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Beaulieu et al. BMC Neuroscience 2014, 15:56
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BMC Neuroscience

RESEARCH ARTICLE Open Access

Oxidative-stress induced increase in circulating fatty acids does not contribute to phospholipase A_2-dependent appetitive long-term memory failure in the pond snail *Lymnaea stagnalis*

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Abstract

Background: Reactive oxygen species (ROS) are essential for normal physiological functioning of the brain. However, uncompensated increase in ROS levels may results in oxidative stress. Phospholipase A_2 (PLA_2) is one of the key players activated by elevated ROS levels resulting in the hydrolysis of various products from the plasmamembrane such as peroxidized fatty acids. Free fatty acids (FFAs) and fatty acid metabolites are often implicated to the genesis of cognitive impairment. Previously we have shown that age-, and experimentally induced oxidative stress causes PLA_2-dependent long-term memory (LTM) failure in an aversive operant conditioning model in *Lymnaea stagnalis*. In the present study, we investigate the effects of experimentally induced oxidative stress and the role of elevated levels of circulating FFAs on LTM function using a non-aversive appetitive classical conditioning paradigm.

Results: We show that intracoelomic injection of exogenous PLA_2 or pro-oxidant induced PLA_2 activation negatively affects LTM performance in our learning paradigm. In addition, we show that experimental induction of oxidative stress causes significant temporal changes in circulating FFA levels. Importantly, the time of training coincides with the peak of this change in lipid metabolism. However, intracoelomic injection with exogenous arachidonic acid, one of the main FFAs released by PLA_2, does not affect LTM function. Moreover, sequestrating circulating FFAs with the aid of bovine serum albumin does not rescue pro-oxidant induced appetitive LTM failure.

Conclusions: Our data substantiates previous evidence linking lipid peroxidation and PLA_2 activation to age- and oxidative stress-related cognitive impairment, neuronal dysfunction and disease. In addition however, our data indicate that lipid peroxidation induced increased levels of circulating (per)oxidized FFAs are not a factor in oxidative stress induced LTM impairment.

Keywords: Cognitive impairment, Lipid peroxidation, Classical conditioning, Invertebrate, Phospholipase A2, Arachidonic acid, Mollusc, Oxidative stress, Free fatty acid

Background

Reactive oxygen species (ROS) play an important and critical role in synaptic plasticity and memory formation [1,2]. However, high levels of ROS are also shown to cause detrimental effects in the brain that negatively affect neuronal plasticity and memory function [3,4]. For instance, excessive (experimentally) increased levels of ROS are associated with decreased cognitive performance in both vertebrate and invertebrate species [1,2,4-7]. Under normal physiological conditions, the potential damaging effects of ROS is controlled by robust antioxidant defense systems. However, excessive formation of free radicals due to for instance an inflammatory reaction or failing antioxidant capacity as observed in aging, might overwhelm the antioxidant defense capacity of an organism resulting in oxidative stress. Oxidative stress may cause structural damage to nucleotides, lipids and proteins. The neuronal

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plasmamembrane is particularly prone to oxidative insult due to its high poly-unsaturated fatty acid (PUFA) content [8-11]. Many processes participate in the repair of oxidative damage to cellular components. In the case of membrane phospholipids, members of the Phospholipase A_2 (PLA_2) family of fatty acid acylases that catalyze hydrolysis of *sn-2* lipid bonds in glycerophospholipids are key players [12,13]. PLA_2's promote the formation of lysophopholipids and free fatty acids (FFAs) such as arachidonic acid (AA) and docosahexaenoic acid (DHA) [14-16]. Under normal physiological conditions PLA_2, its products and their metabolites play essential roles in regulating signal transduction, various neuronal signaling pathways, ion channel functioning and gene transcription processes [15,17-21]. Under conditions of oxidative stress PLA_2 is one of the mediators through which peroxidized fatty acids are excised from the lipid bilayer matrix [12,15]. Recent evidence increasingly associates deregulation of lipid metabolism due to PLA_2 (over) activation as a cause of nervous system dysfunction and cognitive impairment [7,12,22,23]. Yet, the physiological mechanisms underlying these phenomena remain incompletely resolved.

Our current research on the foundations of learning and memory impairment in the pond snail *Lymnaea stagnalis* (*L. stagnalis; L.*) focuses on the question of lipid-peroxidation dependent facets of neuronal dysfunction [6,7,22]. The present study follows from our recent observations implicating age-, inflammation- and experimental oxidative stress-induced PLA_2 activity in long-term memory (LTM) failure [7,22]. Considering the pivotal role of PLA_2 activation in behavioural plasticity, we investigated the question whether it is the fatty acids released upon PLA_2 activation that causes cognitive impairment in our model system. Here we utilized an established and widely studied classical appetitive reward-conditioning paradigm involving chemosensory conditioning of the animals' feeding behaviour i.e., "rasping" [22,24-27]. Using this model we investigated the effects of experimentally pro-oxidant induced PLA_2 activation on associative appetitive LTM impairment and the role of circulating FFAs therein. We will provide evidence that increased levels of circulating (per)oxidized fatty acids are not a factor in oxidative stress induced LTM impairment.

Results

Enhanced levels of extracellular PLA_2 inhibit LTM formation

To assess the impact of increased levels of extracellular PLA_2 on the formation of appetitive LTM, animals randomly assigned to two test groups were injected with PLA_2 from bee venom or vehicle-only 1 hr before their first training session. Animals were subsequently tested for the presence of conditioned feeding responses 22–24 hrs after their last training session (Figure 1A). These experiments revealed a prominent suppression of conditioned feeding responses in animals that received treatment with PLA_2 (Figure 1B; ANOVA interaction time x treatment; $F_{1,57} = 19.315$, $p < 0.0001$). That is, vehicle-injected conditioned (CS-UCS) animals displayed robust conditioned feeding responses compared to their non-conditioned (CS-DS) peers ($F_{1,57} = 35.672$ $p < 0.0001$). In contrast, PLA_2-injected animals displayed a conditioned feeding response that was significantly reduced compared to the vehicle conditioned animals ($F_{1,57} = 15.634$ $p = 0.0002$) and was statistically indistinguishable from their non-conditioned partners ($F_{1,57} = 0.083$, $p = 0.77$).

