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RESEARCH ARTICLE

Seasonal variation of long-term potentiation at a central synapse in the medicinal leech

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SUMMARY

Long-term potentiation (LTP) is a persistent increase in synaptic transmission that is thought to contribute to a variety of adaptive processes including learning and memory. Although learning is known to undergo circannual variations, it is not known whether LTP undergoes similar changes despite the importance of LTP in learning and memory. Here we report that synapses in the CNS of the medicinal leech demonstrate seasonal variation in the capacity to undergo LTP following paired presynaptic and postsynaptic stimulation. LTP was observed during the April–October period, but no LTP was observed during the November–March period. Application of forskolin, a technique often used to produce chemical LTP, failed to elicit potentiation during the November–March period. Implementing stimulation patterns that normally result in long term depression (LTD) also failed to elicit any change in synaptic strength during the November–March period. These experiments indicate that LTP and LTD can be influenced by circannual rhythms and also suggest a seasonal influence on learning and memory.

Key words: LTP, LTD, leech, circannual, seasonal variation.

INTRODUCTION

Nearly all animals exhibit rhythmic periods of enhanced and reduced behavioral activity over daily (circadian) and seasonal/annual (circannual) time scales. Coordinating physiological processes with these cycles is important to ensure that metabolic resources are available during periods of higher activity and are not inappropriately utilized during reduced activity periods (Bradshaw and Holzapfel, 2007). In a variety of species, learning and memory exhibit both circadian and circannual variation (Catarsi et al., 1990; Gerstner et al., 2009; Sherry and Hoshooley, 2009; Walton et al., 2011). Long-term potentiation (LTP) is a form of neuromodulation in which coordinated activity of the presynaptic and postsynaptic neurons produces a persistent strengthening of synaptic transmission that is thought to be a critical cellular component of learning and memory (Grimwood et al., 2001). Many forms of LTP are mediated by the NMDA receptor (NMDAR), an ionotropic glutamate receptor that requires the coincidence of both ligand binding and postsynaptic depolarization (indicative of presynaptic and postsynaptic activity, respectively) for the NMDAR channel to open (Nowak et al., 1984). LTP has been reported to be modulated by circadian rhythms (Chadhury et al., 2005), but it is not known whether circannual rhythms also influence LTP.

The medicinal leech (*Hirudo verbana*) (Siddall et al., 2007) is a useful model system for carrying out studies on activity-dependent synaptic plasticity. It has a simple and well-characterized CNS (Kristan et al., 2005), which facilitates recording from the same pair of neurons from one preparation to the next. Furthermore, the leech is amenable to behavioral and physiological studies of learning and memory (Crisp and Burrell, 2009), and leech synapses are capable of undergoing LTP and long-term depression (LTD) using mechanisms similar to vertebrate forms of NMDAR-dependent LTP and LTD (Burrell and Sahley, 2004; Grey et al., 2009; Li and Burrell, 2009; Grey and Burrell, 2010). Here, we examined the seasonal variation of LTP between the pressure-sensitive mechanosensory neuron (P cell) and the anterior pagoda neuron (AP cell), a proposed neuromodulatory neuron that projects to the periphery (Gao and Macagno, 1987). The glutamatergic P-to-AP connection is monosynaptic and is useful for studying general properties of synaptic transmission and plasticity in the leech (Wessel et al., 1999; Gaudry and Kristan, 2009; Todd et al., 2010). Additionally, the P-to-AP synapse shares many of the same cellular properties of LTP/LTD observed in mammals, including a requirement for postsynaptic NMDAR activation, downstream stimulation of protein kinases (for LTP) and phosphatases (LTD), and involvement of receptor trafficking (Grey et al., 2009; Grey and Burrell, 2010).

LTP in the P-to-AP synapse was examined over a 12 month period and was found to vary at different times of the year. LTP was observed during the April–October period; however, LTP was not observed during the November–March period, either by coordinated presynaptic and postsynaptic activity or by chemical activation. Additionally, stimulation patterns that typically induce LTD also failed to induce persistent depression during the November–March period. These results suggest that synaptic plasticity is endogenously regulated in a circannual pattern.

