Title: Non-nociceptive afferent activity depresses nocifensive behavior and nociceptive synapses via an endocannabinoid-dependent mechanism

Running Head: Endocannabinoids depress synapses and behavior

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Abstract

Previously, low frequency stimulation (LFS) of a non-nociceptive touch-sensitive neuron has been found to elicit endocannabinoids-dependent long-term depression (eCB-LTD) in nociceptive synapses in the leech central nervous system (CNS) that requires activation of a presynaptic TRPV-like receptor by postsynaptically-synthesized 2-AG. This capacity of non-nociceptive afferent activity to reduce nociceptive signaling resembles gate control of pain, albeit longer lasting in these synaptic experiments. Having observed eCB-LTD at a single sensory-motor synapse, this study examines the functional relevance of this mechanism. Specifically, whether this form of synaptic plasticity has similar effects at the behavioral level in which additional, intersegmental neural circuits are engaged. Experiments were carried out using a semi-intact preparation that permitted both synaptic recordings and monitoring of the leech whole-body shortening, a defensive withdrawal reflex that was elicited via intracellular stimulation of a single nociceptive neuron (the N-cell). The same LFS of a non-nociceptive afferent that induced eCB-LTD in single synapses also produced an attenuation of the shortening reflex. Similar attenuation of behavior was also observed when 2-AG was applied. LFS-induced behavioral and synaptic depression was blocked by tetrahydrolipstatin (THL), a DAG lipase inhibitor, and by SB366791, a TRPV1 antagonist. The effects of both THL and SB366791 were observed following either bath-application of the drug or intracellular injection into the presynaptic (SB366791) or postsynaptic (THL) neuron. These findings demonstrate a novel, endocannabinoid-based mechanism by which non-nociceptive afferent activity may modulate nocifensive behaviors via action on primary afferent synapses.

Keywords: endocannabinoid, nociception, leech, long-term depression
Introduction

It is well-established that nociceptive signaling can be modulated by non-nociceptive afferent activity, a process referred to as gate control (Melzack and Wall 1965). Traditionally, gate control was thought to be a short-term process, lasting as long as the duration of the non-nociceptive afferent activity. Non-nociceptive Aβ fibers activated GABAergic interneurons, which, in turn, attenuate C fiber signaling (D'Mello and Dickenson 2008; Melzack and Wall 1965; Price et al. 2009; Zeilhofer et al. 2009). This gate control process is the basis for analgesic therapies, such as transcutaneous electrical nerve stimulators (TENS) and spinal cord stimulation (SCS). However, both TENS and SCS treatment have been shown to induce analgesic affects that outlast the actual duration of afferent stimulation, suggesting that other processes may be involved (DeSantana et al. 2008; Sluka et al. 2006; Sluka and Walsh 2003). One potential neurophysiological process that could contribute to this persistent decrease in nociceptive signaling is endocannabinoid-mediated synaptic modulation.

Endocannabinoids are lipid based neurotransmitters, such as anandamide and 2-arachidonoyl glycerol (2-AG), involved in short- and long-term forms of synaptic depression (Katona and Freund 2012). In most cases, these transmitters are synthesized postsynaptically and act on receptors on the presynaptic neuron (retrograde signaling). One feature of endocannabinoids that make them an attractive candidate for gate control modulation is that they are synthesized in an activity-dependent manner. Another attractive feature is that eCB-LTD is not necessarily restricted to the activated synaptic pathway, but can elicit heterosynaptic LTD in other inputs that converge onto the same postsynaptic target (Chevaleyre et al. 2007; Huang
et al. 2008). It is possible that the stimulation of non-nociceptive afferents (e.g., Aβ fibers) induces endocannabinoid synthesis in the postsynaptic targets in the spinal cord that receive input from both nociceptive and non-nociceptive afferents. The resulting activity-induced endocannabinoid synthesis and/or release could subsequently depress both non-nociceptive and nociceptive afferent (e.g. C fibers) synapses.

Invertebrates are known to possess an active endocannabinoid system that includes the same transmitters and many of the biochemical pathways found in vertebrates (Elphick 2012; Leung et al. 2008; Matias et al. 2001; McPartland et al. 2006). In the medicinal leech, identifiable nociceptive and non-nociceptive sensory afferents, along with their postsynaptic targets, are well-characterized (Blackshaw et al. 1982; Nicholls and Baylor 1968). Previously, our laboratory has found that low frequency stimulation of a single non-nociceptive touch (T) cell can depress nociceptive (N-cell) synapses in an endocannabinoid-dependent manner (Yuan and Burrell 2010; 2012). Both the N- and T-cell synapse onto the same postsynaptic target, the L-motor neuron that innervates the longitudinal muscles (Muller and Nicholls 1974) that mediate defensive whole-body shortening (Kristan et al. 2005; Shaw and Kristan 1995). This eCB-LTD required 2-AG synthesis in the postsynaptic L motor neuron and activation of a putative TRPV-like receptor in the presynaptic nociceptive afferent (Yuan and Burrell 2010; 2012). These previous studies were carried out in isolated ganglia and, therefore, were restricted to only one synapse of the shortening neural circuit. However, the whole body shortening circuit includes intersegmental elements that coordinate activation of motor neurons throughout the CNS (Figure 1a; Shaw and Kristan 1995, 1999).

