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Inhibiting cGMP and elevating NO levels do not affect post-tetanic potentiation at the crayfish neuromuscular junction

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ABSTRACT

Nitric oxide (NO) acts as a retrograde messenger in the central nervous system, and previous research has found it to both depress and facilitate synaptic transmission in synapses. However, this discrepancy leaves the role of NO unclear, specifically in the crayfish neuromuscular junction (NMJ). In an attempt to clarify NO's role, we studied its effect on synaptic plasticity, the strengthening or weakening of synapses over time based on their reactivity, in the crayfish NMJ. Additionally, we studied the role of cGMP, an element of the NO-cGMP pathway, on synaptic plasticity. We applied ODQ, a cGMP inhibitor, sodium nitroprusside (SNP), an NO donor, and a combination of the two drugs to determine whether or not NO has a role in synaptic plasticity independent of the NO-cGMP pathway. We hypothesized that ODQ, would decrease percent change in excitatory postsynaptic potentials (EPSPs) in long-term facilitation (LTF) or long-term depression (LTD), while increasing NO concentration would increase percent change in EPSPs in LTF or LTD, and increasing NO levels while inhibiting cGMP would decrease percent change in EPSPs in LTF or LTD. We compared the control conditions with the experimental conditions and found no significant difference in the percent change of EPSPs in any of the comparisons. Therefore, we cannot confirm the role of the NO-cGMP in synaptic plasticity.

INTRODUCTION

Research has shown nitric oxide (NO) to play an important role in a broad array of physiological functions, including human organ systems, senses of pain and smell, neuromuscular development, and neurotransmission (Miclescu and Gordth 2009). Expanding on its role in synaptic transmission, NO has been shown to be an intricate part of spatial learning and spatial memory (Hölscher 1997).

Regarding its role in synaptic transmission, NO possesses unique qualities making it especially well-suited to serve as a signaling agent. Because it is small and carries no charge, NO can freely diffuse across cell membranes rather than relying on active transport proteins. NO is a retrograde messenger, produced in the postsynaptic neuron, and ultimately modulates neurotransmitter release in the presynaptic neuron (Arancio et al. 2000). Due to its relative uncreactivity, NO is able to complete the journey to the presynaptic neuron without being destroyed (Denninger and Marletta 1999).

NO influences synaptic transmission through the mechanism known as the NO-cGMP pathway (Arancio et al. 1996, Aonuma et al. 1999). First, nitric oxide synthase in the postsynaptic neuron generates NO from L-arginine. NO then diffuses out of the postsynaptic neuron and travels to the presynaptic neuron where it targets guanylyl cyclase which in turn produces a second signal, cGMP. cGMP targets PKG, somehow modulating the concentration of intracellular Ca^{2+}. The change in intracellular Ca^{2+} concentration affects neurotransmitter release, giving rise to different modes of synaptic plasticity.

Arancio et al. (1996) described the mechanisms of the NO-cGMP pathway in cultured hippocampal neurons, demonstrating its existence in vertebrates, but less is known about the NO-cGMP pathway and its role in invertebrates. Aonuma and his colleagues (Aonuma et al. 1999, Aonuma et al. 2000, Aonuma and Newlan 2001) conducted a series of experiments investigating different aspects of the NO-cGMP pathway within crayfish neurons. They first established that L-arginine modulates synaptic transmission and plays a role in plasticity, and since L-arginine is a precursor to NO, they hypothesized that NO would modulate neurotransmission in the same way (1999). In 2000, they conducted a follow-up experiment, to test this hypothesis, finding that NO did in fact modulate neurotransmission. Then in 2001, Aonuma and Newlan found cGMP has a similar effect on transmission, supporting the existence of a NO-cGMP pathway in crayfish neurons.

The goal of our study is to extend research supporting the existence of a NO-cGMP pathway in crayfish neurons and clarify NO's role in synaptic transmission in invertebrates. While Aonuma and his colleagues (Aonuma et al. 1999, Aonuma et al. 2000, Aonuma and Newlan 2001) demonstrated the effects of components of the NO-cGMP pathway on plasticity, their stimulation at 1 Hz to elicit depression differs from the method of tetanic stimulation Arancio et al. (1996) used

in rat hippocampal neurons. Hwang et al. (2014) found that tetanic stimulation at 50 Hz resulted in long-term depression (LTD) while Hochstein (2009) found that stimulation at 50 Hz resulted in long-term facilitation (LTF). A short-term mode of synaptic plasticity called post-tetanic potentiation (PTP) is also evoked by tetanic stimulation. PTP refers to a short lived increase in EPSP amplitude resulting from tetanic stimulation (10 Hz to 200 Hz) for a prolonged period of time (200 milliseconds to 5 seconds) (Malenka 2002). Using plasticity elicited by tetanic stimulation at 50 Hz, we hope to further support the roles of NO and cGMP in plasticity and further support the existence of the NO-cGMP pathway in crayfish neurons.