MATERIALS AND METHODS

Hirudo verbana L. leeches weighing 3 g were obtained approximately monthly from a commercial supplier (Leeches USA, Westbury, NY, USA or Niagara Medicinal Leeches, Niagara Falls, ON, Canada) and kept in pond water (0.52 g l^{-1} H_2O *Hirudo* salt; Leeches USA) at 15°C, under a 12 h:12 h light:dark cycle. According to the suppliers, the leeches were bred and reared indoors, under

controlled conditions, and shipped approximately 5 months after their last feeding. During periods when leeches from the two commercial suppliers were used at the same time, there were no obvious differences in synaptic properties including the capacity to undergo LTP or LTD. Ganglia were dissected and placed in a recording chamber (1 ml) with constant perfusion (~1 ml min^{-1}). Dissections and recordings were carried out in leech saline containing (mmol l^{-1}): 115 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes.

Dual intracellular recordings were made by impaling individual neurons with a glass microelectrode using a micropositioner (Model 1480; Siskiyou Inc., Grants Pass, OR, USA). Electrodes were pulled from borosilicate capillary tubing (1.0 mm o.d., 0.75 mm i.d.; FHC Bowdoinham, ME, USA) to a resistance of 25–35 MΩ and filled with 3 mol l^{-1} potassium acetate. Signals were amplified with a bridge amplifier (BA-1S; National Precision Instruments, Tamm, Germany) and then digitally converted (Digidata 1322A A/D converter; Molecular Devices, Sunnyvale, CA, USA) for viewing and subsequent analysis (Axoscope; Molecular Devices). Individual neurons were identified based on their position, size and action potential shape. Current pulses were delivered to individual neurons using a programmable stimulator (MultiChannel Systems STG 1004; Reutlingen, Germany). Excitatory postsynaptic potentials (EPSPs) in the AP cell were elicited by brief, 10 ms, 1.5 nA current injections into a contralateral P-cell. To prevent the initiation of action potentials, the AP neuron was hyperpolarized to the same membrane potential during both the pre- and post-tests (–75 mV). Input resistance of the postsynaptic AP cell was measured throughout each experiment by injecting negative currents (0.5 nA, 500 ms). Typically, 4–6 EPSPs (to minimize synaptic depression) and 7–9 input resistance measurements were averaged per recording.

In all experiments, baseline EPSP amplitude and input resistance measurements were taken in normal saline. NMDAR-dependent synaptic plasticity requires the co-agonist glycine (Burrell and Sahley, 2004; Grey and Burrell, 2010); therefore, 1 μmol l^{-1} glycine was perfused into the bath during pairing sessions. The pairing protocol, based on studies in *Aplysia* (Lin and Glanzman, 1997), consisted of 25 Hz P cell stimulation (1.5 nA for 10 ms, 10 pulses) and a simultaneous depolarization of the AP cell (2 nA for 500 ms). Paired activation of the P and AP cells was repeated 5 times with an inter-trial interval of 2 min. The pairing protocol was followed by a 45 min consolidation period in normal saline, followed by a post-test of the P-to-AP EPSP and AP input resistance. A no-stimulation control group consisted of a 10 min perfusion of saline + 1 μmol l^{-1} glycine followed by a 45 min consolidation period in normal saline, and post-test of the P-to-AP EPSP. Across all experiments, the mean AP cell resting potential was approximately –40 mV, and the mean input resistance was 12±0.1 and 11.7±0.2 MΩ for the pre-test and post-test, respectively. EPSP amplitude and input resistance measurements were taken at the conclusion of each 60 min experiment, normalized to their initial values (% of baseline), and presented as the mean ± s.e. Cells were excluded if input resistance changed >30% from baseline. In addition, only cells with initial EPSPs <7 mV were studied, as synapses >7 mV did not potentiate, consistent with LTP observed in other leech and vertebrate synapses (Grey and Burrell, 2008; Bi and Poo, 1998; Montgomery et al., 2001). Experiments were conducted over the period from 27 June 2008 to 16 July 2009.

