 2). BSA itself had no effect on CaV3.1 current amplitude (Figure 2, current was 109 ± 2% of initial after 10 minutes in BSA, n=6). The potency of NA-5HT inhibition of CaV3 channels was determined by superfusing single concentrations of drug onto cells repetitively stepped from –86 mV to –26 mV. NA-5HT inhibited CaV3.1 with \( pEC_{50} \) of 7.36 ± 0.09 (~ 40 nM), CaV3.2 with a \( pEC_{50} \) of 6.59 ± 0.15 (~ 250 nM) and CaV3.3 with a \( pEC_{50} \) of 6.79 ± 0.06 (~ 160 nM) (Figure 3). N-oleoyl serotonin (18:1ω9, 10 µM) inhibited CaV3 channels to similar degree as NA-5HT (10 µM), but the unsaturated N-palmitoyl serotonin (C16, 10 µM) was much less effective (Figure 4).

**NA-5HT effect on channel activation and inactivation**

Arachidonic acid and its conjugates inhibit CaV3 channels in part by increasing steady state inactivation and thus reducing the numbers of channels available to open during a depolarization. We examined whether the inhibition of CaV3 channels by NA-5HT could be due to effects on channel availability or activation (Figure 5). For CaV3.1 and 3.2 activation curves were constructed by stepping cells from –106 mV to potentials between –86 and +59 mV, and then were repeated after 5 minutes perfusion of submaximally effective concentrations of NA-5HT. In the presence of NA-5HT there were small (2-3 mV) shifts in the potential at which half the channels were activated, these shifts were not different from those seen with time matched...
vehicle controls in (Figure 5, Table 1). The voltage-dependence of activation for 

CaV3.3 was determined by measuring tail currents at -90 mV immediately following 

steps to positive voltages (see Methods). The V_{0.5} for activation of CaV3.3 was not 

different from predrug in the presence of NA-5HT (300 nM) however the small time 

dependent shift in V_{0.5} seen in parallel control experiments (~2 mV) was not 

observed in the presence of NA-5HT (Figure 5, Table 1).

Steady state inactivation was determined by holding cells at –106 mV and then 

stepping them for 5s to test potentials between –126 mV and –51 mV before 

measuring the current following a step to –26 mV. This was repeated after 5 minutes 

in sub-maximally inhibitory concentrations of drug. NA-5HT produced a significant 

hyperpolarizing shift in the membrane potential at which 50 % of the channels were 

available for activation for each channel (Figure 5, Table 1). The shifts in steady 

state inactivation in cells exposed to vehicle alone for 5 minutes were less than 2 mV 

(Table 1). The increase in steady state inactivation is likely to make a major 

contribution to the inhibition of CaV3 channel currents by NA-5HT.

If the effects of NA-5HT on channel inactivation contributed significantly to the 

inhibition of the CaV currents then applying NA-5HT to cells voltage clamped at 

potentials significantly more negative than -86 mV would be expected to produce less 

inhibition of channel currents. The inhibition by 50 nM NA-5HT of CaV3.1 currents 

elicited by a step to -26 mV was significantly less when cells were held at -106 mV 

(20 ± 7 %, P = 0.007) or -126 mV (25 ± 7 %, P = 0.014) than when they were held at 