AAPH injection elevates circulating FFA levels and causes PLA_2-dependent LTM failure

Prior studies in *L. stagnalis* implicate natural and experimentally (AAPH) induced non-enzymatic lipid peroxidation to memory deficiencies in an aversive operant conditioning of aerial respiratory behaviour [7]. We also demonstrated this phenomenon can be fully reversed through treatment with the general PLA_2 inhibitor aristolochic acid [7]. Therefore, we now proceed with testing whether AAPH treatment also affect non-aversive appetitive classical conditioning. To this end, we first evaluated the temporal characteristics of AAPH induced free fatty acid (FFA) release into the circulatory system. FFA content of haemolymph extracted immediately or 12, 24, 48, 96 and 168 hours after intracoelomic injection of AAPH were quantified with ADIFAB-FFA assay. A significant increase in haemolymph FFA levels developed over the first 24–48 hours after AAPH injection (Figure 2; One-way ANOVA $F_{6,44} = 7.798$, $p < 0.0001$; Planned comparison 0 hr vs 24 hrs $F_{1,44} = 21.538$, $p < 0.0001$ and 0 hr vs 48 hrs $F_{1,44} = 23.536$, $p < 0.0001$). No such increase was observed when haemolymph was collected 24 hrs after vehicle injection (Planned comparison 0 hr vs vehicle 24 hrs $F_{1,44} = 0.0025$, $p = 0.96$). FFA levels returned to control levels 2–4 days after AAPH injection. These data support the notion that AAPH injection can cause significant temporal changes in lipid metabolism within the whole animal.

Next we tested the effect of AAPH induced oxidative stress on non-aversive appetitive classical conditioning and the involvement of PLA_2 in this process. To this end animals were injected 24 hrs before the first training trial with either vehicle or AAPH. Subsequently, animals were injected 1 hr before the start of the first training session with either aristolochic acid or vehicle only (Figure 3A). We found that treatment with AAPH caused a significant reduction in the conditioned feeding response and that treatment with the PLA_2 inhibitor aristolochic acid counteracted AAPH's negative effect on LTM formation (Figure 3B; ANOVA interaction training x treatment,

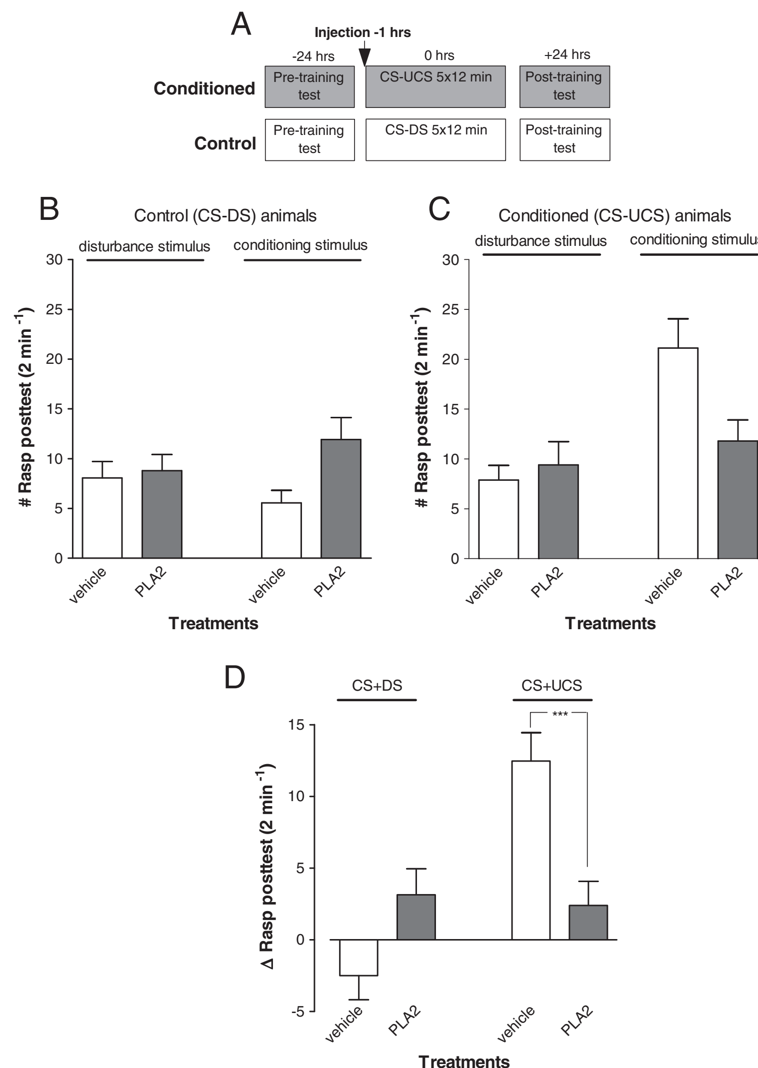

Figure 1 Long-term memory assessments after bee venom PLA_2 injection. A. Protocol indicating timing of injection and start of pre- and post-training tests with respect to the start of the first training session for both the conditioned (CS-UCS) and control (CS-DS) animals. **B-D** LTM assessment in animals injected 1 hr before training. In all control animals, rasping movements were similar upon application of the disturbance stimulus or conditioning stimulus **(B)**. In contrast, in conditioned vehicle injected animals the number of rasping movements increased after application of the conditioning stimulus **(C)**. The difference between the number of rasps induced by the conditioning stimulus minus the disturbance stimulus shows that there was a robust response to conditioning but only in the vehicle injected animals. None of the unconditioned animals or the PLA_2 injected conditioned animals responded with significant feeding movements in the post-training test **(D)**. These results indicate LTM impairment in snails injected with exogenous PLA_2 1 hr before training. ***$p < 0.001$.

$F_{3,85} = 2.847$, $p < 0.05$). That is, AAPH treated animals had a lowered feeding response compared to vehicle treated conditioned animals ($F_{1,85} = 11.668$, $p < 0.001$) and their response was statistically indistinguishable from the non-conditioned peers (Figure 3B; $F_{1,85} = 0.6016$, $p = 0.44$). In contrast animals treated with aristolochic acid only learned well ($F_{1,85} = 13.805$, $p = 0.0003$) in a manner that was statistically indistinguishable from that of vehicle treated animals (Figure 3B; $F_{1,85} = 0.841$, $p = 0.36$). Moreover, animals treated with the combination of AAPH and aristolochic acid did display a significant conditioned feeding response (Figure 3B black bars; $F_{1,85} = 20.047$, $p < 0.0001$) that was significantly different from the AAPH injected animals (Figure 3B; $F_{1,85} = 17.219$, $p < 0.0001$). Note that training and testing trials were performed when AAPH-induced haemolymph FFA levels were at or close to their peak.