Also, while Aonuma and Newlan (2001) demonstrated that NO and cGMP have similar effects on neurotransmission and plasticity, they did not investigate how NO modulates transmission independent of NO-cGMP pathway. We explored whether NO would still modulate neurotransmission if cGMP production is inhibited. We also considered the possibility of alternative pathways, independent of the NO-cGMP pathway, NO may take in order to modulate neurotransmission. We hypothesized that by inhibiting cGMP with ODQ, the percent change of EPSPs will decrease in either LTF or LTD, by increasing NO levels with sodium nitroprusside (SNP), the percent change in EPSPs will increase in either LTF or LTD, and by increasing NO levels while inhibiting cGMP, the percent change in EPSPs will decrease in either LTF or LTD. However, our findings did not confirm our hypotheses. There were no significant differences in the percent change of EPSPs between the standard control condition and elevated NO levels condition, no significant differences between the DMSO control condition and the inhibited cGMP condition, and no significant difference between the DMSO control condition and the inhibited cGMP in the presence of elevated NO levels condition.

MATERIALS AND METHODS

Crayfish Tail Preparation

We used the crayfish genus, *orconectes*, for this study, and it was anaesthetized in an ice bath before dissection. The abdominal region of the crayfish was dissected by cutting along the left and right side of the abdomen towards the distal end of the tail. The exoskeleton was removed, followed by the removal of the ventral muscles and the digestive tract to expose the deep extensor muscles and nerve connections. After dissection, we submerged the crayfish in the solution and pinned it down to Sylgard in a petri dish for data collection. We performed our experiments at room temperature.

Solutions

To preserve the integrity of the crayfish, we used a crayfish Ringer's solution consisting of 5.4mM KCl, 196mM NaCl, 2.6mM $MgCl_2$, 10mM HEPES, and 6mM $CaCl_2$ with a pH of 7.4. To prepare the 100 μM concentration of ODQ solution, we used a micropipette (Pipetman, Gilson Inc.) to measure and obtain 50μl of 50mM ODQ from aliquots stored in a fridge. This ODQ solution was diluted with 25ml crayfish ringer solution in a capped bottle (250ml, PYREX, Corning Inc.). The ODQ drug concentration was chosen based on Hall et al. (2010) experiment. To prepare the 100μM Sodium nitroprusside solution, we obtained 50μl of 100mM Sodium nitroprusside from an aliquot and diluted it with 50ml crayfish ringer solution. To prepare the 100μM ODQ with 100μM Sodium nitroprusside solution, 50μl ODQ and 25μl Sodium nitroprusside were mixed with 25ml ringer solution. To prepare the DMSO control solution, 50μl of DMSO was added to 25ml of crayfish solution. Under our experimental conditions, the crayfish was submerged in the appropriate solution to prepare for data collection.

Microelectrode Preparation

With a PUL-1 microelectrode puller by World Precisions Instruments, we pulled microelectrodes from borosilicate glass capillary tubes, 1.2 millimeters in diameter. We filled the recording electrode with 3M KCl and we made a suction electrode by grinding down the tip with sandpaper so that it was large enough to suck a crayfish nerve bundle. After we put the reference electrodes into the crayfish solution and connected the electrodes to their respective electrode holders (the blunted electrode connected to a syringe), we checked the resistance of the recording electrode tip to be >13MΩ. The crayfish muscle was observed under the microscope Leica Zoom 2000.

