

SHORT COMMUNICATION

Linking the ‘why’ and ‘how’ of ageing: evidence for somatotrophic control of long-term memory function in the pond snail *Lymnaea stagnalis*

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ABSTRACT

Organisms live on a budget; hence, they cannot maximize all their activities at the same time. Instead, they must prioritize how they spend limiting resources on the many processes they rely on in their lives. Among others, they are thought to economize on the maintenance and repair processes required for survival in favour of maximizing reproduction, with ageing as a consequence. We investigate the biological mechanisms of neuronal ageing. Using *Lymnaea stagnalis*, we have previously described various aspects of age-associated neuronal decline and appetitive long-term memory failure. In view of postulated trade-offs between somatic maintenance and reproduction, we tested for interactions between resource allocation mechanisms and brain function. We show that removal of the lateral lobes, which are key regulators of energy balance in *L. stagnalis*, increases body mass and enhances appetitive learning, raising the possibility that the lateral lobes are one of the sites where the ‘why’ and ‘how’ of (neuronal) ageing meet.

KEY WORDS: Invertebrate, Insulin-like peptides, Mollusc, Neuronal plasticity, Life-history optimization, Energy budget

INTRODUCTION

Starting with gestation and ending with death, the life cycles of all species proceed along characteristic timetables with typical patterns of development, growth, maturation, reproduction and characteristic life expectancies that, according to life history theory, are the result of evolutionary optimization of how organisms work around constraints and distribute limiting resources between their various body systems and activities to maximize fitness (Stearns, 1992; Roff, 2002; Flatt and Heyland, 2011). In most species, including our own, this optimized state includes a more or less gradual functional decline during the last stages of life, i.e. most species age. Life history theory and other closely related theories such as the dynamic energy budget theory and the disposable soma theory of ageing treat ageing, either implicitly or explicitly, as a price paid for optimizing the balance of growth, maturation and reproduction at a cost with the

maintenance and repair processes required for individual survival (e.g. Kirkwood, 1977, 2011; Kirkwood and Austad, 2000; Kooijman, 2010; Perlman, 2013).

Ageing is a complex, multi-faceted process that manifests itself in many different ways at all levels of biological organization. Despite substantial effort, many aspects of ageing are still not fully understood. One of those puzzles is how the ultimate causes of ageing connect to its proximate exponents and how ultimate evolutionary theories of ageing (the ‘why’ of ageing) translate to proximate mechanisms (the ‘how’ of ageing). In this study, we embarked on putting our previous findings on age-associated memory failure in an invertebrate model system, in the broader evolutionary perspective of life history theory.

Ageing affects all animal tissues and organ systems. Likewise, neurons and brains tend to deteriorate with time, even when they are not affected by disease. Much has been learned about biological ageing of the nervous system. Yet much more needs to be learned before we truly understand all its many intricacies and facets, including, for example, the issue of how neurophysiological changes occurring with age relate to the symptoms of behavioural and cognitive decline manifest in many healthy ageing metazoans, including humans. Using the pond snail *Lymnaea stagnalis* (Linnaeus 1758), we have gathered biochemical, physiological and behavioural evidence that point towards alterations in (membrane) lipid metabolism as a key factor in the declining neuronal excitability and associative long-term memory (LTM) function that characterize ageing in this model system (Hermann et al., 2007, 2013, 2014; Watson et al., 2012a,b). Here, we investigate how experimental interference with one of the key life history budget control systems of *L. stagnalis* affects LTM function.