Although the identity of the neuron(s) that form the intersegmental pathway is unknown,
it is possible to ascertain changes at this level using semi-intact preparations in which whole body is elicited via intracellular stimulation of a single N-cell. Using this preparation, it was found that touch afferent activity reduced shortening in parallel with synaptic depression in the intrasegmental portion of the shortening circuit (the N-to-L synapse). Furthermore, this behavioral depression was mediated by the same endocannabinoid-dependent process observed in N-to-L synaptic depression.
Materials and Methods

Animal Preparation

Leeches (*Hirudo verbana*; 3g) were obtained from commercial suppliers (Niagara Medicinal Leeches, Cheyenne, WY; Leeches USA, Westbury, NY) and maintained in artificial pond water (0.52 g/L H$_2$O *Hirudo* salt) and kept on a 12 hour light/dark cycle at 18º C. The dissections and recordings were carried out in normal saline solution (in mM: 114 NaCl, 4 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 5 NaOH, and 10 HEPES; pH = 7.4). Drugs for pharmacology experiments were dissolved in leech saline solution from frozen stock solutions and final concentrations were made just prior to each experiment. Dimethyl sulfoxide (DMSO) and Orlistat (tetrahydrolipstatin, THL) were obtained from Sigma-Aldrich (St. Louis, MO). Drugs from Tocris (Ellisville, MO) included 2-arachidonoylglycerol (2-AG) and SB3667912. All drugs were prepared from frozen stocks (dissolved in saline or DMSO where appropriate) and the final concentrations were prepared on the day of the experiment. Vehicle control experiments were carried out in saline that contained equivalent levels of DMSO (0.01%).

Semi-Intact Preparation

The semi-intact preparations used in these experiments were similar to those used in previous studies (Burrell and Sahley 2005) and consisted of a dissected portion of the CNS where intracellular recordings were made and an intact portion in which behavior was monitored (Figure 1a). The leech CNS consists of a chain of ganglia linked by a connective nerve (Muller et al. 1981). Similar to the spinal cord, each ganglion has the same compliment of sensory, motor and interneurons (notable exceptions are the head...
and tail ganglia which were not used in this study). Sensory and motor neurons project to the periphery via two bilateral pairs of segmental nerves. Dissections were carried out in frozen Sylgard™-filled dishes containing ice cold leech saline. The leeches were pinned ventral side up and an incision was made along the midline from segments 1 to 7, with the head ganglion removed to minimize response variability (Shaw and Kristan 1997; 1995). This portion of the CNS was dissected from the skin, but remained connected to segments 8 through 10, which were left intact and could still shorten (Figure 1a). The semi-intact preparation was pinned into a 3mm Sylgard™-lined dish and Teflon coated sliver wire (0.008”; A-M Systems, Inc., Carlsborg, WA) that were bared at the tips and inserted into the longitudinal muscle layer just beneath the skin in the intact portion of the preparation (Lewis and Kristan 1998). These electromyogram (EMG) wires were connected to a differential amplifier (A-M System model 3600; Everett, WA) to record the magnitude of the shortening reflex. EMG activity was quantified by measuring the area under the EMG signal during the 1.5 second period beginning at the point where the N-cell stimulus train started. To validate that the EMG measurements reflected the magnitude of the shortening reflex, a pilot study was carried out in which shortening was measured using both EMG recordings and an isometric tension transducer (Model 72-4481, Harvard Apparatus, Holliston, MA). A nylon monofilament (Surgical Specialties Corporation, Reading, PA) was tied to the intact portion of the preparation and then connected to the tension transducer (Figure 1a). EMG and tension transducer recordings of the shortening reflex were made during intracellular stimulation of a single lateral N-cell in segment 4 with a train of 10 action potentials at frequencies of 2, 5, 10 and 25 Hz. For the remaining experiments, whole-
body shortening was elicited by intracellular stimulation of a single lateral N-cell in segment 4 (10 action potentials at 10 Hz) which was found to produce a reliable shortening response in the intact portion of the preparation based on EMG and tension transducer recordings as well as visible observation.

In these same semi-intact preparation, current clamp (bridge balanced) intracellular recordings were made using sharp glass microelectrodes (25-40 MΩ) fabricated from borosilicate capillary tubing (1.0mm OD, 0.75mm ID; FHC, Bowdoinham, ME) using a horizontal puller (Sutter Instruments P-97; Novato, CA) and were filled with 3M K+ acetate. Impalement of individual neurons at ganglia 4 was carried out by a manual micropositioner (Model 1480; Siskiyou Inc., Grants Pass, OR). Current pulses were delivered to the microelectrodes through via a programmable stimulator (STG 1004; Multi-Channel Systems; Reutlingen, Germany) and signals were recorded using a bridge amplifier (BA-1S; NPI, Tamm, Germany). The signals were digitally converted (Digidata 1322A A/D converter) for observation and analysis (Axoscope; Molecular Devices, Sunnyvale, CA).