Statistical tests were conducted using Statistica analysis software (www.statsoft.com). A threshold of $P<0.05$ was used to determine statistical significance for independent *t*-tests and linear regression analyses. Data from pairing- and forskolin-induced LTP experiments conducted between April and October have already been presented (Grey and Burrell, 2008; Grey and Burrell, 2010) and are reused here for comparison with comparable experiments conducted between November and March.

RESULTS

Pairing P and AP cell activity elicited LTP in only 7 out of 12 months (Fig. 1, left *y*-axis, filled circles). LTP was observed during the April–October period, although there was some variation in the level of potentiation observed. In the November–March period no LTP was observed, and there was a brief period in December in which the same pairing protocol that normally induces LTP actually resulted in synaptic depression (LTD).

To further examine these data, experiments were classified as either plastic (>20% change in EPSP amplitude, either potentiation or depression) or non-plastic (<20% change) and the percentage of plastic synapses was calculated over each month. In general, the monthly percentage of plastic synapses paralleled changes in LTP over the year (Fig. 1, right *y*-axis, filled triangles). That is, the periods in which synapses exhibited LTP also exhibited a high percentage of synapses that experienced a significant (>20%) change in EPSP amplitude. In addition, during periods when LTP was not observed, the reliability of synapses demonstrating any substantial change in amplitude also declined (both plastic and non-plastic synapses were included in the percentage change in AP EPSP analysis). These results indicate that periods of reduced LTP were not the result of increased variability in the level of synaptic change, but instead reflect a period in which synapses consistently exhibited decreased plasticity following presynaptic/postsynaptic pairing. The two notable exceptions to this pattern are in experiments conducted during November and December, in which the reliability of synapses undergoing a >20% change in amplitude was relatively high, but no potentiation was observed. In November, 8 out of 10 synapses tested exhibited >20% change; however, this group consisted of both

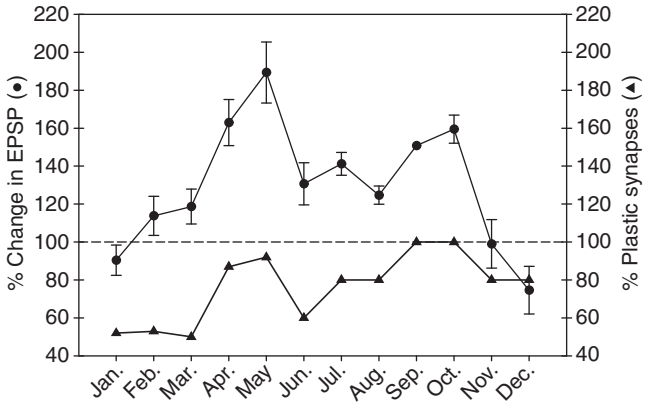

Fig. 1. Seasonal variation of pairing-induced long-term potentiation (LTP). Experimental data were normalized to the corresponding pre-test EPSP, grouped by month, and presented as means ± s.e. following presynaptic/postsynaptic pairing (filled circles). LTP was observed in the April–October period. The results for the percentage change in EPSP groups include all experiments (both plastic and non-plastic synapses) conducted in that month, only excluding experiments in which the post-test input resistance (R_{input}) changed more than 30% from the pretest R_{input}. The percentage of synapses exhibiting >20% change (filled triangles) fluctuates in a seasonal manner in parallel with the level of LTP. The number of pairing experiments recorded per month is as follows: January N=23, February N=15, March N=20, April N=15, May N=12, June N=17, July N=5, August N=5, September N=1, October N=2, November N=10, December N=10.

potentiated and depressed synapses (3 potentiated, 5 depressed), yielding a mean of no potentiation for that period. In December, the reliability of synapses undergoing >20% change in EPSP amplitude was also high but, as previously mentioned, these synapses reliably depressed despite undergoing a pairing protocol that normally elicits LTP.