Lymnaea stagnalis provides great opportunities for studying this type of problem: its brain provides great practical advantages in linking whole-animal behavioural (dys)functions to their exact neuronal underpinnings and experimental interference with the animals energy budget control can be carried out in a straightforward by extirpation of the so-called ‘lateral lobes’ (LLs), which are small accessory ganglia controlling the animals somatotrophic/gonadotropic balance (Geraerts, 1976a,b, 1992; Roubos et al., 1980). The LLs operate by inhibiting the light green cell (LGC), clusters of neuroendocrine cells located in the cerebral ganglia that produce a set of seven insulin-like peptide homologs (ILPs) called molluscan insulin-like peptides (MIPs; Geraerts, 1976a,b, 1992; Roubos et al., 1980; Geraerts et al., 1992; Smit et al., 1998). In particular, two neuroendocrine neurons, the canopy cells (CCs) located in the LLs, are (based on immunochemical, histochemical and ultrastructural studies) considered to be ectopic specialized LGCs that are implicated in the regulation (i.e. inhibition) of the LGCs (Roubos et al., 1980; Geraerts et al., 1992; Hatakeyama et al., 2000).

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ILPs are an evolutionarily ancient class of peptide hormones that play vital role in the regulation of carbohydrate metabolism, growth, cell survival and a great many other body functions in a wide range of animal species. In recent years, ILPs have been increasingly implicated in brain function. For example, in the mammalian brain, ILPs exert beneficial effects on processes such as growth and development, cell survival, adult neurogenesis, synaptic plasticity and cognition (Isgaard et al., 2007; Åberg, 2010; Fernandez and Torres-Alemán, 2012; Chambers et al., 2015). *Lymnaea stagnalis* appears to form no exception to this rule; several studies report that MIP genes are upregulated during LTM consolidation and that insulin/MIP signalling is necessary for LTM formation (Azami et al., 2006; Murakami, et al., 2013; Mita et al., 2014; Pirger et al., 2014; Kojima et al., 2015). ILPs could, however, also have a ‘dark’ side. They are, paradoxically, implicated with increasing regularity in cell damage and accelerated ageing in many animal model systems and tissues’ and have been linked to aggravation of ageing conditions (Zhao et al., 2004; Cohen and Dillin, 2008; Fernandez and Torres-Alemán, 2012; Gkikas et al., 2014).

Surgical removal of LLs in *L. stagnalis* releases the LGCs from inhibition, leading to an increase in MIP release, changes in carbohydrate homeostasis and an increased rate of body growth at a cost of reduced female reproductive output, i.e. their extirpation causes substantial shifts in the balance of two of the most important resources of the animal (Geraerts, 1976a,b; Roubos et al., 1980; Geraerts, 1992; Geraerts et al., 1992). Hence, manipulating the LLs provides a unique opportunity with which to explore relationships between the domains of life history optimization and nervous system performance.

MATERIALS AND METHODS

Animals and housing conditions

Animals were bred and raised in the laboratory under constant and strictly controlled ambient conditions, as previously described (Hermann et al., 2007; Watson et al., 2012a) (12 h:12 h light:dark, ambient temperature 18–19°C). To maintain tight control over the snails aquatic environment, the animals were kept in artificial pond water (pH 7.4–7.6) reconstituted in-house from purified reverse osmosis (RO) water reconditioned to a conductivity of ~450 $\mu\text{S cm}^{-1}$ by the addition of Instant Ocean salts (Aquarium Systems, Mentor, OH, USA) at 0.26 g per litre and calcium to a saturating level of ~60 mg l^{-1} in the form of calcium carbonate (light powder; EMD analytics, Gibbstown, NJ, USA) to the tanks. In addition, animals had continuous access to sterilized cuttlefish (*Sepia officinalis*) bone (two or three per tank). Together, these additions chemically recondition the RO source water to that of water within the range of that reported for the natural environments of the species (concentrations for main ion species in mg l^{-1} : Na^+ 56; K^+ 2; Ca^{2+} 60; Mg^{2+} 6.7; Cl^- 98; SO_4^{2-} 14). Animals were fed *ad libitum* with a standard diet consisting of Romaine lettuce and Aquamax-carniverous Grower 600 trout pellets (Purina Mills, St Louis, MO, USA). For the present study, fully sexually mature snails (age 7–9 months; shell length 2.5–3.0 cm) were taken at random from an age-synchronized population displaying survival characteristics of healthy ageing populations (see Hermann et al., 2014 for further details on population survival assessment).