Individual cells were identified based on their position and action potential shape. The nociceptive (N) and longitudinal L-motor (L) cells have two bilateral pairs, while the touch (T) cells have three bilateral pairs (Nicholls and Baylor 1968). The touch and nociceptive cell soma are located on the ventral side of the ganglion, while the motor neurons are found on the dorsal side. For these experiments, the nerve cord (segments 1-7) of the semi-intact preparation was pinned dorsal side up in the Sylgard™ chamber. This permits recordings from the L-motor neuron and the lateral-most touch and nociceptive neurons at ganglia 4 (Figure 1a, b). The N-to-L synapse is mediated by a
monosynaptic, chemical synapse, while the T-to-L synapse consists of both an electrical component and a polysynaptic chemical component (Figure 1c).

Heterosynaptic long-term depression of the nociceptive synapse (the N-to-L synapse) was elicited by low frequency stimulation (LFS) of the T-cell (1Hz for 900 seconds) as previously reported (Yuan and Burrell 2010). Recordings of the N-to-L EPSPs were made prior to LFS (pre-test) and 60 minutes after LFS ended (post-test; Figure 1d). Separate electrode impalements of the same N- and L-cell were made for the pre- and post-test recordings because chronic recordings of leech neurons results in the progressive rundown of the EPSP, likely due to damage from the chronic impalements (Eliot et al. 1994). Postsynaptic (L motor neuron) input resistance was recorded at the pre- and post-test levels and only stable recordings were included in the data analysis. For all experiments, the mean pre-test input resistance was 24 ± 2.4 MΩ and post-test input resistance was 22 ± 2.8 MΩ. The peak EPSP amplitude was recorded every 10 seconds and calculated by averaging ≈10 EPSPs during both the pre- and post-test.

Drugs were bath-applied during LFS or for 15 minutes without LFS during control experiments. Bath application of drugs was isolated to the CNS, with a Sylgard™ wall surrounding the semi-intact skin portion of the preparation. Iontophoretic injection was applied for 5 minutes prior LFS and consisted of -1 nA, 500-msec current pulses delivered at 1 Hz. In experiments with iontophoresis, injection of the drug was either applied on the presynaptic N-cell or postsynaptic L-cell (Figure 1d).

Statistics
EPSP amplitude measurements of the pre- and post-test recordings were normalized and presented as means ± standard error. Statistical analyses using a one-way analysis of variance (ANOVA) were performed to determine main effects with Newman-Keuls post-hoc tests to confirm the ANOVA results. Linear regression analysis was used in experiments examining the relationship between EMG and tension transducer measurements of the shortening response. The analyses were carried out with Statistica analysis software (Statsoft). All significance was determined at an alpha level of at least p<0.05.
Results

EMG recordings accurately reflect strength of the shortening response

In order to determine whether the EMG recordings provided a reliable measure of the strength of the whole body shortening reflex, experiments were carried out in which shortening was monitored using EMG electrodes and a tension transducer simultaneously. Measurements were made of the shortening in response to increasing frequency of N-cell stimulation. Based on the tension transducer recordings, no shortening was observed when the N-cell was stimulated at the lower frequencies (2Hz and 5Hz), but significant shortening was observed when the N-cell was stimulated at 10Hz and 25Hz (N=3). There was a statistically significant correlation between the EMG recording and the amplitude of the response measured with the tension transducer (Figure 2). These results indicate that the EMG electrodes provide a reliable measure of the magnitude of whole body shortening.

Non-nociceptive stimulation depressed both the shortening behavior and the nociceptive synapse

Previously, we found that LFS of the non-nociceptive T-cell resulted in depression of nociceptive synapses in isolated ganglia (Yuan and Burrell 2010; 2012). To determine whether this depression was behaviorally relevant, experiments were carried out to monitor changes in shortening following T-cell LFS. N-cell stimulation in ganglion (10 spikes at 10Hz) was used to induce shortening in the semi-intact preparation. Pre-test recordings of the N-to-L synapse and shortening response were carried out prior to T-cell LFS (15 minutes; 1Hz). After a 60 minute rest period, post-test recordings were
carried out to determine any changes in synaptic transmission and behavior. T-cell LFS
induced significant depression in the nociceptive N-to-L synapse, consistent with
previous research (Figure 3). In the same preparations, a significant decrease in the
shortening reflex was also observed (Figure 3). Control experiments without LFS found
no change at the synaptic or behavioral level. These data indicate that a single
nociceptive neuron in single ganglia can effectively induce the shortening reflex, but that
this reflex can be attenuated through LFS activation of a single non-nociceptive neuron.
In isolated ganglia, this process was observed to be endocannabinoid-dependent;
therefore, experiments were carried out to determine whether these transmitters
mediated depression of the shortening behavior.