Arachidonic acid does not affect LTM performance

The above results clearly implicate PLA_2 as a critical factor in oxidative stress induced appetitive LTM deficiency. PLA_2 is responsible for the hydrolysis of membrane phospholipids resulting in the release of FFAs including

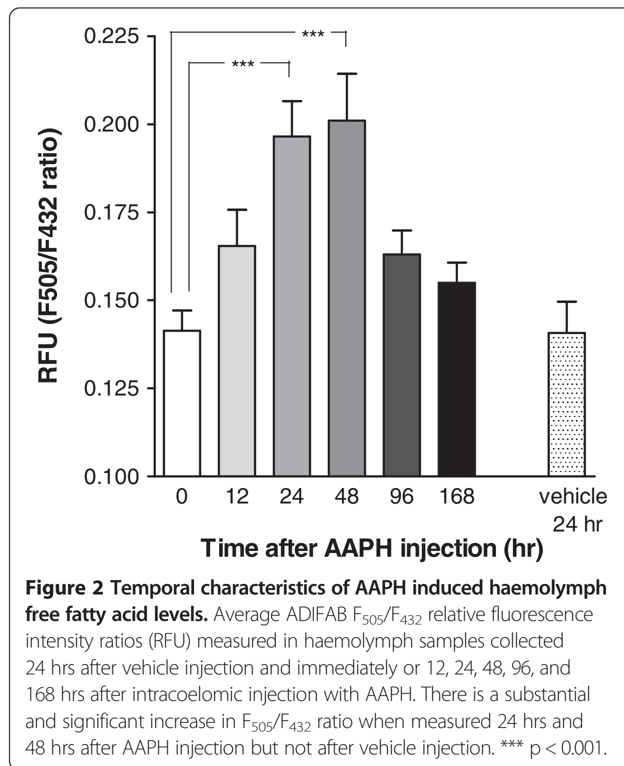

Figure 2 Temporal characteristics of AAPH induced haemolymph free fatty acid levels. Average ADIFAB F_{505}/F_{432} relative fluorescence intensity ratios (RFU) measured in haemolymph samples collected 24 hrs after vehicle injection and immediately or 12, 24, 48, 96, and 168 hrs after intracoelomic injection with AAPH. There is a substantial and significant increase in F_{505}/F_{432} ratio when measured 24 hrs and 48 hrs after AAPH injection but not after vehicle injection. *** $p < 0.001$.

arachidonic acid as one of its most prominent products. Arachidonic acid is the primary substrate for the cyclooxygenase (COX) branch of the eicosanoid pathway. We previously showed that systemic immune challenges cause oxidative stress and induces a PLA_2- and COX-dependent appetitive LTM failure in *L. stagnalis* [22]. Thus, to test the hypothesis that elevated haemolymph arachidonic acid levels underlie LTM impairment, animals were injected with exogenous arachidonic acid or vehicle-only before their first training session and tested for the presence of conditioned feeding responses 22–24 hrs after their last training session (Figure 4A). These experiments revealed no significant negative effect of arachidonic acid injection on appetitive LTM (ANOVA interaction time x treatment; $F_{1,48} = 0.001$, $p = 0.99$; planned comparison treatment vs training $F_{1,48} = 9.4584$, $p = 0.003$).

Binding of free fatty acids to BSA does not rescue LTM failure

Above we showed that injection with exogenous arachidonic acid prior to training has no effect on appetitive LTM function. However, arachidonic acid is likely not the only PLA_2-hydrolysis product released with neuromodulatory or signaling capabilities. Defatted bovine serum albumin (BSA) is considered a high affinity FFA binding protein [28,29]. Thus, we first verified whether BSA is an effective tool for haemolymph FFA manipulation in *L. stagnalis.* Animals were injected with AAPH or vehicle-only and 24 hrs later subsequently injected with defatted BSA or vehicle-only. Two hours after BSA injections, haemolymph was collected and FFA levels were determined immediately using the ADIFAB assay. As before, AAPH injection induced elevated haemolymph FFA (Figure 5; One-way ANOVA $F_{3,19} = 23.695$, $p < 0.0001$; vehicle vs. AAPH $F_{1,19} = 16.522$, $p = 0.0006$). Moreover our data showed that BSA effectively binds FFAs in *L. stagnalis* haemolymph. That is, naturally occurring FFAs levels are significantly lower after BSA injection (vehicle vs BSA $F_{1,19} = 19.385$, $p = 0.0003$) and animals subjected to injections of both AAPH and BSA have similar haemolymph FFA levels as vehicle-only injected animals (vehicle vs AAPH-BSA $F_{1,19} = 1.519$, $p = 0.23$).

Next we tested whether BSA-injection could reverse AAPH induced appetitive LTM failure. Animals were injected with AAPH or vehicle-only 24 hrs before training and subsequently injected 1 hr before training with defatted BSA or vehicle (Figure 6A; note that the injection regime was identical as used for the previous experiment). We found that injection with BSA did not counter AAPH's negative effect on LTM formation (Figure 6). As before, treatment with AAPH caused a significant reduction in the conditioned feeding response compared to vehicle treated conditioned animals (ANOVA interaction training x treatment, $F_{3,102} = 2.748$, $p < 0.05$; vehicle vs AAPH $F_{1,102} = 8.7611$, $p = 0.003$). In contrast, animals treated with BSA-only learned well ($F_{1,102} = 13.805$, $p = 0.0003$) in a manner that was statistically indistinguishable from that of vehicle treated conditioned animals (Figure 6B; $F_{1,102} = 0.0949$, $p = 0.76$). However, animals treated with the combination of AAPH + BSA did not display a significant conditioned feeding response (Figure 6B black bars; $F_{1,102} = 0.5285$, $p = 0.47$). This conditioned feeding response was different compared to vehicle or BSA treated conditioned animals ($F_{1,102} = 12.717$, $p < 0.0001$ and $F_{1,102} = 10.26107$, $p = 0.002$ for vehicle and BSA respectively) but not significantly different from the AAPH-only injected animals (Figure 6B; $F_{1,102} = 0.0183$, $p = 0.89$).

Discussion

This study's primary purpose was to test the hypothesis that FFAs released by oxidative stress-induced PLA_2 activity are a factor in learning and memory dysfunction in *L. stagnalis.* Our primary observations are: 1- Increasing the level of extracellular PLA_2 activity through injection of venom-derived extracellular PLA_2 evoked LTM impairment. 2- Experimental induction of oxidative stress with the lipid peroxidation-inducing free radical generator AAPH triggers an increase in circulating FFA levels that peaks within 24–48 hrs and attenuates LTM performance. 3- Experimental elevation of circulating arachidonic acid through intracoelomic injection of exogenous arachidonic