-86 mV (55 ± 8 % n = 6-7 for each).
The effects of NA-5HT on Ca\textsubscript{V}3 channels were not significantly affected when GTP was omitted from the pipette (Figure 6), implying a lack of G-protein involvement in the channel inhibition. We further tested this by examining the effects of NA-5HT on Ca\textsubscript{V}3.1 when the competitive inhibitor of G-protein activation, GBP\textbeta\textsubscript{S} (1.2 mM) or the irreversible activator of G-proteins GTP\textgamma\textsubscript{S} (1.2 mM) were included in the pipette. In the presence of internal GTP\textgamma\textsubscript{S}, inhibition of Ca\textsubscript{V}3.1 by 50 nM NA-5HT was 61 ± 8% compared with 64 ± 4% in cells with GTP-containing internal (n=6 for each). In a separate set of experiments, the inhibition of Ca\textsubscript{V}3.1 by 50 nM NA-5HT was 38 ± 8% in the presence of GDP\textbeta\textsubscript{S} and 51 ± 12% in GTP (n=6 for each). Neither nucleotide derivative significantly affected the inhibition of Ca\textsubscript{V}3.1 channels by NA-5HT. In control experiments, GDP\textbeta\textsubscript{S} strongly reduced 5-HT\textsubscript{1B} receptor mediated activation of G protein-gated inwardly rectifying K channels (GI\textsubscript{RK}) in AtT-20 cells. Current produced by application of 100 nM 5-HT was -180 ± 10 pA in control, and -13 ± 3 pA in the presence of GDP\textbeta\textsubscript{S} (n=4). Conversely, addition of GTP\textgamma\textsubscript{S} to the internal solution produced a rapidly developing spontaneous inward current that peaked at -330 ± 130 pA (n=5). This current occluded the effect of any subsequent application of 5-HT.

We examined the effects of NA-5HT on Ca\textsubscript{V}3 channel kinetics by comparing the effects of 5 minute applications of compound with time matched controls. Currents were elicited from a holding potential of −106 mV and we measured the time to peak and time constant of channel inactivation from an open state. Because the voltage steps were long enough to capture most of the channel inactivation, tail currents on repolarization were very small, so channel deactivation was examined in a separate series of experiments (see below). NA-5HT did not affect time to peak or inactivation
from an open state for CaV3.1 (50 nM) or CaV3.2 (300 nM) (Figure 7). However, NA-5HT (300 nM) significantly accelerated both the time to peak and rate of inactivation from an open state for CaV3.3 over a wide range of test potentials (Figure 8, ANOVA). In a separate series of experiments where we examined the activation of CaV3.3 using only permeant monovalent cations (see above), the time to peak for the currents was also significantly accelerated. The time to peak for a step from -90 mV to 0 mV was 23.4 ± 0.7 ms, after 5 minutes in 300 nM NA-5HT it was 20.0 ± 0.5 ms (P = 0.0001, paired t-Test, n=7), in parallel control experiments the time to peak was 20.4 ± 1.2 ms, 5 minutes later it was 19.4 ± 0.9 ms, (P = 0.18, paired t-Test, n=7).

The effects of NA-5HT on accelerating the activation kinetics of CaV3.3 were concentration dependent. We analysed the time to peak of the CaV3.3 recordings used to generate the concentration response curves described above. Currents were evoked from a holding potential of -86 mV to a test potential of -26 mV. Significant acceleration of channel activation was apparent at 1 µM NA-5HT (P < 0.001 t-Test, vs parallel controls, Figure 9), by contrast significant inhibition of the CaV3.3 current was apparent at concentrations of NA-5HT greater than 100 nM. We determined the effects of 1 µM NA-5HT on the time to peak of CaV3.1 and CaV3.2 using the same voltage protocol, with measurements made either at a steady state inhibition (if inhibition was less than 90%) or when inhibition of the current was at approximately 90 %, in cells where 1 µM NA-5HT produced a greater than 90 % inhibition. We found that in contrast to the effects seen on CaV3.3 currents, NA-5HT did not affect the time to peak for either CaV3.1 or CaV3.2 Figure 9).
We examined the effects of NA-5HT (1 µM) on tail currents resulting from repolarization of cells from -26 mV to -86 mV. The tail currents were best fit by a 2 component exponential. Data was collected from time points where the currents were maximally inhibited or inhibited by about 90 % of control, which ever was the larger current. NA-5HT did not significantly affect any of the kinetic parameters of the CaV3.1 or CaV3.2 tail currents (n=6-8, paired t-Test, Figure 10). However, in the presence of 1 µM NA-5HT the major component of the CaV3.3 channel deactivation was significantly accelerated, from 1.83 ± 0.30 ms to 1.29 ± 0.28 ms (P < 0.001, paired t-Test, n=7, Figure 10). The proportion of the tail current accounted for by this component did not change (0.80 ± 0.02 in control, 0.76 ± 0.04 in drug), and there was no significant change in the properties of the minor component of the tail current. There were no changes in the kinetics of the tail currents of time matched control recordings.
Discussion