Data Collection

We used 12 crayfish and stimulated nerves to achieve a postsynaptic response, EPSP. We stimulated the nerve using the Grass Instruments SD9 Stimulator by inserting a microelectrode into a crayfish muscle tissue yielding a resting membrane potential between -60mV and -80mV. We used LabChart to record the crayfish's resting membrane potential and used Analog Digital Instruments PowerLab 4/25 to measure the resulting EPSPs. We used a syringe to suck a nerve into the blunted electrode. In each experiment, we stimulated at a repeated frequency of 0.5 Hz for 1 minute through the reference electrode which yielded a baseline of 30 EPSP measurements. The voltage varied between each trial to reach threshold and achieve an EPSP without making the muscle twitch. Then after collecting these baseline

measurements, we stimulated the nerve at a high frequency of 50 Hz for 10 seconds to stimulate synaptic plasticity and record the resulting EPSPs amplitudes at 0.5 Hz for 8 minutes as done in Hochstein's (2009) and Hwang and colleagues' (2014) studies. This procedure was applied to each experimental condition.

Data Analysis

To take into consideration variation across individual crayfish cells, we normalized all the EPSP amplitudes to the baseline. To do this we averaged the 30 baseline measurements taken in the first minute at 0.5 Hz. We normalized the EPSP amplitudes relative to the baseline average to find percent change by using the equation: Percent change = $[(EPSP - EPSP_{baseline}) / EPSP_{baseline})] \times 100$ (Badhwar et al. 2006).

For post-tetanic potentiation (PTP), a short-term plasticity, we quantified the strength of plasticity by averaging the first 6 EPSP amplitudes measured, or the first 12 seconds, after tetanic stimulation ended. We took the mean of the first few seconds after potentiation because the first measurement alone is not necessarily indicative of the strength of potentiation, since EPSPs decay quickly after stimulation ends. The strength of PTP was compared across different experimental conditions with a Student's T-test.

For long-term plasticity, we hoped to quantify the strength of plasticity by averaging the 30 EPSP amplitudes measured within the 8th minute after stimulation. However, since we found both long-term depression and long-term facilitation in our experimental trials, sometimes within the same experimental condition, we ultimately only investigated the role of the NO-cGMP pathway on short-term plasticity.

DMSO, the solvent of ODQ, may affect plasticity instead of or in addition to ODQ, so data from experiments involving ODQ were compared to the DMSO control data, while the data from the SNP condition were compared to the ringer control condition. In summary, the ODQ data was compared to DMSO control data, ODQ + SNP data was compared to DMSO control data, and SNP data was compared to ringer solution control data.

RESULTS

We investigated the influence of the NO-cGMP pathway on synaptic plasticity in the crayfish neuromuscular junction (NMJ) by elevating nitric oxide (NO) levels with sodium nitroprusside (SNP) and by inhibiting cGMP formation with the addition of ODQ to the crayfish ringer solution. Using intracellular recording, we measured EPSP amplitudes for five conditions (control with 0 µM ODQ, 0 µM SNP; DMSO control with 25 µM DMSO; 100 µM SNP; 100 µM ODQ; and 100 µM SNP with 100 µM ODQ) for one minute before and for eight minutes after applying a tetanic stimulation at 50 Hz for 10 seconds.

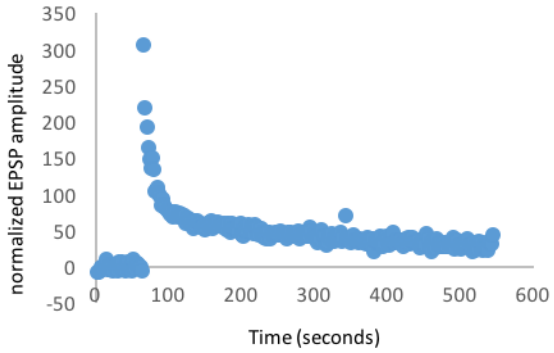

Figure 1. The first 60 seconds represent the baseline of EPSPs before tetanus. Tetanic stimulation (50Hz, 10s) at 60 seconds results in post-tetanic potentiation.

Tetanic stimulation at 50 Hz for 10 seconds results in post-tetanic potentiation (PTP)

Unlike previous *Pioneering Neuroscience* journals using tetanic stimulation at 50 Hz for 10 seconds, we observed that tetanic stimulation led to PTP. As seen in Figure 1, tetanic stimulation increases EPSP magnitudes, and once stimulation ceases, EPSPs decay into long-term plasticity, in the case of Figure 1, long-term facilitation (LTF).