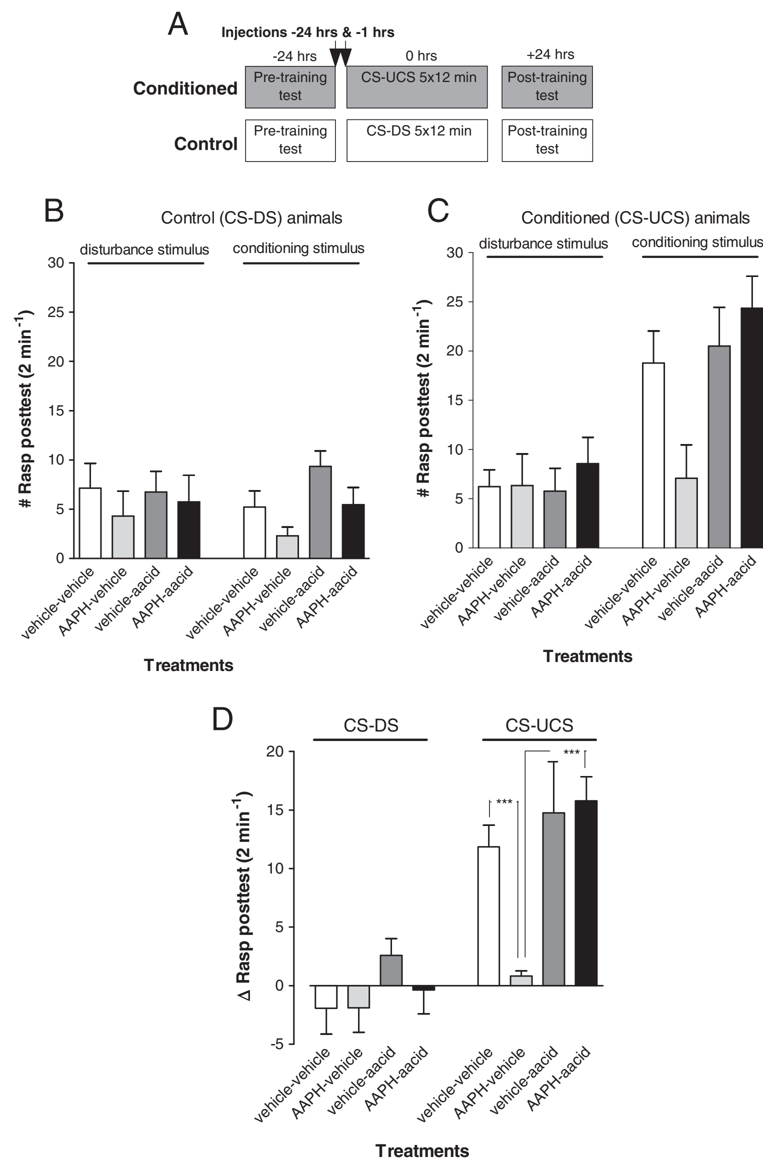

Figure 3 PLA_2 inhibition restores LTM deficiency in AAPH injected animals. A. Protocol indicating timing of injection and start of pre- and post-training tests with respect to the start of the first training session for both the conditioned (CS-UCS) and control (CS-DS) animals. **B-D**. LTM assessment in animals injected with either vehicle only (vehicle-vehicle), AAPH only (AAPH-vehicle), aristolochic acid only (vehicle-aacid) or injected with both AAPH and aristolochic acid (AAPH-aacid). In all control animals, rasping movements were similar upon application of the disturbance stimulus or conditioning stimulus **(B)**. In contrast, conditioned animals, either vehicle injected, aristolochic acid only or AAPH + aristolochic acid injected, showed an increase in the number of rasping movements after application of the conditioning stimulus **(C)**. Thus, the AAPH injected conditioned group, showed a significant reduction in their Δrasp values in the post-training test compared to all other conditioned groups. Animals injected with aristolochic acid only or AAPH + aristolochic acid were not different in their conditioned response than the vehicle injected animals **(D)**. This suggests that co-treatment of AAPH with a general PLA_2 inhibitor reverses the AAPH induced adverse effect on appetitive LTM performance. ***$p < 0.001$.

acid does not affect LTM performance. 4. Experimentally induced oxidative stress-associated LTM impairment can be rescued with the general PLA_2 inhibitor aristolochic acid but not by normalizing the level of circulating FFAs with defatted BSA.

The present data substantiate previous evidence where we link lipid peroxidation and PLA_2 activation to age- and oxidative stress-associated LTM impairment in another, aversive operant conditioning learning and memory paradigm [7]. In both our learning models age- and oxidative stress associated LTM failure is associated with reduced electrical excitability of key interneurons in the circuits underlying the respective behaviours [6,7,22,30]. Both behavioural and electrophysiological facets of age-associated

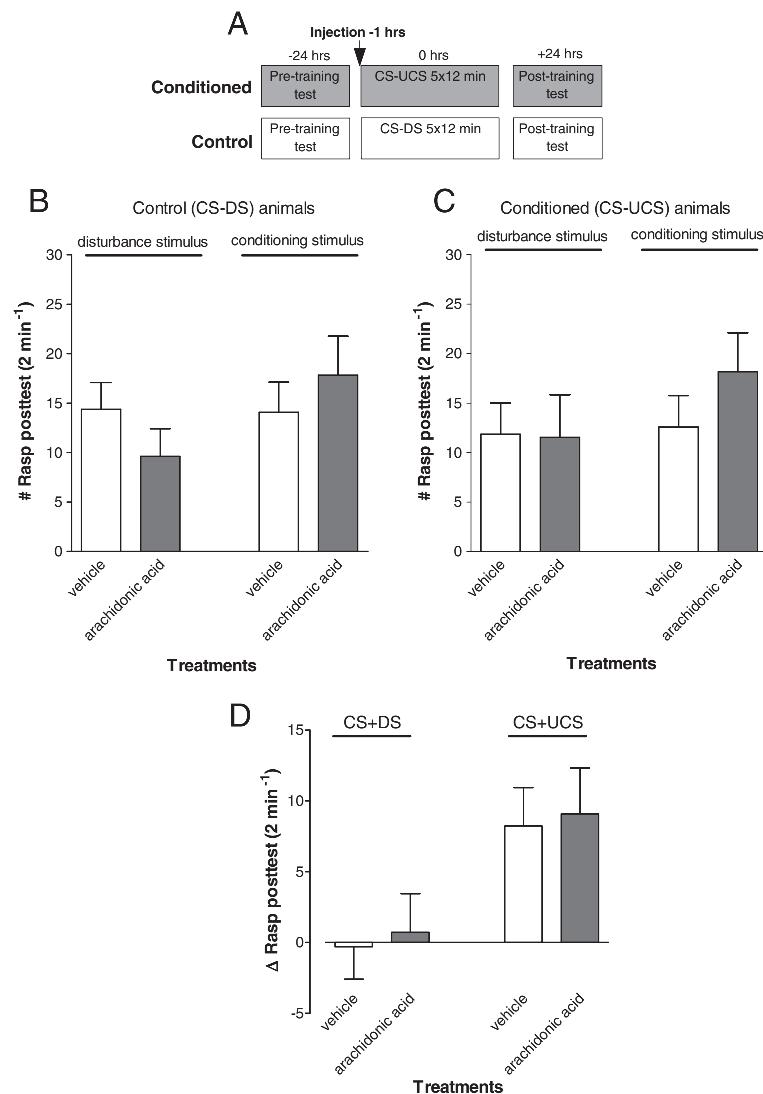

Figure 4 Long-term memory assessment after injection with arachidonic acid. A. Protocol indicating timing of injection and start of pre- and post-training tests with respect to the start of the first training session for both the conditioned (CS-UCS) and control (CS-DS) animals. **B-D**. LTM assessment in animals injected 1 hr before training. There was a robust response to conditioning in both vehicle and arachidonic acid injected animals. Thus all conditioned animals responded with significant feeding movements in the post-training test upon application of the conditioning stimulus. These results indicate that injection with exogenous arachidonic acid does not induce LTM impairment in snails.