Surgery and lateral lobe extirpation

Both LLs were extirpated using a method modified from Scheibenstock et al. (2002). These lobes can be extirpated without destruction of any other part of the cerebral and other ganglia (see also Geraerts, 1976b). Animals were allowed to recover for at least

10 days before being tested. Untreated (no surgery) and sham-operated animals from the same populations were included as controls. The latter animals were handled in exactly the same way as described above, except that the lateral lobes were kept intact. Sham-operated and LL-extirpated animals were kept in the same tank separated by a perforated screen dividing the tank in half. For identification purposes, each animal was provided a unique code on its shell with an indelible marker. One set of animals was used to monitor survival rate and changes in total wet body mass. A separate set of animals was used to determine the effect of LL extirpation on sensory and motor control, and LTM formation.

Each LL contains a single CC with each cell having an axonal projection in the contralateral median lip nerve (van Minnen et al., 1979; Benjamin et al., 1980). To verify the success of the LL extirpation, the left or right median lip nerve was retrogradely back-labelled with nickel-lysine (for detailed procedures, see Hermann et al., 2000). After back-labelling for 24–36 h, the preparations were developed with rubeanic acid and examined for the absence or presence of CC staining and photographed. All successful backfills should show staining of at least one contralateral LGC underneath the dorsal body. Therefore, preparations without contralateral LGC staining were deemed incomplete and excluded from analysis.

Sucrose response testing

To assess whether LL extirpation alters chemo-sensation and/or motor control, feeding response to sucrose (0.4% w/v; i.e. 11.7 mmol l^{-1} final concentration in artificial pond water) were tested in LL-extirpated, sham-operated and no-surgery animals as per established protocol (Watson et al., 2012b; Hermann et al., 2013). Tests were performed using 100-ml translucent polystyrene beakers (4.5 cm diameter), filled with 80 ml of artificial pond water that allowed for full submergence of the animals. 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 three consecutive periods of 2 min, the second period starting with gentle administration of artificial pond water (10 ml) [which is a disturbance stimulus (DS)] and the third period starting with the administration of sucrose solution (0.4% w/v final concentration). The disturbance 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. $\Delta\text{Rasp disturbance} = \text{rasps after DS} - \text{rasps after pond water}$). The sucrose response was calculated by taking the difference between the number of rasps counted during the third period from the number counted during the second period (i.e. $\Delta\text{Rasp sucrose} = \text{rasps after sucrose} - \text{rasps after DS}$).

Appetitive classical conditioning paradigm

LTM performance was assessed 10–12 days after surgery using an established single-day, multi-trial appetitive classical conditioning protocol (Hermann et al., 2007, 2013; Watson et al., 2012b; Beaulieu et al., 2014). Snails were sampled at random and marked for identification purposes with indelible marker. Food was withheld starting 48 h prior to the first pre-training test and for the remainder of the training and post-training testing.

Pre- and post-test procedures

On day 1, prior to behavioural conditioning, snails were individually tested for their natural response to the administration of artificial pond water, the disturbance stimulus (DS) and the conditional stimulus (CS) n-amyl acetate (‘pre-training test’). Tests were performed using 100 ml translucent polystyrene beakers

(4.5 cm diameter), filled with 80 ml of artificial pond water (this would provide a water column of ~4.5 cm). 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) and the second period starting with the administration of the CS (10 ml *n*-amyl acetate in artificial pond water; 4 ppm final concentration). To facilitate observation, the test beakers were elevated on translucent plastic stands surrounded by mirrors. The *n*-amyl acetate rasping response was calculated by taking the difference between the number of rasps counted during the second period from the number counted during the first period (i.e. $\Delta\text{Rasp} = \text{rasps after CS} - \text{rasps after DS}$). To correct for both differences in background rasping activity and potential application artefacts, the pre-training tests were performed in duplicate at least 1 h apart, and behavioural responses were calculated as the average of both ΔRasp (i.e. $\Delta\text{Rasp}_{\text{pre-test}} = \text{average } \Delta\text{Rasp}_{\text{pre-test1}} \text{ and } \Delta\text{Rasp}_{\text{pre-test2}}$). After completion of a test, snails were gently rinsed with artificial pond water and returned to their home tanks. A single post-training test was performed on day 3 (22–24 h after the last training) following identical procedures described for the pre-testing above.