The major finding of this study is that NA-5HT inhibits T-type calcium channels with a similar potency to its antagonist actions at native TRPV1 receptors and with a much greater potency than its inhibitory actions at FAAH. Thus inhibition of T-type calcium channels, which have a well established role in neuronal activity related to nociception in both the central and peripheral nervous system (Shin et al., 2008), is likely to mediate at least some of the anti-nociceptive effects of NA-5HT.

The $EC_{50}$ of NA-5HT to inhibit Ca$_{v}$3.1 under our experimental conditions is about 50 nM, which compares favourably with compounds being developed by the Pharmaceutical Industry as T-type calcium channel inhibitors (Giordanetto et al., 2011). NA-5HT is the most potent fatty acid-derived inhibitor of Ca$_{v}$3.1 and Ca$_{v}$3.3 identified (Chemin et al., 2001; Barbara et al., 2009; Ross et al., 2009). NA-5HT also inhibited Ca$_{v}$3.2 ($EC_{50} = 250$ nM) at concentrations similar to $N$-arachidonoyl GABA hydroxide (NA-GABA-OH), the most potent NAAN previously examined ($EC_{50} = 200$ nM, Barbara et al., 2009). Given the voltage dependence of NA-5HT actions, its effects at more depolarized membrane potentials are likely to enhanced, as previously demonstrated for other lipophilic modulators of these channels (Chemin et al., 2001; Ross et al., 2008). The other major pharmacological targets of NA-5HT identified to date are TRPV1 and FAAH. NA-5HT inhibits capsaicin or anandamide activation of TRPV1 with an $EC_{50}$ between 40 and 100 nM (Maione et al., 2007) while inhibition of FAAH activity occurs with $EC_{50}$s between 1 and 10 µM (Bisogno et al., 1998; Jonsson et al., 2001; Fowler et al., 2003). Given that inhibition of FAAH is likely to modulate the concentration of anandamide and related compounds that are
ligands for both TRPV1 and CaV3 channels, the overall effects of endogenous or exogenously applied NA-5HT are likely to be complex. Delineating which effects of NA-5HT are mediated directly on target channels and which occur secondary to inhibition of FAAH will require careful experimental design. It is, however, unlikely that NA-5HT was inhibiting CaV3 I_{Ca} secondary to inhibition of FAAH and generation of AEA in our experiments. Firstly, the concentrations of NA-5HT needed to inhibit each channel were at least 5- to 20-fold lower than those reported to inhibit FAAH (Bisogno et al., 1998, Jonsson et al., 2001; Fowler et al., 2003). More pertinently, the characteristics of NA-5HT inhibition differ significantly from those of AEA, with NA-5HT only significantly affecting CaV3.3 channel kinetics, which is in contrast to the effects of AEA on the channel opening and open state inactivation (Chemin et al., 2001, Ross et al., 2009) of all CaV3 types, particularly CaV3.1. 2-Acyl glycerol, which may also be metabolized by FAAH, does not inhibit CaV3 channels (Chemin et al., 2001).