Depression of the nociceptive synapse and behavior is 2-AG-dependent

Previous research has shown that depression of the nociceptive synapse by non-
nociceptive LFS could be mimicked by 2-AG or occluded by prior 2-AG treatment (Yuan
and Burrell 2010; 2012). Therefore, the ability of 2-AG to alter the shortening response
was also examined. Following pre-test recordings of N-cell elicited shortening and the
N-to-L synapse, 2-AG (60 μM) was bath-applied selectively to the CNS portion of the
preparation (ganglia from segments 1-7) for 15 minutes. Following 2-AG treatment and
a subsequent 60 minute washout period, post-test recordings of the N-to-L synapse and
shortening behavior were measured. Similar to the LFS-induced depression, 2-AG
produced depression in the nociceptive synapse that was accompanied by a decrease
in shortening (Figure 4a). No change was observed in vehicle controls (0.01% DMSO).
This suggests that 2-AG attenuates N-cell elicited shortening, in parallel, with depression in the N-to-L synapse.

Given that T-cell LFS depresses shortening and that N-cell elicited shortening is sensitive to 2-AG, the question as to whether T-cell LFS-induced depression requires 2-AG was addressed. 2-AG synthesis from DAG requires DAG lipase (Bisogno et al. 2003). In previous experiments in the leech, multiple inhibitors of DAG lipase were found to inhibit N-to-L eCB-LTD in isolated ganglia, specifically RHC-80267, OMDM-188, and tetrahydrolipstatin (THL) (Yuan and Burrell 2010; 2012). To determine whether inhibiting DAG lipase blocked behavioral depression following T-cell LFS, experiments were carried out with THL treatment. Pre-test recordings of the N-to-L EPSP and N-cell stimulated shortening were followed by bath-application of THL (10 μM) for 15 minutes onto the isolated CNS portion of the preparation during T-cell LFS. THL was washed out and post-test recordings were carried out 60 minutes later. THL treatment during LFS blocked the depression normally observed in the nociceptive synapse as well the attenuation of the shortening reflex (Figure 4b). Control experiments in which THL was applied without LFS did not reveal any non-specific effects of drug treatment. These findings support the hypothesis that LFS-induced depression of synaptic transmission and shortening is endocannabinoid-dependent, specifically requiring 2-AG.

In previous experiments in which THL was selectively injected into either the pre-(N-cell) or postsynaptic (L motor neuron) cell, only postsynaptic injection blocked LFS-induced eCB-LTD indicating that the L motor neuron was the site of 2-AG synthesis (Yuan and Burrell 2010). Postsynaptic injection of THL (10 μM) was repeated in the semi-intact preparation and the effects on both the nociceptive synapse and the elicited
shortening response were tested. As in previous experiments, postsynaptic THL injection blocked LFS-induced depression in the N-to-L synapse (Figure 4c). Surprisingly, N-cell elicited shortening was also prevented indicating that 2-AG synthesis in the L motor neuron affected the intersegmental neural circuit mediating whole-body shortening. This result suggests that blocking 2-AG synthesis in the L motor neuron is sufficient to block eCB-LTD of other nociceptive synapses that contribute to the intersegmental shortening neural circuit.

As mentioned earlier, pre-treatment with 2-AG occludes subsequent LFS-induced depression at the N-cell synapse (Yuan and Burrell 2010). These experiments were repeated in the semi-intact preparations, however rather than bath-applying 2-AG as was done previously, 2-AG (60 μM) was injected into the N-cell prior to pre-test recordings of the behavior and synapse. In these pre-treated preparations, subsequent LFS failed to elicit depression of either the N-cell synapse or the shortening reflex (Figure 4c). These results are consistent with 2-AG occluding subsequent LFS-induced synaptic and behavioral depression and extend previous occlusion experiments by focusing 2-AG pre-treatment onto the N-cell.

Depression of the nociceptive synapse and behavior involves a presynaptic TRPV-like receptor

Previous pharmacological studies in our laboratory have indicated that a TRPV-like receptor was involved in eCB-LTD in the leech. Specifically, eCB-LTD could be mimicked and/or occluded by the TRPV activators capsaicin and resiniferatoxin and could be blocked by the TRPV antagonists capsazepine or SB366791 (Li and Burrell
2011; Yuan and Burrell 2010; 2012). Therefore, the role of this TRPV-like receptor was examined during endocannabinoid-dependent depression of the shortening reflex. The selective TRPV1 antagonist, SB366791 (10 μM), has previously been shown to block eCB-LTD in the leech (Yuan and Burrell 2010; 2012). Bath-applied SB366791 to the CNS portion of the preparation prevented 2-AG-induced depression of both the nociceptive synapse and of N-cell elicited shortening (Figure 5a). The ability of SB366791 to block LFS-induced depression of shortening was also examined. Bath-application of SB366791 to the CNS portion of the preparation blocked LFS-induced depression at the behavioral and synaptic level, suggesting that this activity-induced depression required the TRPV-like receptor (Figure 5b).