respiratory and appetitive LTM impairment can be reproduced in young animals through treatment with AAPH, a water-soluble free radical generator commonly used to induce FA peroxidation (present study and [7]). In addition, in both our two models, all behavioural, electrophysiological and biochemical symptoms of aging and experimental oxidative stress can be reversed by treatment with aristolochic acid, a broad spectrum PLA_2 inhibitor (present study and [6,7,11]). No evidence was found of significant age-associated impairment or experimental oxidative stress-induced repression of short/intermediate term forms in either of the two behavioural conditioning paradigms [7,27], suggesting that transcription-independent forms of memory are relatively impervious to aging or oxidative stress. Intriguingly, selective appetitive LTM impairment could also be induced in young *Lymnaea* through systemic activation of their cellular immune system and, as before in aged and oxidation-stressed young animals, LTM could be rescued by means of PLA_2 inhibition [22]. Thus, although, the possibility exists that the cellular and molecular mechanisms underlying oxidative stress induced PLA_2 activation dependent LTM impairments in our two learning models are different, this seems unlikely. The brain and its neurons are due to their high polyunsaturated fatty acids (PUFA) content inordinately sensitive to free radical

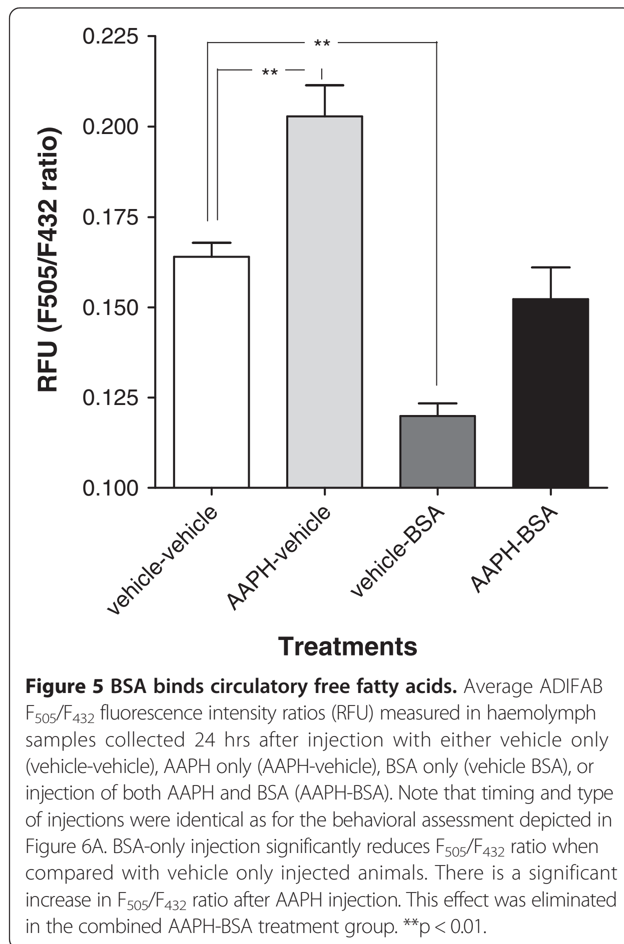

Figure 5 BSA binds circulatory free fatty acids. Average ADIFAB F_{505}/F_{432} fluorescence intensity ratios (RFU) measured in haemolymph samples collected 24 hrs after injection with either vehicle only (vehicle-vehicle), AAPH only (AAPH-vehicle), BSA only (vehicle BSA), or injection of both AAPH and BSA (AAPH-BSA). Note that timing and type of injections were identical as for the behavioral assessment depicted in Figure 6A. BSA-only injection significantly reduces F_{505}/F_{432} ratio when compared with vehicle only injected animals. There is a significant increase in F_{505}/F_{432} ratio after AAPH injection. This effect was eliminated in the combined AAPH-BSA treatment group. $^{**}p < 0.01$.

attack, oxidative stress and subsequent peroxidation, a process that if not properly contained can severely disrupt membrane architecture and lipid signaling processes [31-33]. Neurons defend themselves against lipid-peroxidation through various mechanisms, one that involves excision of (per)oxidized FA by PLA_2 [12,15]. As will be discussed below, PLA_2, (per)oxidized FA and their various metabolites all can alter signal transduction, various neuronal signaling pathways, ion channel functioning and gene transcription processes and ultimately behavioral plasticity such as learning and memory processes. Thus, although to our knowledge our studies are the first to show these aspects in molluscs, it is not surprisingly that similar conclusions are drawn with increasing frequency in studies linking oxidative stress related PLA_2 activation with cognitive impairment, neuronal dysfunction and disease not only in other, non-molluscan, invertebrate species but also in mammals and humans [12,16,34-38]. Together this suggests that *Lymnaea's* LTM functions in general are sensitive to lipid peroxidation and oxidative membrane damage, and that lipid peroxidation-dependent activation of PLA_2 is a fundamental and evolutionary conserved factor in the decline in behavioral and neuronal plasticity widely observed across the animal kingdom.

Activation of PLA_2 by reactive oxygen species in *L. stagnalis* results in the release of extracellular (per)oxidized FFAs (present study and [7]). Many FFAs, including AA and DHA the two FAs most commonly found at the glycerophospholipid *sn-2* position in neurons, have various demonstrated biological activity in the nervous system. For instance, under normal conditions they can regulate membrane fluidity and other aspects of phospholipid membrane microarchitecture, may engage in modulatory interactions with various ion channels, affect alterations in membrane protein clustering, modify receptor sensitivity and signal transduction pathways as well as affect gene transcription processes [17-21,39-43]. However, under conditions of oxidative stress, causes (over) activation of PLA_2 resulting in peroxidized FA excision from the lipid bilayer matrix [12,15]. Recent evidence increasingly associates deregulation of lipid metabolism due to PLA_2 (over) activation as a cause of nervous system dysfunction and cognitive impairment [7,12,22,23]. In addition, AA is the substrate for many potentially neuroactive lipids metabolized including those generated by the eicosanoid metabolic pathways. Each of the three main branches of eicosanoid metabolism, the epoxygenase or CYP-450, the lipoxygenase (LOX) and the cyclooxygenase (COX) pathways, has the potential to generate a variety of lipid-derived neuromodulatory substances that may engage with either intracellular or extracellular targets [16,18,20,41,44]. In addition, (per)oxidized FFAs can undergo further metabolic processing resulting in the production of highly cytotoxic aldehydes such as 4-hydroxynonenal and malondialdehyde [1,14].