T-type calcium channels are involved in a wide range of physiological processes (Perez-Reyes, 2003, Shin et al., 2008), including many that are also potentially affected by the elevated levels of endocannabinoids resulting from NA-5HT inhibition of FAAH or NA-5HT inhibition of TRPV1. In particular, CaV3.1 channels have been reported to have an important role in neurons involved in the endogenous anti-nociceptive circuits originating in the periaqueductal grey (Park et al., 2010), a region where NA-5HT administration potentiates endogenous anti-nociception effects (Suplita et al., 2005). However, demonstrating the relative contribution of T-type calcium channels compared with potential targets of NA-5HT such as TRPV1 and FAAH is further complicated because other drugs used to probe these mechanisms
are themselves T-type $I_{\text{Ca}}$ blockers; for example the CB₁ receptor antagonists SR 141716A (Chemin et al., 2001) and AM251 (Ross et al., 2008) and the TRPV1 antagonist capsazepine (Docherty et al., 1997), all inhibit native or recombinant T-type channels.

Intriguingly, NA-5HT inhibits the proliferation of C6 glioma cells (Jacobsson et al., 2011) via mechanism(s) not involving CB₁ receptors or TRPV1 and also slows growth of K-ras-transformed rat thyroid cells by a CB₁-independent process (Bifulco et al., 2004). There is a strong correlation between the expression of T-type calcium channels and growth in some cancer cells, and inhibition of these channels slows growth significantly (reviewed in Panner & Wurster, 2006). The expression of Caᵥ3 channels in C6 or K-ras-transformed rat thyroid cells is incompletely characterized (Bertolesi et al., 2002), however, it is tempting to speculate that the effects of NA-5HT on growth of these cells may be mediated via inhibition of T-type calcium channels.

The effects of NA-5HT Caᵥ3 channels are quite similar to that of other NAAN such as NA-DA, NA-Gly and NA-GABA-OH (Barbara et al., 2009, Ross et al., 2009). All 4 compounds produce strong hyperpolarizing shifts in the membrane potential at which Caᵥ3 channels inactivate, which would have the effect of reducing the number of channels available to open from all but the most negative membrane potentials. This mechanism for modulation of Caᵥ3 is shared with arachidonic acid (Zhang et al., 2000; Talavera et al., 2004; Chemin et al., 2007) and the phytocannabinoids $\Delta^9$-tetrahydrocannabinol and cannabidiol (Ross et al., 2008). In addition to effects on steady state channel inactivation, anandamide (and arachidonic acid) both have
effects on the kinetics of Ca\textsubscript{v}3 channels, manifest by an acceleration of channel opening and open state inactivation, although the effects of AEA on Ca\textsubscript{v}3.2 kinetics only occur at quite high concentrations (Chemin \textit{et al.}, 2001, 2007; Talavera \textit{et al.}, 2004). These kinetic effects are absent or minimal with NA-Gly and NA-DA modulation of Ca\textsubscript{v}3 channels (Ross \textit{et al.}, 2009) and NA-5HT did not affect the channel opening or open state inactivation of Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 in the present study. However, NA-5HT accelerated the apparent activation, inactivation from an open state and deactivation of Ca\textsubscript{v}3.3. In the presence of NA-5HT, the kinetics of Ca\textsubscript{v}3.3 activation are accelerated to a degree that approximates a 10 mV positive shift in membrane potential (Figure 8). This acceleration occurs in the absence of any significant effect on the voltage-dependence of channel activation, suggesting that NA-5HT may be affecting a transition between open and inactivated state(s) of the channel. NA-5HT modulation of Ca\textsubscript{v}3 channel kinetics resembles that of AEA in that the Ca\textsubscript{v}3.3 channels are the most strongly affected, and neither drug dramatically affects Ca\textsubscript{v}3.2 kinetics. The acceleration of entry into an inactivated state, coupled with an enhanced deactivation, would contribute to a reduction of calcium entry through any channels that opened in the presence of NA-5HT.