Training procedure

Snails were trained in a single-day, multi-trial forward-delay conditioning format. Sucrose dissolved in artificial pond water (to a final concentration of 0.4% w/v) served as the unconditioned stimulus (UCS) and *n*-amyl acetate (4 ppm final concentration) served 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. artificial pond water) was implemented. Snails were randomly assigned to either the CS–UCS ('conditioned') or the CS–DS ('non-conditioned') group and trained 'en masse'. Training was performed in 1 l polypropylene beakers (9 cm in diameter) containing 480 ml artificial pond water providing a water column of ~9 cm. 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 artificial 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 of clean artificial pond water. After 11 min and 45 s, the training procedure was repeated. Snails received a total of five training procedures on a single day. The snails were at all times fully submerged during training and testing. Care was taken to ensure that pre-training tests, training and post-training tests commenced at the same time of day for each group and always occurred in the same location. 'Conditioned' and 'non-conditioned' snails were always tested and trained concurrently.

Our training assay and protocol are direct adaptations from protocols developed by Audesirk et al. (1982) and Alexander et al. (1982, 1984). They differ slightly from related appetitive training protocols commonly used by other research groups (e.g. Kemenes et al., 2011; Marra et al., 2013) in that we fully submerged the animals in training and testing solutions, employed multi-trial rather than single-trial training protocols, and used slightly lower CS (0.0004% *n*-amyl acetate) and UCS (0.4% sucrose) concentrations than were previously shown to provide optimal behavioural responses under our experimental conditions (see Hermann et al., 2007; Watson et al., 2012b; Hermann et al., 2013; Beaulieu et al.,

2014). The use of 0.4% sucrose as an UCS creates a safe margin with the very low UCS concentration (0.067% sucrose) previously reported to induce memory lapses in this learning model system (Marra et al., 2013).

Statistical analysis

Data were analysed using Statistica version 7.1 (StatSoft, Tulsa, OK, USA), using repeated measures ANOVA for body growth data and a multifactorial ANOVA for LTM and sucrose response data. Compliance with parametric assumptions was confirmed for each data set analysed using 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). Log-rank (Mantel–Cox) test for survival data and contingency test of backfill success rate were performed in Graphpad Prism version 5.0b (Graphpad Software, La Jolla, CA, USA). Throughout the text, average and data dispersion are expressed as arithmetic means and, unless indicated otherwise, standard error of the mean (s.e.m.). All graphs were made using Graphpad Prism.

RESULTS AND DISCUSSION

Verification of surgical procedures

Each LL contains a single CC with an axonal projection in the contralateral median lip nerve (van Minnen et al., 1979; Benjamin et al., 1980). Thus, effectiveness of LL extirpation procedures can be evaluated by retrograde nickel-lysine labelling of the contralateral median lip nerve: i.e. no soma labelling of the CC will be observed when extirpation of the LL was successful. Fig. 1A shows an example of a sham-operated (Ai) and LL-extirpated (Aii) backfilled preparation. Despite the labelling in both preparations of a contralateral LGC (arrowhead), which is indicative of a complete backfill of the median lip nerve, staining of the CC is absent in the LL-extirpated CNS (compare the labelled cell circled in Ai with the absence of a labelled cell circled in Aii). Like most other neuronal tracing techniques, retrograde labelling with nickel lysine is not perfect. Although none of the 20 LL-extirpated preparations showed labelling, a low, statistically insignificant, number of false negatives were observed in the no surgery (2 of 15) and sham-operated control preparations (5 of 19; $\chi^2 = 31.76$, $P < 0.0001$; Fig. 1B). Thus, we are confident that LL extirpation procedures were effective.