In many synapses, including those in the leech, the capacity to undergo LTP is inversely proportional to the initial amplitude of the EPSP; that is, large EPSP synapses tend to undergo little or no potentiation or even undergo depression (Bi and Poo, 1998; Montgomery et al., 2001; Burrell and Sahley, 2004; Grey and Burrell, 2010). The mean size of the P-to-AP EPSP from November to March (4.5±0.2 mV) was significantly larger than EPSPs tested from April to October (3.3±0.2 mV; *t*-test=4.94, d.f.=77, $P<0.0001$). However, the greater size of the EPSP in the November–March synapses does not appear to play a significant role in their ability to undergo LTP. First, April–October synapses exhibited a negative correlation between initial EPSP amplitude and the percentage change in EPSP size after pairing (Fig. 2A; $r^2=0.15$, $F=5.71$, $P<0.05$), but no such correlation was observed in synapses tested in November–March ($r^2=0.008$, $F=0.32$, $P>0.05$). Second, April–October synapses with higher initial EPSPs still exhibited significant potentiation when compared with November–March synapses of a similar size. This was confirmed when the level of potentiation between the two seasonal groups was compared in synapses arbitrarily divided into those <4 mV and those >4 mV (Fig. 2B). The April–October synapses exhibited significant LTP compared with the November–March synapses in both the <4 mV (*t*-test=5.2, d.f.=39, $P<0.0001$) and >4 mV groups (*t*-test=4.3, d.f.=36, $P<0.001$).

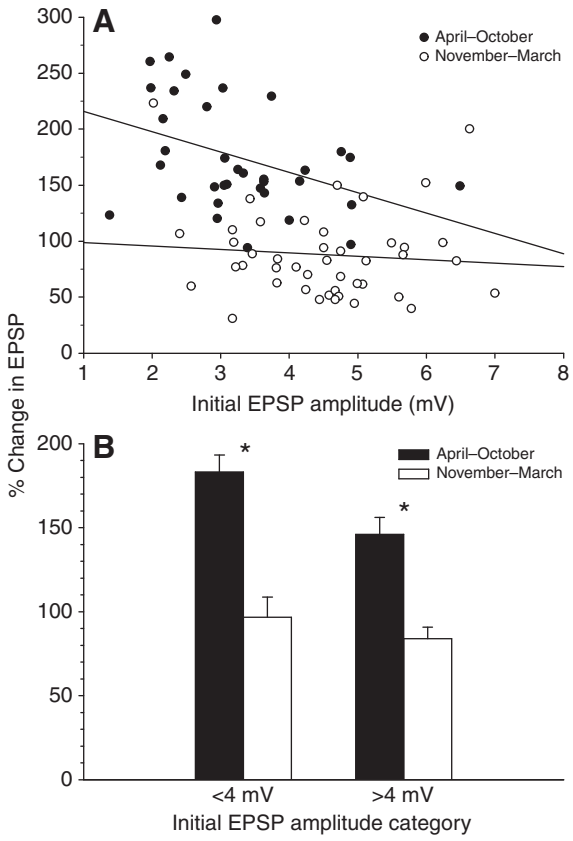

Fig. 2. Relationship between the EPSP amplitude prior to pairing and LTP. (A) In synapses tested between April and October, there was a negative correlation between the initial EPSP amplitude and the level of LTP. No such correlation was observed in synapses tested between November and March. (B) When P cell to AP cell (P-to-AP) EPSPs were divided into 'small' (<4 mV) and 'large' (>4 mV) groups, LTP was observed in both the <4 mV and >4 mV groups in synapses tested during April–October while no LTP was observed in either group in synapses tested in November–March. *Statistically significant difference between April–October and November–March synapses.