Interestingly, we recently showed that challenging the immune system impairs LTM function in *L. stagnalis* that seems to involve PLA_2 and COX activity [22]. In addition, non-enzymatic lipid peroxidation (including AAPH induced), has been shown to result in the production of AA and DHA derived iso- and neuroprostanes including the LOX series [45,46]. PLA_2- and/or LOX-dependent arachidonic-acid modulated background K^+ channels have been described in *Lymnaea* and various other molluscs [47-51]. Opening of these 4TM2P channels generates an outward current. It is therefore conceivable that elevated levels of PLA_2-activity associated with inflammation, oxidative stress and aging induce a decline in neuronal excitability through activation of these 4TM2P background K^+ channels. In this respect the 12-LOX product 12-HPETE, first described in the gastropod *Aplysia californica*, is quite interesting. 12-HPETE has long been known as an activator of *Aplysia's* S-type K^+-channels [52]. These K^+ channels, later identified to belong to the TREK-1 family, are instrumental in non-synaptic forms of plasticity underlying behavioral modification of *Aplysia*'s gill

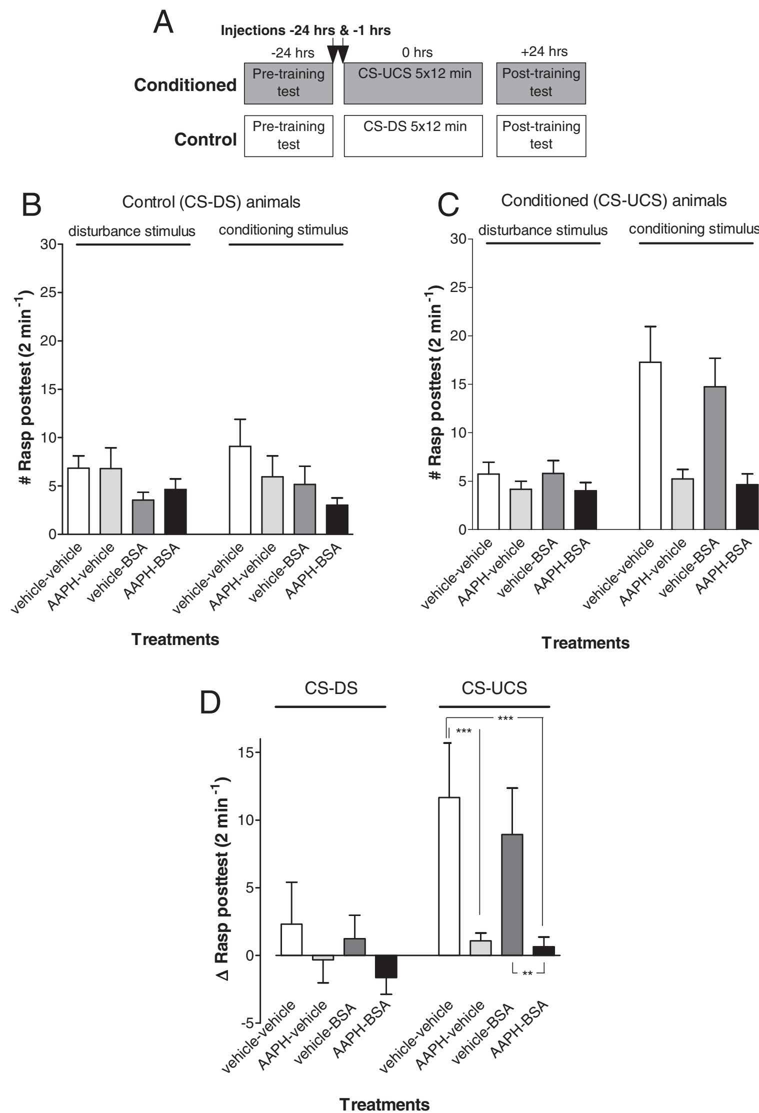

Figure 6 Sequestering circulating FFAs with BSA does not restore LTM failure in AAPH injected animals. A. Protocol indicating timing of injection and start of pre- and post-training tests with respect to the start of the first training session for both the conditioned (CS-UCS) and control (CS-DS) animals. **B-D**. LTM assessment in animals treated with vehicle-only (vehicle-vehicle), AAPH only (AAPH-vehicle), BSA only (vehicle-BSA) or injections of both AAPH and BSA (AAPH-BSA). In all control animals, rasping movements were similar upon application of the disturbance stimulus or conditioning stimulus **(B)**. In contrast, conditioned animals, either vehicle injected or BSA only injected, showed an increase in the number of rasping movements after application of the conditioning stimulus **(C)**. Thus, both the AAPH-only and AAPH-BSA injected conditioned groups, showed a significant reduction in their Δrasp values in the post-training test compared to the other conditioned groups. Animals injected with aristolochic acid only were not different in their conditioned response than the vehicle injected animals **(D)**. This suggests that co-treatment of AAPH with BSA, does not reverse the AAPH induced adverse effect on appetitive LTM performance. $^{**}p < 0.01$; $^{***}p < 0.001$.

withdrawal reflex [53] and are implicated as synaptic modulator in *Aplysia* and various vertebrate model systems [54-58]. Alterations in receptor sensitivity, ion channels and signal transduction pathways as well as affect gene transcription by PLA_2, it's products and their metabolites will most likely affect activity,- transcription- and protein synthesis-dependent processes such as LTM formation.

Based on the evidence described above it seems therefore very plausible that lipid-peroxidation evoked elevated levels of FFAs through activation of PLA_2 are the source of the observed LTM dysfunction in *L. stagnalis* (present study and [7,22]). However, in the present study we show that increasing extracellular levels of AA does not affect LTM performance in *L. stagnalis*. Moreover, we also show in the current study that removing AAPH-induced elevated levels of circulating peroxidized FFA with BSA does not rescue LTM failure in our model system.

So, if not extracellular FFAs then what might be a source of the lipid-peroxidation dependent LTM failure observed in *L. stagnalis*? The first possibility is that PLA_2 itself is the main culprit. The PLA_2 family is a family of heterogeneous enzymes acting in different cellular locations and to some extent different activation profiles usually classified into intracellular located cytosolic PLA_2 ($cPLA_2$), calcium-independent PLA_2 ($iPLA_2$), extracellular acting secretory PLA_2 ($sPLA_2$) and the recently identified lysosomal and adipose-specific PLA_2's [15,34,59]. Upon activation, the various PLA_2 family members such as cPLA2 and sPLA2 can induce FA release and eicosanoid production by themselves [60-62]. In addition, recent evidence also indicates the existence of cross-talk and trans-activation between the various intracellular and cytosolic PLA_2 family members [63-65]. Thus it is quite conceivable that AAPH, a water-soluble extracellular free radical generator used to induce lipid peroxidation in the present study activates various intracellular and extracellular PLA_2 enzymes.