LL extirpation does not affect survival

Survival rate of animals was monitored in no surgery, sham-operated and LL-extirpated animals for 4 weeks following surgery. Significant differences in survival were found in the three cohorts ($\chi^2 = 8.512$, $P = 0.0142$; Fig. 2A). Whereas no deaths occurred in the no-surgery group, 33% and 34% of the animals in, respectively, the sham-operated and LL-extirpated groups died before post-surgery day 9. Survival of these two groups stabilized after this time point (Fig. 2A). Multiple comparison analysis revealed significant differences in survival between both sham-operated and no-surgery snails ($\chi^2 = 9.423$, $P = 0.0021$) and LL-extirpated and no-surgery snails ($\chi^2 = 8.083$, $P = 0.0045$). There was no difference in survival between sham-operated and LL-extirpated snails ($\chi^2 = 0.140$, $P = 0.71$). Thus, whereas surgery affects survival for 1–2 weeks post-surgery, LL extirpation itself does not appear to have further effects on survival within the first month post-surgery.

LL extirpation induces increase in total body mass

In view of earlier findings that LLs control body growth of juvenile sexually immature animals (Geraerts, 1976a,b; Roubos et al., 1980), we examined whether LL extirpation also affected body mass in the

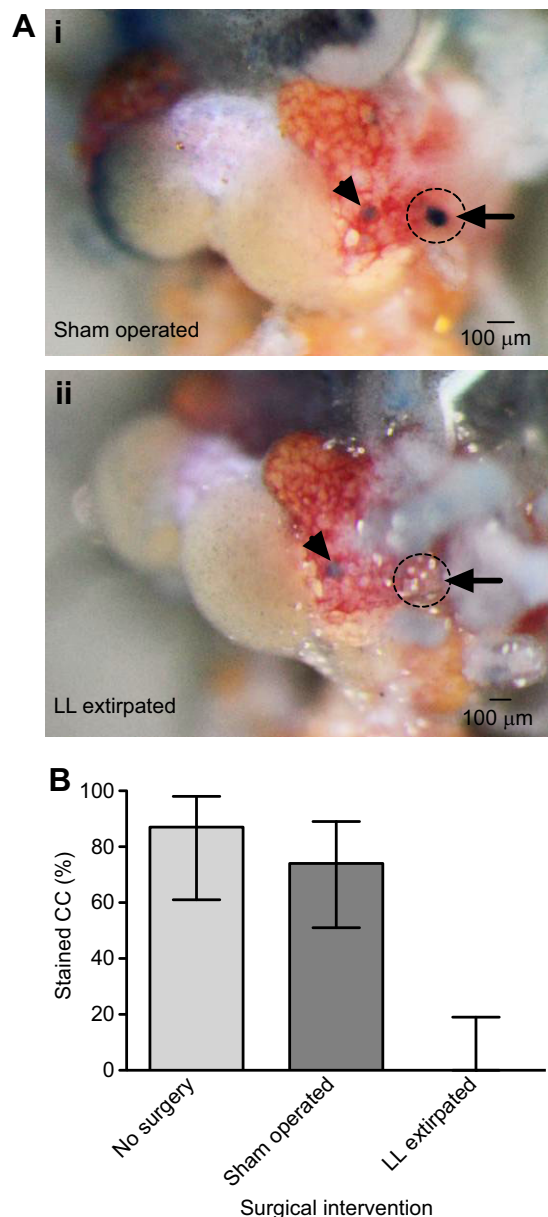


Fig. 1. Verification of lateral lobe (LL) extirpation. Nickel-lysine backfill of medium lip nerve was used to determine success of LL extirpation. (A) Photographic examples of backfill of the left medium lip nerve and contralateral staining in the right cerebral ganglion and attached LL (circle) of sham-operated (i) and LL-extirpated (ii) preparations. Note the presence and absence of a labelled canopy cell (CC) soma in the LL (arrow) in, respectively, sham-operated and LL-extirpated central nervous systems, which are indicative of successful LL extirpation. In both preparations, a single light green cell (LGC) soma is visible (arrowhead), demonstrating complete staining of the medium lip nerve. (B) Average percentage ($\pm 95\%$ confidence interval) of nickel-lysine stained CCs in no-surgery, sham-operated and LL-extirpated preparations.