The leeches used in this study were reared in a controlled environment by the suppliers, fasted for approximately 5 months and then shipped to the laboratory. As a leech's feeding state significantly influences the osmolality of the hemolymph (Zerbst-Boroffka, 1973) and osmolality has been shown to affect input resistance in leech Retzuis neurons (Coulon et al., 2008), it is possible that the AP cell's input resistance (R_{input}), and thus excitability, was dependent on the supplier's feeding schedule and not due to seasonal differences in LTP. In order to investigate this possibility, the percentage change in P-to-AP EPSP was plotted as a function of the number of days the animals spent in the laboratory. As shown in Fig. 3A, we analyzed the interval between shipment arrival and percentage change in EPSP by linear regression and found no effect on the change in EPSP amplitude ($F_{1,137}=0.09$; $R^2=0.001$; $P<0.77$). These results suggest that, for the animals used in these experiments, differences in feeding state or other factors related to how long the animals had been in the laboratory did not affect the capacity for LTP.

We also examined the relationship between initial postsynaptic (AP cell) R_{input} and changes in EPSP amplitude. There was a weak, but statistically significant correlation between the AP cell R_{input} and the percentage change in EPSP amplitude ($F_{1,137}=3.99$; $R^2=0.03$; $P=0.05$; Fig. 3B). Although postsynaptic R_{input} may have had some small influence on the level of LTP, the magnitude of the effect did not appear to be enough to determine whether LTP was elicited. In addition, there was no obvious seasonal variation in postsynaptic R_{input} (Fig. 3C). These findings indicate that the observed changes in P-to-AP LTP were not the result of variation in postsynaptic R_{input}.

In the P-to-AP synapse, pairing-induced LTP requires that activity in the P cell and in the AP cell occurs simultaneously (0 ms interstimulus interval, ISI), similar to what is observed during LTP in *Aplysia* (Lin and Glanzman, 1997). However, it is possible to elicit LTD in the P-to-AP synapse by stimulating the postsynaptic AP cell 1 s prior to the P cell (–1 s ISI) (Grey and Burrell, 2010). Given the variation observed with pairing-dependent LTP, the effects of altering the presynaptic and postsynaptic ISI were examined during the months in which LTP was absent (December–March). As with LTP, LTD normally observed following the –1 s ISI protocol was absent in synapses tested during December–March (Fig. 4; *t*-test=5.87, $P<0.0001$, d.f.=14). The effects of –500 ms, +500 ms and +1 s ISI protocols were also tested during this period. These pairing protocols normally do not elicit LTP or LTD and this was also observed in synapses tested during December–March (Fig. 4).

It is also possible to elicit LTP 'chemically' in both mammalian and leech synapses *via* bath application of the adenylyl cyclase activator forskolin (Otmakhov et al., 2004; Grey and Burrell, 2008). Application of 50 μmol l^{-1} forskolin plus 0.1 μmol l^{-1} rolipram (a phosphodiesterase inhibitor) in Mg^{2+}-free saline for 15 min has previously been shown to elicit persistent and robust LTP (164±15%) in the leech in experiments carried out between May and October (Grey and Burrell, 2008). These experiments were repeated during the months of December–February, and here we