Besides being present and acting on the cellular plasmamembrane both $cPLA_2$ and $iPLA_2$ can localize and/or target organellar membranes including mitochondrial and nuclear membranes [34,66-68]. For instance, it has been shown that activation of PLA_2 can induce mitochondrial dysfunction due to a loss of mitochondrial membrane potential, swelling of mitochondria and/or the production of superoxide from mitochondria [69]. Furthermore, interactions between $cPLA_2$ and NADPH-oxidase complexes in the plasmamembrane have been implicated in the redox-pathology of various neurodegenerative diseases [34,70]. In addition to their potential interference with numerous signaling processes, PLA_2 enzymatic activity may impact various membrane architecture-sensitive processes. For example, PLA_2 may alter phospholipid-packing causing membranes to become more molecularly ordered and affect processes involved in vesicle fusion, exocytosis and actin-dependent processes [71-75]. Moreover, there is evidence that architectural changes induced by PLA_2 may alter gating states for a variety of ion channels including members of the K2P two-pore "leak" potassium family [76,77]. At this point in time we cannot say if and which of these scenarios underpins LTM impairment in our model system. Nor can we pinpoint which PLA_2 is involved. However, the observation in the current and one of our previous studies [7], that treatment with aristolochic acid, commonly considered a general PLA_2 inhibitor and inhibiting various classes of PLA_2 [68,78-81], reverses the AAPH-induced LTM failure is consistent with PLA_2's involvement.

Another intriguing potential scenario to consider as explanation for the current results is the possibility that other products released extracellular after PLA_2 activation are causing LTM failure in *L. stagnalis*. For instance, hydrolysis of oxidized membrane phospholipids by PLA_2 will not only liberate FFAs but also produce lysophospholipids (LPLs). Involvement of LPLs in membrane-associated processes such as membrane budding, ruffle formation, protein complex assembly and ion channel gating has been reported [34,59,82-85]. However, extracellular LPLs, like FFAs, can bind to BSA [86-88]. Therefore, we currently interpret BSA's inability to reverse AAPH-induced LTM deficiency as evidence against the idea that circulating LPLs play a major role in the current LTM failure model. Alternatively, extracellular (per)oxidized FAs can undergo further metabolic processing resulting in the production of highly cytotoxic aldehydes such as 4-hydroxynonenal and malondialdehyde [1,14]. It is important to note that BSA is not cell permeable. Thus, indirect activation of intracellular PLA_2 might cause elevated levels of intracellular FFAs and LPLs thereby affecting various intracellular targets [14,89,90]. For instance, it has been demonstrated that increased levels of intracellular FFAs can directly affect NADPH-oxidase [90,91] resulting in a further increase of ROS and lipid peroxidation [91].

In addition to FFAs and LPLs, PLA_2 activation results in the extracellular release of lyso-platelet-activating factor (lyso-PAF), the platelet-activating factor (PAF) precursor [92-94]. PAF is a bioactive phospholipid that under normal conditions is thought to be involved in the regulation of synaptic plasticity, memory and neuronal protection [92-94]. In addition, PAF is a transcriptional activator of the cyclooxygenase-2 (COX-2) gene [92]. As noted before, AA is the primary substrate for the COX branch of the eicosanoid (inflammatory) pathway. Importantly, ROS can also be generated as a by-product of COX activity, thus creating a positive feedback loop that potentially can cause escalation of PLA_2, PAF and COX-activity thereby causing substantive deregulation of lipid homeostasis [92-94]. In this respect it is interesting to note that we recently showed that systemic immune challenges in *L. stagnalis* induces a PLA_2-dependent LTM failure that could be rescued with treatment of indomethacin, a putative COX inhibitor [22].

One of the intriguing questions still remaining is why inhibition of PLA_2 24 hrs after its activation is sufficient to reverse the oxidative stress induced LTM failure? We show in the present study that circulating FFA levels are their highest level 24–48 hrs after PLA_2 activation suggesting that some of the PLA_2 and FFA-dependent and associated pathways are initiated in the first 24 hrs. after induction of oxidative stress. At present we cannot definitively provide a mechanism to what causes the PLA_2-dependent LTM failure within this time frame. However based on the actions of PLA_2, FFA and their various products as outlined above there are some potential

explanations. For instance, it is conceivable that by inhibiting PLA_2 activity, even though FFAs are already released for some time, we stop the positive feedback loop discussed above, thereby preventing further escalation of PLA_2, its products and their pathways. Notwithstanding, further investigations are clearly needed to resolve this issue.

Conclusion

The current results support our earlier work where we link oxidative stress, lipid metabolism and age-associated LTM impairment in *L. stagnalis*. In the present study, we provide evidence that lipid peroxidation induced elevated levels of circulating FFA do not impact LTM performance. This is of particularly interest considering the increasing focus on the interplay between ROS, PLA_2, COX, their substrates and metabolites as a potential factor in the deregulation of lipid metabolism and cell- and neuronal dysfunction and cognitive performance.

Methods

Animals

Animals were bred and raised under constant and strictly controlled ambient conditions as previously described [6,7,11,22,27,30,95]. Water used in the facility was sourced from a reverse osmosis system and reconditioned to a conductivity of ~450 Ω.cm through the addition of Instant Ocean salts at 1 g/US Gallon (i.e., artificial pond water; Aquarium Systems USA). Calcium concentration was kept at saturation level (~60 mg/L as $CaCO_3$) through the addition of calcium carbonate (light powder; EMD analytics, Gibbstown, New Jersey) to the tanks. In addition, animals had continuous access to sterilized cuttlefish (*Sepia officinalis*) bone (2–3 per tank). Animals were fed a routine diet consisting of Romaine lettuce and Aquamax-carniverous Grower 600 trout pellets *ad libitum* (Purina Mills LLC, St. Louis, Missouri). For the present study, young sexually mature snails (age 7–9 months; shell length 2.5 cm-3 cm; see also [7]) were taken at random from healthy populations. The use and care of animals conformed to the University of Calgary Animal Care and Use Policy which adheres to the guidelines, policies and standards of the Canadian Council on Animal Care (CCAC), the Canadian Association of Laboratory Animal Medicine (CALAM), standards of Veterinary Care, and the Alberta Veterinary Association (AVMA) professional codes and standards.

Training and testing procedures

Preparation

Behavioural conditioning was done using a non-aversive appetitive classical conditioning protocol [22,27]. Snails were sampled at random and marked, for identification purposes with indelible marker. Food was withheld starting 48 hrs prior to the first pre-training test and for the remainder of the training and post-training testing.

Testing procedures

On day 1, prior to behavioural conditioning, each snail was individually tested for their natural response to the administration of pond water, the disturbance stimulus (DS), as well as the conditional stimulus (CS) n-amyl acetate ("Pre-training test") [22]. Tests were performed using 100-ml translucent polystyrene beakers (4.5 cm diameter), filled with 80 mL of water taken from the snail's home tank. After transfer into the beakers, the snails were allowed to acclimatize for 15 min before testing commenced. Testing involved counting the number of rasps over two consecutive periods of 2 min, the first period starting with gentle administration of the DS (10 ml artificial pond water), the second period starting with the administration of the CS (10 ml n-amyl acetate solution; 4 ppm final concentration). To facilitate observation, the test beakers were elevated by translucent plastic stands and were surrounded by mirrors to ensure continuous view of the buccal mass and radula of the snails during testing. A test response was calculated by taking the difference between the number of rasps counted during the second period minus the number counted during the first period (i.e., ΔRasp = rasps after CS – rasps after DS). To correct for both differences in background rasping activity and potential application artefacts, the pre-training tests were performed in duplicate with >1 hr interval, and behavioural responses were calculated as the average of both ΔRasp (i.e., $\Delta Rasp_{pre\text{-}test}$ = average $\Delta Rasp_{pre\text{-}test}1$ and $\Delta Rasp_{pre\text{-}test}2$). After completion of a test, snails were gently rinsed and returned to their home tanks. A single post-training test was performed on day 3 (22–24 hrs after training) following identical procedures as described for the pre-testing above.