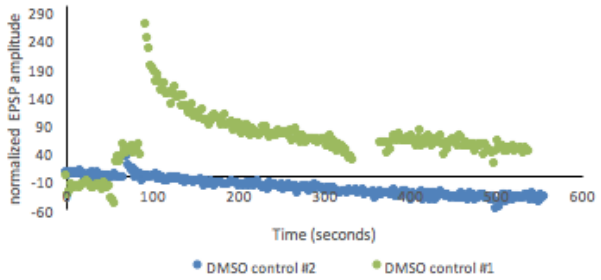

Figure 2. Two trials of the DMSO control condition. The first 60 seconds represent the baseline of EPSPs before the tetanic stimulation (50Hz, 10s). Both trials show the post-tetanic potentiation after tetanus, but DMSO control #1 shows LTF, while DMSO control #2 shows LTD.

Tetanic stimulation at 50 Hz for 10 seconds does not result in a definite long-term plasticity

While Figure 1 shows LTF, we also observed long-term depression (LTD) after stimulation as seen in Figure 2. Long term plasticity did not correlate with the experimental condition and could differ within the same experimental condition. For example, in the first trial of our DMSO condition we observed LTF with a mean

percent change in EPSP amplitudes of +46% in the eighth minute after stimulation. In the second trial of our DMSO condition, we observed LTD with a mean percent change in EPSP amplitudes of -40.6%. The variance in long-term plasticities led us to analyze the effect of the NO-cGMP pathway on strength of PTP, a short-term plasticity all experimental trials exhibited, rather than LTF or LTD.

Elevating NO levels has an indeterminate effect on PTP.

In our control condition, EPSPs increased 50.2% (SE=14.8, n=3). Whereas in the 100 µM SNP condition, EPSPs increased 44.0% (SE=10.0, n=2) (Fig. 3). We conducted a Student's t-test to analyze the difference in percent change of EPSPs between the control condition and 100 µM SNP condition for the first twelve seconds after tetanic stimulation. The mean percent change in EPSP in the control condition was not significantly different from the 100 µM SNP condition ($p > 0.05$). Thus we are unable to conclude that elevating NO levels increases synaptic plasticity in the crayfish NMJ.

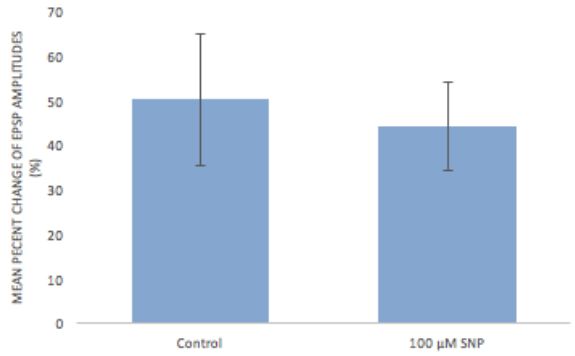

Figure 3. Mean percent change in EPSP amplitudes in the 12 seconds following tetanic stimulation (50Hz, 10 s) for the control condition and the SNP (100 µM) condition. There was no significant difference between the two conditions. $p = 0.75 > 0.05$. Error bars represent the standard error of the mean. n = 3 for the control and n = 2 for SNP.

Inhibiting cGMP has an indeterminate effect on PTP

In our DMSO control condition, EPSPs increased 125.1% (SE=111.5, n=2), whereas in the 100 µM ODQ condition, EPSPs increased 108.6% (SE=85.8, n=2) (Fig. 4). We analyzed our data in the same manner as before. The mean percent change in EPSP in the control condition was not significantly different from the 100 µM ODQ condition ($p > 0.05$). Thus we are unable to conclude that inhibiting cGMP inhibits synaptic plasticity in the crayfish NMJ.

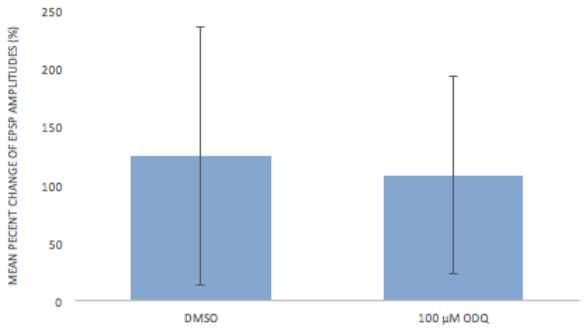

Figure 4. Mean percent change in EPSP amplitudes in the 12 seconds following tetanic stimulation (50 Hz, 10s) for the DMSO condition (100 µM) and the ODQ condition (100 µM). There was no significant difference between the two conditions. $p = 0.9 > 0.05$. Error bars represent the standard error of the mean, n=2 for two conditions.