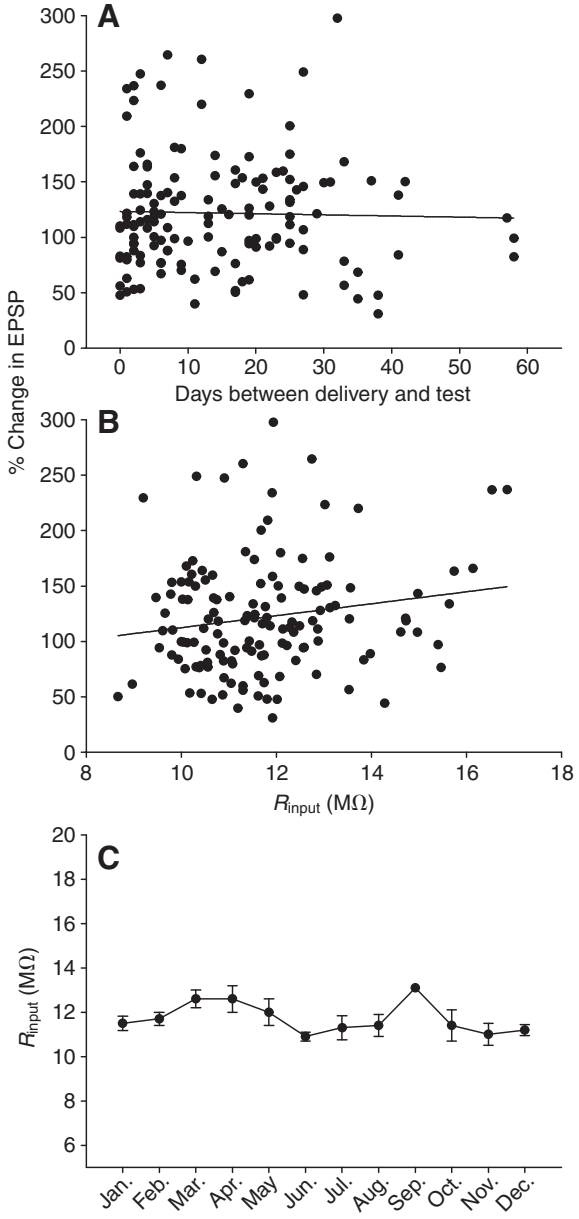

Fig. 3. Number of days that animals spent in the laboratory and input resistance do not account for variation in LTP. (A) The percentage change in EPSP for each experiment was plotted in relation to the number of days from delivery of the leeches to the lab to when the experiments were conducted. No correlation was observed between these two factors. (B) The percentage change in EPSP for each experiment was plotted in relation to the postsynaptic input resistance (R_{input}). Although there was a small effect of R_{input} on the level of LTP there was no obvious seasonal variation in R_{input}. (C) R_{input} plotted by month. The R_{input} measurements corresponding to EPSP measurements presented in Fig. 1A were averaged by month and are presented means ± s.e. There is no obvious seasonal variation in AP cell R_{input}.

report forskolin failed to elicit potentiation during this period (Fig. 5; 89±10%; *t*-test=4.07, $P<0.001$, d.f.=16). In synapses tested between December and February, there was no difference between those treated with forskolin and the untreated vehicle controls (n.s., $P>0.05$). This chemical LTP result parallels the activity-induced LTP findings and suggests that the capacity for NMDAR-dependent LTP is fundamentally altered.

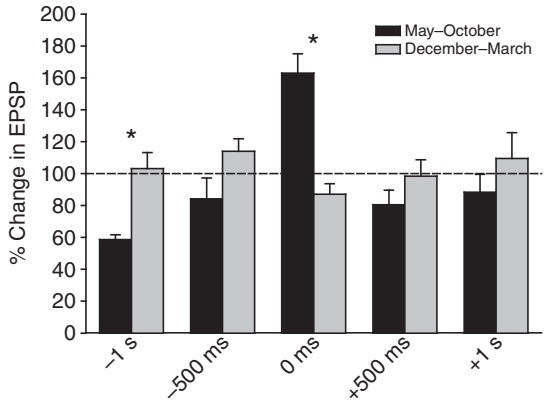

Fig. 4. Alteration of the temporal order of paired P and AP cell activation does not produce bidirectional plasticity during December–March. Shifting the relative onset between presynaptic and postsynaptic stimulation in December–March does not produce bidirectional plasticity, as seen in the months of May–October. Specifically, the two windows of plasticity normally observed during May–October, one at the 0 ms interstimulus internal (ISI) time point that resulted in LTP and a second at the –1 s ISI that resulted in LTD, are absent during December–March. –1 s: 101±7%, *N*=7; –500 ms: 114±8%, *N*=4; +500 ms: 98±10%, *N*=11; +1 s: 97±11%, *N*=7). *Statistically significant difference between May–October and December–March synapses.