Ca\textsubscript{v}3 subtype-specific effects have been observed with several modulators of the channels including Zn\textsuperscript{2+} and \Delta\textsuperscript{9}-tetrahydrocannabinol (Traboulsie \textit{et al.}, 2007, Ross \textit{et al.}, 2008). In contrast to the effects of NA-5HT, Zn\textsuperscript{2+} selectively slows the inactivation from an open state and deactivation of Ca\textsubscript{v}3.3, while \Delta\textsuperscript{9}-tetrahydrocannabinol has similar effects to Zn\textsuperscript{2+} on Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 but not Ca\textsubscript{v}3.3. It is tempting to speculate that these compounds are acting to modulate channel activity through common binding sites on the channels that have slightly
different ligand recognition properties and which can speed or slow the transitions between different states of the channels. However, the site(s) of action for lipophilic ligand modulation of CaV3 channels is not known, and there is limited information about where any drugs that modulate CaV3 channels bind to affect channel function. NA-5HT is an endogenous compound (Verhoeckx et al., 2011) and it is regularly used as pharmacological probe for cannabinoid-related (patho)physiological processes. Our data suggest a novel role for NA-5HT as an endogenous modulator of cellular excitability through its actions on T-type calcium channels, although confirmation of this awaits the development of selective inhibitors of NA-5HT metabolism or compounds that selectively affect the binding site(s) for NA-5HT on the channels.
Acknowledgements

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Statement of conflicts of interest

None.
References


Figure Legends

**Figure 1.** *N*-arachidonyl serotonin inhibits recombinant human T-type calcium channels. Whole cell patch clamp recordings were made from human Ca\(_{V}3.1\), Ca\(_{V}3.2\) and Ca\(_{V}3.3\) channels stably expressed in HEK 293 cells. Currents were evoked by stepping from –86 mV to –26 mV every 10s. The effect of NA-5HT on each of Ca\(_{V}3.1\), Ca\(_{V}3.2\) and Ca\(_{V}3.3\) are illustrated, with a representative time plot and example traces. Each trace is an example of at least 6 similar experiments. NA-5HT was applied for the duration of the bar. The dotted line represents zero current.

**Figure 2.** *N*-arachidonyl serotonin inhibition of Ca\(_{V}3.1\) is reversed by bovine serum albumin. Whole cell patch clamp recordings were made from human Ca\(_{V}3.1\) HEK 293 cells. Currents were evoked by stepping from –86 mV to –26 mV every 10s. Representative time plots of the effect of washing off *N*-arachidonyl serotonin with A) buffer alone and B) bovine serum albumin (BSA), are illustrated. C) BSA alone had no effect of Ca\(_{V}3.1\) currents. BSA significantly (P < 0.001, Student’s t-Test) enhanced the reversal of NA-5HT inhibition of Ca\(_{V}3.1\), summarized in D), with closed circles representing reversal of current inhibition by wash with buffer alone and open circles wash with BSA. The bars represent the mean ± s.e.m.

**Figure 3.** Concentration response plot for *N*-arachidonyl serotonin inhibition of Ca\(_{V}3\) channels. Whole cell patch clamp recordings were made from human Ca\(_{V}3.1\), Ca\(_{V}3.2\) and Ca\(_{V}3.3\) channels stably expressed in HEK 293 cells. Currents were evoked by stepping from –86 mV to –26 mV every 10s. A single concentration of drug was superfused over each cell, each point represents the mean ± s.e.m. of at
NA-5HT inhibits Cav3 channels with a rank order of Cav3.1 (50 nM) > Cav3.3 (200 nM) > Cav3.2 (250 nM).

**Figure 4.** Unsaturated N-acyl serotonin analogs inhibit recombinant human T-type calcium channels more effectively than the saturated NP-5HT. Whole cell patch clamp recordings were made from human Cav3.1, Cav3.2 and Cav3.3 channels stably expressed in HEK 293 cells. Currents were evoked by stepping from −86 mV to −26 mV every 10s. A summary of the effect of 300 nM and 10 µM N-arachidonyl serotonin (NA-5HT), N-oleoyl serotonin (NO-5HT) and 10 µM N-palmitoyl serotonin (NP-5HT) on each of Cav3.1, Cav3.2 and Cav3.3 is illustrated. The bars are the mean ± s.e.m of 5-7 cells for each drug on each channel, control experiments for each represent superfusion of vehicle alone.