Elevating NO levels and inhibiting cGMP together has an indeterminate effect on PTP

In our DMSO control condition, EPSPs increased 125.1% (SE=111.5, n=2), whereas in our SNP with ODQ condition, EPSPs increased 97.2% (SE=18.1, n=2) (Fig. 5). We analyzed our data in the same manner as before. The mean percent change in EPSP in the DMSO control condition was not significantly different from the 100 µM ODQ + 100 µM SNP condition ($p > 0.05$). Thus we are unable to conclude that inhibiting cGMP overpowers elevated NO levels to inhibit plasticity.

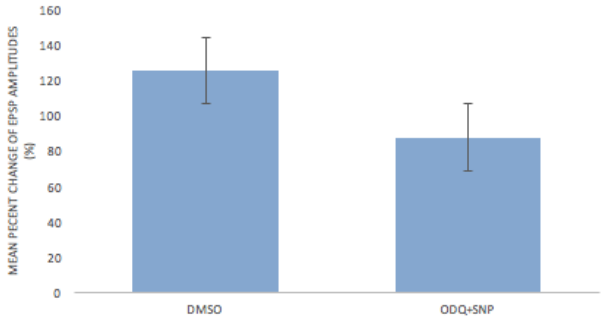

Figure 5. Mean percent change in EPSP amplitudes in 12 seconds following tetanic stimulation (50 Hz, 10s) for the DMSO control condition (100 µM) and the ODQ (100 µM) + SNP (100 µM) condition. There was no significant difference between these two conditions. $p = 0.79 > 0.05$. Error bars represent the standard error of the mean. n = 2 for two conditions.

DISCUSSION

To determine the role of the NO-cGMP pathway in synaptic plasticity at the crayfish neuromuscular junction (NMJ), we applied the nitric oxide (NO) donor sodium nitroprusside (SNP) and the cGMP inhibitor ODQ to the extracellular solution of crayfish muscles cells independently and together. Ultimately, we found no significant difference in the strength of plasticity between

our experimental and control conditions. Contrary to our hypotheses, elevating NO levels with SNP did not significantly increase plasticity, inhibiting cGMP with ODQ did not significantly decrease plasticity, and inhibiting cGMP did not overpower elevated NO to significantly decrease plasticity.

Although Hwang et al. (2014) and Hochstein (2009) both used tetanic stimulation at 50 Hz for 10 seconds, they found different types of long-term plasticity. Hwang et al. found long-term depression (LTD) while Hochstein found long-term facilitation (LTF). A secondary objective of our research was to resolve this discrepancy, but curiously, we found both LTD and LTF after tetanic stimulation, even within the same experimental condition. Tetanic stimulation reliably leads to potentiation in rat hippocampal neurons (Arancio 1996), but in the crayfish NMJ, long-term plasticity appears to be sensitive to small changes that biology students have little control over.

Although the long-term plasticity we observed varied, we consistently observed post-tetanic potentiation (PTP). PTP refers to a short lived increase in EPSP amplitude resulting from tetanic stimulation (10 Hz to 200 Hz) for a prolonged period of time (200 milliseconds to 5 seconds), consistent with both our methods and our findings (Malenka 2002). Mulkeynand Zucker (1992) found that PTP depends on the concentration of intracellular Ca^{2+}, and Arancio et al. (1996) found that NO prompts cGMP production, modulating the concentration of intracellular Ca^{2+}. Thus, it would be reasonable for the NO-cGMP pathway to play an important role in facilitating PTP, similar to how we originally expected the NO-cGMP pathway to play an integral role in LTF and LTD.

Although our experimental conditions did not make significant impacts on synaptic plasticity, our results do not strongly support that the NO-cGMP pathway does not play a role in plasticity in the crayfish NMJ. In all, only 11 experimental trials were conducted, and each experimental condition had a maximum of 3 trials. This small sample size offers a low statistical power and has a reduced capability of predicting a true effect. Even if a statistically significant difference was found, it would still not be strongly supported and may be discredited with additional experimentation. In our experiments, the standard error for the ODQ condition was 85.82 indicating variation across trials leading to problems in statistical analysis. One of the ODQ experimental trials exhibited stronger PTP than the DMSO control, while another exhibited a much weaker PTP than the DMSO control. Because of our small sample size, we are not confident in how ODQ affects PTP at all. More experimental trials would better indicate whether or not our experimental conditions affect synaptic plasticity.