DISCUSSION

In this study we have shown that NMDAR-dependent LTP at the leech P-to-AP glutamatergic synapse undergoes seasonal variation consistent with a circannual rhythm. LTP was observed in April–October (spring to autumn), while no potentiation was observed in synapses tested during November–March. In addition, application of the adenylyl cyclase activator forskolin, which normally elicits chemical LTP (Otmakhov et al., 2004; Grey and Burrell, 2008), failed to elicit potentiation during the December–February period. Finally, NMDAR-dependent LTD elicited by negative pairing (postsynaptic before presynaptic) was also absent during December–March. The changes in capacity for LTP were not related to the initial size of the EPSP, the postsynaptic R_{input} or the time since the leech was last fed.

Circadian cycles modulate LTP, possibly *via* melatonin, which is secreted in a circadian pattern and contributes to the regulation of daily rhythms for a number of physiological functions in mammals (Chaudhury et al., 2005; Wang et al., 2005; Ozcan et al., 2006). A recent study has shown that changes in photoperiod can also modulate LTP and learning in mice (Walton et al., 2011). Specifically, these authors found that short day length led to decreases in the level of LTP and learning performance. It is not known how seasonal/photoperiod changes alter LTP, although melatonin levels can reflect day length (Bartness et al., 1993) and melatonin has been shown to inhibit LTP (Fukunaga et al., 2002; Wang et al., 2005; Ozcan et al., 2006). Melatonin is present in invertebrates and appears to regulate activity patterns (Hardeland and Poeggeler, 2003; Tanaka et al., 2007); however, no studies on the presence or effects of melatonin in the leech have been carried out to date. A study performed in hamsters showed that LTP increases during hibernation (Spangenberger et al., 1995) and it is likely that the seasonal influences on neural plasticity depend on whether the animal enters a distinct hibernation state or goes through seasonally regulated periods of reduced activity.

It is unclear what mediates the seasonal effects of LTP in the leech P-to-AP synapse. The fact that both LTP and LTD are impaired

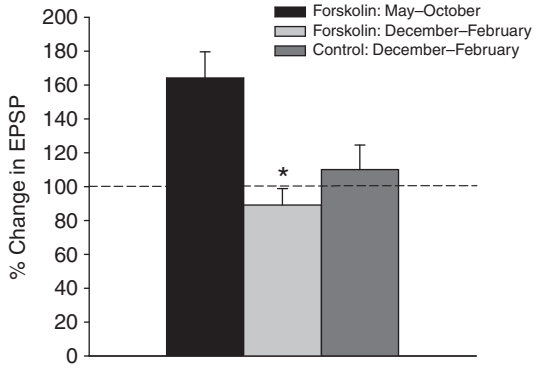

Fig. 5. Forskolin-induced 'chemical' LTP also exhibits seasonal variation. In synapses tested between May and October, LTP was elicited by forskolin, an adenylyl cyclase activator. However, no forskolin-induced LTP was observed in synapses tested during the months of December to February. *Statistically significant difference from May–October forskolin-treated synapses.

during the winter months and that both forms of synaptic plasticity are NMDAR dependent (Grey et al., 2009; Grey and Burrell, 2010) may indicate that NMDAR levels are reduced during the winter. Walton and colleagues have suggested a similar mechanism to explain the effects of photoperiod on hippocampal LTP (Walton et al., 2011). It is also possible that downstream protein kinases or phosphatases are altered by these seasonal changes (Fukunaga et al., 2002). The initial P-to-AP EPSP was significantly larger during November–March than during April–October, and a number of studies have noted that large EPSPs have a reduced capacity to undergo LTP (Bi and Poo, 1998; Montgomery et al., 2001; Burrell and Sahley, 2004). Perhaps the larger EPSP size during the winter is responsible for the impaired LTP; this, however, did not appear to be the case for our experiments. Although there was a significant negative correlation between initial EPSP amplitude and level of LTP in April–October synapses, significant potentiation still occurred regardless of the initial size of the synapse. Furthermore, no correlation between initial synapse size and LTP level was observed in the November–March synapses, indicating that these synapses were unable to undergo LTP regardless of their initial size. Finally, even when synapses from both seasonal periods were grouped into smaller (<4 mV) and larger (>4 mV) subgroups, LTP was still observed in the April–October synapses regardless of initial EPSP size and was not observed in the November–March synapses.