**Figure 5.** N-arachidonyl serotonin affects steady state inactivation but not activation of Cav3 channels. Whole cell patch clamp recordings were made from human Cav3 channels stably expressed in HEK 293 cells, 5 minutes after breaking into the cell and then again after 5 minutes in NA-5HT. To measure channel activation, cells expressing Cav3.1 and Cav3.2 were voltage clamped at -106 mV and stepped to potentials above -86 mV in 5 mV increments. The peak current at each test potential is plotted for Cav3.1 and Cav3.2 (A, B). Cells expressing Cav3.3 were voltage clamped at -106 mV, stepped briefly to potentials between -86 mV and + 74 mV and stepped back to -106 mV (see Methods). The amplitude of the tail current was plotted for Cav3.3 (D). To measure steady state inactivation, cells were voltage clamped for 5s at potentials between -126 mV and -46 mV, and then stepped to a test potential of 26 mV. The current at -26 mV following 5 seconds at the indicated
holding potential is plotted for inactivation. Curves are a Boltzmann fit of the data (see Methods). NA-5HT did not affect the voltage-dependence of channel activation, but produced a significant hyperpolarizing shift in the membrane at which 50% of the channels are inactivated for each Ca\textsubscript{v}3 subtype (A, B, C; Table 1).

**Figure 6. The effect of omission of GTP from the pipette solution on inhibition of Ca\textsubscript{v}3 channels by N-arachidonyl serotonin.** Whole cell patch clamp recordings were made from human Ca\textsubscript{v}3 channels stably expressed in HEK 293 cells with an internal solution that did (open circles) or did not (closed circles) contain 0.3 mM GTP. Currents were evoked by stepping from −86 mV to −26 mV every 10s. For each channel type, NA-5HT was superfused at low (30 nM) or high (300 nM) concentrations. Omission of GTP had no discernable effect on the NA-5HT inhibition of Ca\textsubscript{v}3 channels. The bars are the mean ± s.e.m of 6 cells for condition, control experiments for each represent superfusion of vehicle alone.

**Figure 7. N-arachidonyl serotonin does not affect Ca\textsubscript{v}3.1 or Ca\textsubscript{v}3.2 channel kinetics.** Whole cell patch clamp recordings were made from human Ca\textsubscript{v}3 channels stably expressed in HEK 293 cells. Channel activation and inactivation from the open state was measured by stepping cells from a holding potential of −106 mV to potentials above -86 mV. Currents were measured 5 minutes after breaking into the cell and then again after 5 minutes in NA-5HT. The plots illustrate the time to peak and time constant of current decline after peak before (closed circles) and after 5 minutes in NA-5HT. The small shifts seen were not different to those seen in parallel experiments where solvent alone was superfused. Each point represents the mean ± s.e.m. of at least 6 cells.
Figure 8. *N*-arachidonyl serotonin affects Ca\textsubscript{V}3.3 channel kinetics. Whole cell patch clamp recordings were made from human Ca\textsubscript{V}3.3 channels stably expressed in HEK 293 cells. Channel activation and inactivation from the open state was measured by stepping cells from a holding potential of –106 mV to potentials above -86 mV. Currents were measured 5 minutes after breaking into the cell and then again after 5 minutes in NA-5HT (300 nM). The plots illustrate (A) the time to peak and (B) time constant (τ) of current decline after peak before (closed circles) and after 5 minutes in NA-5HT. Each point represents the mean ± s.e.m. of at least 6 cells. NA-5HT accelerated channel activation and inactivation (P < 0.05, ANOVA). (C) Example trace of currents before (thin line) and in the presence of NA-5HT (thicker line), normalized to the peak current for each.