Training procedure

Snails were trained in a single day, multi-trial forward-delay conditioning format [22]. Sucrose (final concentration of 0.4% wt/vol) served as the unconditioned stimulus (UCS) and n-amyl acetate (4 ppm final concentration) as the conditioned stimulus (CS). To control for potential behavioural effects of fluid addition, a disturbance control in which the UCS was paired with the DS (i.e., pond water) was implemented. Snails were randomly assigned to either the CS–UCS ("conditioned") or the CS–DS (unconditioned "control") group and trained "en masse". Training was performed in 1-L polypropylene beakers containing 480 ml clean artificial pond water. After transfer into the training beakers, the snails were allowed to acclimatize for 60 min. Both "control" and "conditioned" groups received 120 mL of the CS solution,

followed 15 s later by 120 ml of the UCS ("conditioned" group) or 120 ml of the DS ("control" group). After 2 min, the beakers containing the snails were drained and gently rinsed with clean pond water and the snails were readied for their next training trial by re-placing them in the 1-L polypropylene beakers holding 480 ml clean artificial pond water. After 11 min and 45 sec the training procedure was repeated. Snails received a total of 5 training procedures on a single day before being returned to their "home" tank. Snails were at all times fully submerged during training and testing. Care was taken to ensure that pre-testing, training and post-testing commenced at the same time of day for each group and training and testing always occurred in the same location. "Conditioned" and "control" snails were always tested and trained concurrently.

Chemicals and drug injections

All chemicals used in this study i.e., defatted bovine albumin serum (BSA; fatty acid free), aristolochic acid, arachidonic acid, Phospholipase A_2 from honey bee venom and 2,2-azobis (2-methylpropion-amidine) dihydrochloride (AAPH) were obtained from Sigma Aldrich (St. Louis, MO). All drugs, except arachidonic acid, were dissolved in sterile ultrapure water. Arachidonic acid was dissolved in dimethyl sulfoxide (DMSO). To prevent premature oxidation of dissolved AA, the drug was either used immediately after dissolving or stored under nitrogen at −20°C. Drug or vehicle-only treatments were delivered by means of intracoelomic injection into the snail (note: this will introduce drugs directly into the animal's blood and circulatory system) in a manner avoiding whole-body withdrawal responses and voiding of haemolymph [7,22,30]. Using body weight measurements injected amounts of drugs were calculated to achieve the following approximate concentrations of drugs in the snails' haemolymph: 1 mM of AAPH, 2 mg/ml of BSA, 20U of PLA_2, 10 μM of both arachidonic acid and aristolochic acid. Haemolymph DMSO concentrations never exceeded 0.05%. As control snails were injected with equivalent volumes vehicle only (vehicle control). Except for AAPH injected 24 hrs before training, all other injections were given 1 hour prior to the start of the first training trial. AAPH is a water-soluble slowly dissociating azo-compound used extensively as a free radical generator in studies of lipid peroxidation and characterization of antioxidants in various model systems including *L. stagnalis* [6,7,96,97]. All solutions were injected through the foot directly into the haemocoel with a microliter syringe and a 25G needle. Animals were behaving normally within 1–2 minutes after injection.

Haemolymph extraction and free fatty acid detection

Snails were injected with AAPH, BSA, AAPH + BSA or vehicle-only using the same injection schedule as for the behavioural assays. Haemolymph was extracted by head–foot retraction [98]. Haemolymph collected of 3 snails/condition was pooled. Free fatty acid levels were quantified using standard protocols involving acrylodan labeled intestinal fatty acid binding protein (ADIFAB; FFA Sciences, San Diego, CA) [7,99,100]. Haemolymph plus ADIFAB (0.2 μM) was loaded on a 96-well plate. Fluorescent signal was measured immediately using a Spectromax 2Me multidetection microplate reader (Molecular Devices, Sunnyvale, CA) with and excitation wavelength of 386 nm and emission wavelength of 432 and 505 nm representing respectively unbound and bound states of ADIFAB. Data are expressed in dimensionless fluorescence intensity units (RFU) determined from intensity ratios measured at 505 and 432 nm (F_{505}/F_{432}; see [99]). An increased F_{505}/F_{432} ratio is indicative of increased haemolymph free fatty acid levels [99,100]. Six or more independent experiments with each 3 replicates/condition were performed.

Statistical analysis

Behavioural data was analyzed by means of a factorial or repeated measure ANOVA. Explicit hypotheses were tested using planned comparisons unless indicated differently in the text. Effect of drugs on FFA production was analyzed by one-way ANOVA. Compliance with parametric assumptions was confirmed for each data set submitted to ANOVA using both graphical (probability plots applied to raw data and residuals) and analytical techniques (Kolmogorov-Smirnov one-sample test for normality, F-max test). Throughout the text, average and data dispersion are expressed as arithmetic means and standard error of the mean (SEM). Figures were constructed using Graphpad Prism version 4.03 (Graphpad Sofware Inc., La Jolla, CA).

Abbreviations

BSA: Bovine serum albumin; CNS: Central nervous system; COX: Cyclooxygenase; CS: Conditioned stimulus; DMSO: Dimethyl sulfoxide; DS: Disturbance stimulus; FFA: Free fatty acid; HBS: Hepes buffered saline; LTM: Long-term memory; PAF: Platelet-activating factor; PLA2: Phospholipase A2; ROS: Reactive oxygen species; UCS: Unconditioned stimulus.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived research program WCW. Conceived and designed the experiments: PMH, WCW. Performed the experiments: EB, JI, SNW, PMH. Analyzed the data EB, JI, SNW, PMH, WCW. Wrote the paper: EB, PMH, WCW. All authors read and approved the final manuscript.

Acknowledgements

The work was supported by a National Science and Engineering Research Council (NSERC) Canada operating grant to WCW. The authors thank Dr. A. Buret for the use of a Spectromax micro plate reader.

Received: 25 October 2013 Accepted: 23 April 2014
Published: 1 May 2014

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doi:10.1186/1471-2202-15-56
Cite this article as: Beaulieu *et al.*: Oxidative-stress induced increase in circulating fatty acids does not contribute to phospholipase A_2-dependent appetitive long-term memory failure in the pond snail *Lymnaea stagnalis*. *BMC Neuroscience* 2014 **15**:56.

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