Next, experiments were carried out in which SB366791 was injected into the N-cell that was stimulated to elicit shortening. In previous experiments, injection of a TRPV1 antagonist into the presynaptic, but not postsynaptic neuron, blocked eCB-LTD (Yuan and Burrell 2010; Li and Burrell 2011). Injection of SB366791 (10 μM) into the N-cell not only blocked LFS-induced depression in the nociceptive synapse, but also prevented attenuation of the shortening behavior following LFS (Figure 5c). No changes in synaptic signaling or behavior were observed when the N-cell was injected with SB366791, but the LFS was omitted. In control experiments in which SB366791 was injected into the T-cell, LFS-induced depression of both the nociceptive synapse and N-cell elicited shortening was still observed providing further evidence that the effects of SB366791 were restricted to the N-cell. These results suggest that a presynaptic TRPV-like receptor mediated nociceptive signaling at both the synaptic and behavioral level.
Discussion

Previous research in our lab has shown that, in isolated ganglia, non-nociceptive (T-cell) LFS depresses the nociceptive (N-to-L) synapse. Based on pharmacological studies, this heterosynaptic depression was mediated through postsynaptic 2-AG synthesis in the L-motor neuron, where 2-AG travels in a retrograde manner to bind to a putative TRPV-like receptors on the presynaptic nociceptive neuron (Yuan and Burrell 2010; 2012). This eCB-LTD required presynaptic calcineurin activation and both pre- and postsynaptic increases in intracellular Ca^{2+}, similar to TRPV1-mediated eCB-LTD in the hippocampus (Jensen and Edwards 2012; Yuan and Burrell 2012). Novel gene expression was also necessary, specifically RNA synthesis in the presynaptic neuron and protein synthesis in the pre- and postsynaptic neurons (Yuan and Burrell 2013). In the present study, the potential functional relevance of this heterosynaptic eCB-LTD was examined using the whole-body shortening reflex.

Whole-body shortening in the leech is a defensive withdrawal reflex in response to noxious mechanosensory stimuli that involves a near-synchronous contraction of all body segments (Shaw and Kristan 1999; Stuart 1970). The reflex is initiated by activation of the P- or N-cells and while it appears that activity in multiple P-cells is required to elicit shortening (Shaw and Kristan 1995), the present study shows that activation of a single N-cell is sufficient to induce shortening, at least in these semi-intact preparations. These primary afferents have direct input onto motor neurons responsible for shortening that are in the same or neighboring segments and it is in this intrasegmental component of the shortening circuit that eCB-LTD has been previously observed using single ganglion experiments. However, these same afferents are also
thought to activate an intersegmental pathway that carries the signal to shorten throughout the leech CNS (Shaw and Kristan 1999). The identity of the neuron or neurons that make up this intersegmental pathway is unknown, but its presence can be inferred from the activation of shortening-related motor neurons down the length of the nerve cord (Arisi et al. 2001; Shaw and Kristan 1999). Although it is not possible to directly record from the N-to-shortening interneuron synapse, it is possible to observe changes in this signaling pathway by direct intracellular stimulation of a single nociceptive afferent and monitoring the subsequent level of shortening.

LFS of the non-nociceptive T-cell attenuated the leech shortening reflex and this was accompanied by eCB-LTD at the N-to-L synapse. Application of the DAG lipase inhibitor THL blocked LFS-induced attenuation of N-cell elicited shortening and depression of the N-to-L synapse. THL was applied only to the CNS of the preparation, therefore the observed effects were at the level of N-cell input to the intersegmental neuron(s) and not where these interneurons synapse onto the motor neurons.

Exogenous 2-AG application to the CNS reduced N-cell elicited shortening and induced depression at the N-to-L synapse, mimicking the effects of LFS. Injection of 2-AG into the N-cell also occluded subsequent LFS-induced synaptic and behavioral depression. Together, these findings are consistent with the hypothesis that the endocannabinoid 2-AG mediates LFS-induced behavioral and synaptic depression.

Previous studies have indicated that T-cell LFS stimulates 2-AG synthesis in the L-motor neuron which then presumably travels in a retrograde manner to modulate the presynaptic N-cell (Yuan and Burrell 2010) similar to most other examples of eCB-LTD (Katona and Freund 2012). When these postsynaptic THL injection experiments were
repeated here, not only was synaptic depression prevented, but LFS-induced
depression of shortening was blocked as well. This is a surprising finding because it
implies that 2-AG synthesis and release from the L motor neuron not only alters
synaptic input that it receives, but that it also acts on other synaptic targets of the N-cell,
e.g. those associated with carrying the intersegmental signal to shorten (see shortening
circuit diagram in Figure 1b). One possible explanation is that 2-AG is being released
extensively within the neuropil by the L motor neuron so that all synaptic targets of the
N-cell (and presumably other synapses sensitive to 2-AG) are affected. Alternatively, it
is possible that this change in all the N-cell synapse occurs through an intracellular
signal that is initiated at the N-to-L synapses and propagates throughout the N-cell to
other connections made by this afferent. eCB-LTD in this neuron is known to require
new protein synthesis in the presynaptic N-cell (Yuan and Burrell 2013) which could
conceivably contribute to a signal that spreads intracellularly to other presynaptic
terminals. Such a process would represent an interesting and novel element to eCB-
mediated synaptic plasticity.