Figure 9. *N*-arachidonyl serotonin selectively affects Ca\textsubscript{V}3.3 channel activation. Whole cell patch clamp recordings were made from human Ca\textsubscript{V}3.1, Ca\textsubscript{V}3.2 and Ca\textsubscript{V}3.3 channels stably expressed in HEK 293 cells. Currents were evoked by stepping cells repetitively from -86 mV to –26 mV every 10s and time to peak determined in Axograph. Each point represents the mean ± s.e.m. of at least 6 cells. A) Illustrates the comparative potency of NA-5HT inhibition of Ca\textsubscript{V}3.3 currents (open circles) with its effects on channel time to peak (closed circles). B) Even at a high concentration (1 µM, closed circles), NA-5HT selectively affects the time to peak of Ca\textsubscript{V}3.3 but not Ca\textsubscript{V}3.1 or Ca\textsubscript{V}3.2. The bars are the mean ± s.e.m of at least 6 cells per condition, control experiments (open circles) represent superfusion of vehicle alone. * P < 0.05, Student’s T-test.
Figure 10. *N*-arachidonyl serotonin selectively affects Ca\(_V\)3.3 channel
deactivation. Whole cell patch clamp recordings were made from human Ca\(_V\)3.1,
Ca\(_V\)3.2 and Ca\(_V\)3.3 channels stably expressed in HEK 293 cells. Currents were
evoked by stepping cells repetitively from -86 mV to –26 mV every 10s and tail
current kinetics measured by fitting to a 2 component exponential curve in Axograph.

A) Illustrates the effects of NA-5HT (1 µM) on Ca\(_V\)3.1 and Ca\(_V\)3.2 tail currents; (i) are
example traces of tail currents before and during NA-HT application, (ii) shows the \(\tau\)
for the major component of decay before (closed circles) and during (open circles)
NA-5HT superfusion. The bars are the mean ± s.e.m of at least 6 cells per condition.
There were no differences in the \(\tau\) for decay for Ca\(_V\)3.2 and Ca\(_V\)3.3 in the presence
and absence of NA-5HT (paired T-test). B) Illustrates the effects of NA-5HT(1 µM)
on Ca\(_V\)3.3 tail currents. (i) example traces of tail currents before and during NA-HT
application, (ii) shows the \(\tau\) for the major component of decay before (closed circles)
and during (open circles) NA-5HT superfusion and in vehicle controls. The bars are
the mean ± s.e.m of at least 6 cells per condition. There was a significant difference
in the \(\tau\) for decay for Ca\(_V\)3.3 during NA-5HT superfusion. * P < 0.05, Paired
Student’s T-test.
Table 1. The effects of NA-5HT on the parameters of steady state activation and inactivation of CaV3 channels. Cells expressing recombinant CaV3 channels were voltage clamped at –106 mV and then stepped to potentials above –86 mV (activation) or stepped for 5s to potentials between –126 and –36 mV before stepping to the test potential of –26 mV. CaV3.3 activation curves were determined from tail current analysis, as outlined in the Methods. The resulting peak currents were fitted to a Boltzmann equation. Changes in the voltage for half activation/inactivation ($V_{0.5}$) of the curves are reported below, no drug represents time dependent changes under our recording conditions. Data is illustrated in Figure 5. ** = $P < 0.001$ from control. ND is not determined.

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<th>Drug</th>
<th>CaV</th>
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<td>50 nM NA-5HT</td>
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<tr>
<td></td>
<td></td>
<td>-7 ± 1**</td>
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<tr>
<td>300 nM NA-5HT</td>
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<td></td>
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<td>-12 ± 1**</td>
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<tr>
<td>300 nM NA-5HT</td>
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249x733mm (300 x 300 DPI)
Cav3.1
Inhibition (%)

Cav3.2
Inhibition (%)

Cav3.3
Inhibition (%)

193x428mm (300 x 300 DPI)
A

Inhibition of $I_{Ca}$ (%)

$\Delta$ Time to peak (ms)

[NA-5HT] log M

B

$\Delta$ Time to peak (ms)

CaV3.1 CaV3.2 CaV3.3

Control NA-5HT, 1 µM

124x184mm (300 x 300 DPI)