Future research with SNP and ODQ conditions is needed to better determine the role of the NO-cGMP pathway on plasticity. More trials are necessary to determine whether or not affecting the NO-cGMP pathway can cause a significant difference in the strength of neurotransmission. Additionally, more research should be done with tetanic stimulation at 50 Hz for 10 seconds. We found LTF and LTD, Hochstein (2009) found LTF and, Hwang et al. (2014) found LTD after tetanic stimulation. Future research could investigate why LTF occurs in some instances but LTD occurs in others. More information regarding the sensitivity of long-term plasticity would make tetanic stimulation at 50 Hz for 10 seconds a more dependable method to elicit long-term plasticity. Ultimately, NO-based studies are important because NO plays an integral role in synaptic plasticity and also has physiological functions such as spatial learning and spatial memory and its role as a retrograde messenger should be continued to be explored.

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REFERENCES

Aonuma, H., T. Nagao, T. Nagayama, and M. Takahata. 1999. Modulatory effects of amino acids on neuromuscular transmission on the crayfish fast flexor muscle. *Journal of Experimental Zoology* 283: 531-540.

Aonuma, H., T. Nagayama, and M. Takahata. 2000. Modulatory effects of nitric oxide on synaptic depression in the crayfish neuromuscular system. *The Journal of Experimental Biology* 203, 3595–3602.

Aonuma, H., and P.L. Newlan. 2001. Opposing actions of nitric oxide on synaptic inputs of identified interneurones in the central nervous system of the crayfish. *The Journal of Experimental Biology* 204: 1319-1332.

Arancio, O., M. Kiebler, C.J. Lee, V. Lev-Ram, R.Y. Tsien, et al. 1996. Nitric oxide acts directly in the presynaptic neuron to produce long-term potentiation in cultured hippocampal neurons. *Cell*. 87: 1025–1035.

Badhwar, A., A. Weston, J. B. Murray, and A. J. Mercier. 2006. A role of cyclic nucleotide monophosphates in synaptic modulation by a crayfish neuropeptide. *Peptides* 27: 1281-1290.

Bon, C.L.M., and J Garthwaite. 2001. Exogenous nitric oxide causes hippocampal synaptic transmission during low-frequency stimulation via the endogenous nitric oxide-cGMP pathway. *European Journal of Neuroscience*. 14: 585-594.

Denninger., J.W., and M.A, Marletta. 1999. Guanylate cyclase and the NO/cGMP signaling pathway. *Biochimica et Biophysica Acta*. 1411: 334-350.

Hall, P., C. Brooks, and C. Kaiser-Nyman. 2010. Octopamine operates through the cGMP pathway at the crayfish neuromuscular junction. *Pioneering Neuroscience*. 11: 13-16.

Hochstein, M. 2008. Nitric oxide inhibition shortens long-term facilitation at the neuromuscular junction of the crayfish. *Pioneering Neuroscience*. 9: 13-16.

Hölscher, C. 1997. Nitric oxide, the enigmatic neuronal messenger: Its role in synaptic plasticity. *Trends in Neurosciences* 20(7): 298-303.

Hwang, H., T. Omura, and B. Clarke. 2014. Nitric oxide synthase inhibitor L-NAME decreases long-term depression at the crayfish neuromuscular junction. *Pioneering Neuroscience*. 10: 5-10.

Malenka, R.C. 2002. Synaptic Plasticity. *In* Davis, K.L, Charrey, D., Coyle, J.T., and Nemeroff, C. (eds). Neuropsychopharmacology: The Fifth Generation of Progress. Lippincott, Williams, & Wilkins, Philadelphia, Pennsylvania, pp. 147-154.

Miclescu, A., and T. Gordth. 2009. Nitric oxide and pain: 'Something old, something new'. *Acta Anaesthesiologica Scandinavica*. 53: 1107-1120.

Mulkey, R.M., and R.S. Zucker. 1992. Posttetanic potentiation at the crayfish neuromuscular junction is dependent on both intracellular calcium and sodium ion accumulation. *The Journal of Neuroscience* 72(11): 4327-4336.