Because these studies utilize controlled stimulation of nociceptive and non-
nociceptive afferents in terms of spike number and frequency, they pose interesting
questions in terms our understanding of this persistent form of gate control modulation.
For example, the observed synaptic depression would appear to mediate a decrease in
nocifensive behavior, but can this decrease in synaptic signaling be overcome by
increased nociceptive afferent activity (e.g. doubling the firing rate) or is nociceptive
signaling effectively blunted across a range of activity levels. Put another way, is the
gate effectively closed or simply harder to open? Another issue is pattern of non-
nociceptive (T-cell) activity that produces depression of nociceptive synapses and
nocifensive signaling. Do different levels of T-cell activity produce proportionate levels
of depression in nociceptive synapses? To what extent do non-eCB mediated forms of
plasticity contribute to this heterosynaptic depression given that T-cell activity can
produce both eCB- and NMDA receptor-mediated homosynaptic depression (Li and
Burrell 2008)? N-cell LFS itself is capable of producing homosynaptic eCB-LTD (Yuan
and Burrell 2010 here) and we have not yet investigated whether such depression also
affects nocifensive shortening.

Invertebrates possess the endocannabinoid neurotransmitters and potentially the
same synthesizing/metabolizing enzymes found in vertebrates, but lack orthologues to
the CB1/CB2 receptors (Burke et al. 2006; Elphick 2012; Leung et al. 2008; Matias et al.
2001; Salzet and Stefano 2002). However, endocannabinoids have been observed to
bind to TRPs (De Petrocellis et al. 2001; Di Marzo and De Petrocellis 2010), including
the TRPV receptor, to mediate synaptic plasticity (Chavez et al. 2010;; Gibson et al.
2008; Grueter et al. 2010; Jensen and Edwards 2012; (Maione et al. 2009). In previous
studies, we have observed that the TRPV1 antagonists, capsazepine and SB366791,
could block eCB-LTD in the leech (Li and Burrell 2011; Yuan and Burrell 2010; 2012). In
this study, bath-application of SB366791 to the CNS portion of the semi-intact
preparation was found to block both 2-AG- and LFS-induced eCB-LTD of the N-to-L
synapse. SB366791 bath-application also prevented 2-AG- and LFS-induced
attenuation of the shortening reflex. In addition, intracellular injection of SB366791 into
the presynaptic N-cell blocked LFS-induced attenuation of shortening and eCB-LTD of
the N-to-L synapse. These results are consistent with the hypothesis that activation of a
presynaptic TRPV-like receptor is required for 2-AG-mediated depression of synapses and behavior. These findings are consistent with evidence that spinal TRPV1 receptors can elicit an analgesic effect, possibly as a result of persistent depression of evoked C-fiber EPSPs (Baccei et al. 2003; Di Marzo and De Petrocellis 2010; Kusudo et al. 2006; Yang et al. 1999).

In this study, we observed that afferent activity that elicits eCB-LTD of a nociceptive synapse also attenuates nocifensive behavior. There is considerable interest in the development of endocannabinoid-based therapies for pain and the present findings are relevant to understanding both the cellular mechanisms of endocannabinoid-mediated modulation of nociceptive circuits and the role of these neurotransmitters in mediating analgesia that is induced by somatosensory afferents activity. For example, a recent study has shown that LFS of spinal afferents produces eCB-LTD in nociceptive synapses (Kato et al. 2012), similar to our own observations. We and others (Kato et al. 2012) have proposed that this endocannabinoid-mediated depression of nociceptive synapses may play a role in TENS and SCS therapies in which the analgesic effects are produced by repetitive stimulation of somatosensory afferents. As discussed in the Introduction, it has been noted that the analgesic effects of TENS and SCS therapies persist long after the afferent stimulation has ended (DeSantana et al. 2008; Sluka et al. 2006; Sluka and Walsh 2003). Endocannabinoid-mediated neuroplasticity has a number of properties that would be consistent with a role in these therapies, specifically activity-dependent transmitter synthesis, persistent modulatory effects and the capacity to alter both active and neighboring inactive synapses. The findings in this study provide the most direct
evidence to date that endocannabinoids may contribute to TENS/SCS-induced analgesia. Endocannabinoids have also been shown to contribute to stress-induced analgesia which, similar to gate control, requires somatosensory stimulus, albeit at noxious levels (Gregg et al. 2012; Hohmann et al. 2005; Nyilas et al. 2009). It would be interesting to test the noxious stimuli used to elicit stressed-induced analgesia in mammals also produces endocannabinoid-dependent decreases in nociceptive synapses and nocifensive behavior in the leech.