The natural range of *H. verbana* is in the temperate zone of the northern hemisphere; specifically, throughout Europe and western Asia (Sawyer, 1986). As ectothermic animals, leeches are likely to have periods of inactivity during periods of low temperature. Although a detailed analysis of *Hirudo* circannual behaviors in their natural environment is lacking, it is known that feeding in preparation for reproduction and deposition of reproductive cocoons occurs mainly between May and August, although this period can last longer at lower latitudes (Sawyer, 1986). During the winter, many freshwater leeches are found buried in the mud and are presumably quiescent during this time (Blair, 1927; Sawyer, 1986). *Hirudo* also exhibits a diurnal activity rhythm (Lotz et al., 1970). Seasonal variation has been observed in a number of behavioral and neurophysiological processes in *Hirudo*, including non-associative learning, serotonin content in the CNS, activity-dependent modulation of afterhyperpolarization and acetylcholine-elicited currents in the serotonergic Retzius cells (Stenzel and Neuhoff, 1976; Catarsi et al., 1990; Szczupak et al., 1993; Scuri et al., 2002).

It is remarkable that such seasonal variation continues despite the fact that these animals were maintained on a constant 12 h:12 h light:dark cycle in the laboratory. During the periods of both intact and impaired LTP, all leeches were obtained from two commercial suppliers (Leeches USA and Niagara Leeches) that rear the animals in a controlled environment with a constant temperature and light:dark cycle (specific details are unavailable from the suppliers). Therefore, it is difficult to hypothesize how a seasonal pattern could be established even though it is consistent with other behavioral and physiological seasonal patterns discussed above. Although the details of what controls leech circannual behavior are not known, one component that may be involved is the neurotransmitter serotonin, given that leech CNS serotonin levels appear to be higher during December–March and lower during April–May (Stenzel and Neuhoff, 1976; Catarsi et al., 1990). Melatonin may also play a role in this process, although no studies concerning the presence or effects of melatonin in the leech CNS have been reported.

Interestingly, although our laboratory has studied NMDAR-dependent and -independent forms of LTP and LTD in other synapses in the leech, some of which were carried out during the same time period as the present P-to-AP study, we have observed no obvious seasonal variation in those connections. It is not clear whether this variation is due to differences intrinsic to the different synapses, differences in the stimulation protocols used to induce LTP/LTD, or both. Our studies in other synapses used either tetanic stimulation or low frequency stimulation to induce LTP and LTD, respectively (Burrell and Sahley, 2004; Burrell and Li, 2008; Li and Burrell, 2009; Yuan and Burrell, 2010), or used more pairings of presynaptic and postsynaptic activity than utilized in the current study [5 in the present study *versus* 10 pairings in another recent study (Li and Burrell, 2011)]. It is possible that these other studies stimulated the synapses in a strong enough manner to overcome any seasonal influences on LTP or LTD.

Circannual rhythms in behavior are complex processes that allow an organism to efficiently utilize favorable conditions and avoid conditions that are unfavorable (Bradshaw and Holzapfel, 2007). The finding that NMDAR-dependent LTP and LTD in the leech may exhibit a circannual rhythm provides a potentially useful experimental system in which to study the interaction between environmental/internal signals that regulate such periodicity with cellular processes of activity-dependent neuroplasticity. Furthermore, given that LTP and LTD are thought to contribute to learning and memory, it may be possible to examine at the cellular level how seasonal factors regulate learning.

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