The physiological and behavioral properties of nociception exhibit a remarkable level of evolutionary conservation, therefore studies using invertebrate nervous systems can provide valuable insights concerning nociception in mammals (Tobin and Bargmann 2004; Walters and Moroz 2009). Similar to vertebrates, nociceptive afferents in the leech can be divided into mechano-nociceptors (the medial N-cells) and polymodal nociceptors (the lateral N-cell used in this study), the latter responding to mechanical stimuli, nociceptive thermal stimuli, low pH, and capsaicin (Pastor et al. 1996). These features combined with the ability to record from both identifiable afferents for light touch or sustained pressure and their postsynaptic targets, and similarities in the endocannabinoid-mediated synaptic plasticity make the leech a useful model system for discovering fundamental biological processes related to nociception.

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**Figure Legends**

**Figure 1.** Experimental protocols and neural circuitry. 

**a.** (top) A semi-intact preparation was used to measure the shortening and synaptic transmission. The ventral nerve cord from segments 1-7 was dissected and pinned onto a Sylgard™ dish, but remained attached to the intact portion of the preparation (segments 8-10). EMG electrodes were inserted under the skin to record neuromuscular activity associated with whole-body shortening. The dotted/dashed line represents a Sylgard™ box placed around the intact portion of the preparation to ensure that bath-applied drugs only acted on the CNS portion of the preparation. Intracellular electrodes were used to impale the T-cell to deliver LFS (not shown), as well as the N-cell, and L-motor neuron for synaptic recordings. Inset: Drawing of an intact leech with the dashed box representing the portion of the animal used in the semi-intact preparation.  

**b.** Simplified model of the leech whole-body shortening reflex. At the local or intrasegmental circuit level, touch (T) and nociceptive (N) primary afferents synapse onto the longitudinal (L) motor neurons (and other motor neurons that are not shown) that innervate the longitudinal muscle fibers responsible for shortening. The N-cell afferent is also thought to activate an as yet unidentified shortening interneuron (S.I.) that carries the signal to shorten throughout the CNS and presumably activates the L and other motor neurons in each ganglion (Shaw and Kristan 1995, 1999) and constitutes the intersegmental circuit. The T-cell may also synapse onto S.I., but T-cell activity alone cannot initiate shortening (Shaw and Kristan 1995). A third primary afferent, the pressure-sensitive (P-) cell also contributes to both the intra- and intersegmental shortening circuits, but has been omitted to simplify the figure.  

**c.** The N-cell has a monosynaptic chemical
(glutamatergic) connection onto the longitudinal L-motor neurons (Yuan and Burrell 2010). The T-cell forms a monosynaptic electrical synapse and a polysynaptic chemical (glutamatergic) connection onto the L-motor neuron (Nichols and Purves 1970; Li and Burrell 2008). **d.** Pre-test recordings of the shortening reflex were tested 3 times at 2 minute intervals. This was immediately followed pre-test measurements of the N-to-L EPSP. Next, LFS (900sec, 1Hz) of the T-cell or drug application was carried out followed 60 mins later by post-test recordings of the shortening reflex and synaptic transmission.

**Figure 2.** EMG recordings provide a reliable measure of whole-body shortening. The scatter plot showed the relationship between the tension transducer and EMG measurements of whole body shortening reflex in response to increasing frequency of N-cell stimulation. The larger, filled circles represent the mean (±SE) EMG measurements as a function of the tension transducer response recorded at the same time. The smaller symbols (filled circles, open circles and filled triangles) represent EMG responses as a function of tension transducer measurements for each of the preparations tested. Regression analysis of data from the 2-25Hz stimulation confirmed a significant correlation between EMG and tension transducer measurements ($r^2=0.56; F=11.26; p<0.01$). (Inset) Representative traces of the behavioral response recorded through the EMG electrodes (top; x-axis) and tension transducer (bottom; y-axis) with 10Hz stimulation.

**Figure 3.** Non-nociceptive stimulation attenuates the nociceptive synapse and N-cell elicited shortening. Top traces are an example of the train of N-cell action potentials
used to elicit shortening (left) and the single action potential used to produce a single EPSP in the L motor neuron (right). The middle traces (left) represent the EMG recording of the shortening reflex during pre-test (gray) and post-test (black) recordings. The middle traces (right) represent the N-to-L EPSP during pre-test and post-test recordings of the synapse. T-cell LFS produced significant depression of both the shortening reflex (N=5; one-way ANOVA $F_{1,10}=24.77; p<0.001$) and the nociceptive synapse (N=5; $F_{1,7}=26.39; p<0.01$) synaptic data. Newman-Keuls post-hoc analyses detected a significant difference in the shortening response between the vehicle control vs. LFS group (*$p<0.001$) and in the EPSP amplitude between the control vs. LFS group ($p<0.01$).

**Figure 4.** Role of 2-AG in behavioral and synaptic depression.  

**a.** Bath-application of 2-AG induced a depression in both the shortening reflex (N=8; one-way ANOVA $F_{1,13}=32.80; p<0.0001$) and the nociceptive synapse (N=5; $F_{1,7}=41.24; p<0.01$), similar to the effects of LFS. Newman-Keuls post-hoc analyses detected a significant difference in the shortening reflex between vehicle control and 2-AG-treated group ($p<0.001$) and in EPSP amplitude between the vehicle control and 2-AG-treated group ($p<0.001$).  

**b.** The DAG lipase antagonist, THL, blocked LFS-induced depression of the shortening reflex ($F_{3,18}=14.64; p<0.0001$) and the nociceptive synapse ($F_{3,15}=12.66; p<0.001$). Newman-Keuls post-hoc analyses detected significant differences in the shortening reflex between the vehicle control (N=7) and LFS groups (N=5; $p<0.001$) and the LFS and LFS+THL groups (N=5; $p<0.001$). Significant differences were also detected in the EPSP amplitude between vehicle control (N=5) and LFS groups (N=5; $p<0.001$) and between the LFS and LFS+THL groups (N=5; $p<0.001$). No significant
differences in shortening or synaptic transmission were observed in the group in which THL was applied, but LFS omitted (N=6) when compared to the vehicle control group. Sample traces show a decrease in EPSP amplitude following LFS (top) and no change in amplitude following LFS with bath-applied THL (bottom). c. Under control conditions, LFS depressed shortening (F2,12=26.60278; p<0.0001) and synaptic transmission (F2,10=28.25846; p<0.0001). Injection of THL into a postsynaptic L-motor neuron (N=5) inhibited depression of the shortening reflex and at the nociceptive synapse. Newman-Keuls post-hoc analyses detected significant differences in the shortening reflex between the LFS (N=5) and LFS+THL injected groups (N=5; p<0.001). Significant differences in the nociceptive synapse were also observed between the LFS (N=5) and LFS+THL injected groups (N=5; p<0.001). Occlusion experiments with N-cell injection of 2-AG prior to the pre-test did not further depress the behavior or synapse after LFS was induced. Post-hoc analyses indicated a differences in shortening behavior with the LFS alone group vs. the 2AG occlusion (OCC) group (N=5; p<0.001) and a difference in the nociceptive synapse between the LFS alone group vs. the 2AG OCC group (N=5; p<0.001). Sample traces observe no changes in EPSP amplitude in the LFS+THL injection (top) or 2-AG occlusion (bottom) groups.

**Figure 5.** Role of the putative TRPV-like receptor. a. Bath-application of SB366791 blocked 2-AG-induced depression of both shortening (F3,19=13.90; p<0.0001) and the nociceptive synapse (F3,14=8.70; p<0.01). Newman-Keuls analysis detected significant differences in the shortening reflex between the vehicle control (N=7) and 2-AG groups (N=8; p<0.001) and between the 2-AG and 2-AG+SB366791 groups (N=5; p<0.001). Significant differences in EPSP amplitude were also detected between the vehicle...
control (N=5) and 2-AG groups (N=5; p<0.01) and between the 2-AG and 2-AG+SB366791 groups (N=5; p<0.01). **b. Bath-application of the TRPV1 inhibitor** SB366791 blocked LFS-induced depression of both the shortening reflex (F_{3,18}=14.89; p<0.0001) and the nociceptive synapse (F_{3,14}=8.92; p<0.01). Newman-Keuls post-hoc analyses detected a significant difference in the shortening reflex between the vehicle control (N=7) and LFS groups (N=5; p<0.001) and between the LFS and LFS+SB366791 groups (N=5; p<0.001). Significant differences in EPSP amplitude were also detected between the between the vehicle control (N=5) and LFS groups (N=5; p<0.01) and between the LFS and LFS+SB366791 groups (N=5; p<0.01). No significant differences shortening or synaptic transmission were observed between the group in which SB366791 was applied, but LFS omitted (N=5) and the vehicle control group. Sample trace showed no changes in EPSP amplitude in the LFS + SB366791 group. **c. Presynaptic (N-cell) injection of SB366791 blocked LFS-induced depression of both the shortening reflex (F_{4,20}=23.53; p<0.000001) and the nociceptive synapse (F_{4,18}=12.93; p<0.00001).** Newman-Keuls analyses found significant differences in the shortening response between the control (N=7) and LFS groups (N=5; p<0.001) and between the LFS and LFS+presynaptic SB366791 (N-cell) groups (N=5; p<0.001). Significant differences in EPSP amplitude were observed between the control (N=5) and LFS groups (N=5; p<0.01) and between the LFS and LFS+presynaptic SB366791 (N-cell) groups (N=5; p<0.01). No significant differences in shortening or synaptic transmission were observed in the group which underwent presynaptic SB366791 injection, but LFS omitted (N=5) when compared to the control group. Presynaptic injection of SB366791 into the T-cell before LFS did not prevent depression in the
shortening behavior or nociceptive synapse. Sample traces show a decrease in EPSP amplitude in the LFS + T-cell SB366791 injection group (top), while no depression is observed with LFS + N-cell SB366791 injection group (bottom).