older sexually mature animals used here. Before surgery there was no difference in total wet body mass of sham-operated and LL-extirpated animals (Student's *t*-test: $t=0.4532$ d.f.=33, $P=0.65$). On average, sham-operated animals weighed 1.60 ± 0.09 g ($n=18$), whereas the LL-extirpated animals weighed on average 1.55 ± 0.06 g ($n=17$). Although both groups showed a steady increase in body mass after surgery, body mass increased significantly faster in the LL-extirpated animals (repeated-

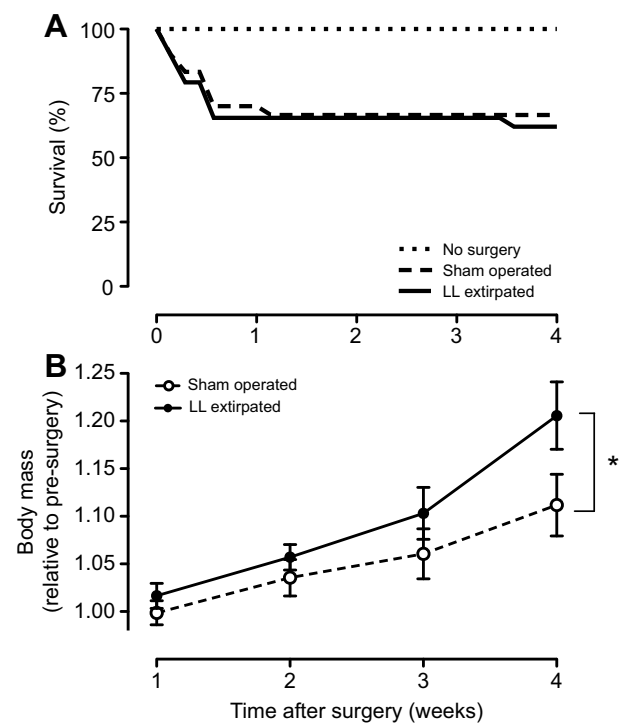


Fig. 2. Effect of LL extirpation on survival and body mass. (A) Survival rate for 4 weeks after surgery of the no-surgery, sham-operated and LL-extirpated animals. Surgery in general appears to have a small acute effect on survival rate, but there is no additional deleterious effect of LL extirpation on survival. (B) Relative changes in whole body mass of sham-operated and LL-extirpated animals. LL-extirpated animals significantly increased their body mass. But the growth-stimulating effect of LL removal does not become apparent until after 3 weeks. * $P < 0.05$.

measures ANOVA, treatment \times time, $F_{4,132}=3.2727$, $P=0.014$; Fig. 2B). Four weeks after surgery the LL-extirpated animals had gained significantly more body mass (21% mass gain relative to pre-surgery mass) than the sham-operated animals (11% mass gain relative to pre-surgery mass; planned comparison, $F_{1,33}=5.707$, $P=0.02$; Fig. 2B). Thus, we conclude that, as previously reported for sexually immature juveniles (Geraerts, 1976a,b), LL extirpation accelerates somatic growth in sexually mature animals. In congruence with the latter studies, we found that the growth-stimulating effect of LL removal does not become apparent until after 3 weeks. Together, these data support the notion that the effects of LL on body mass are mediated by altering the (neurosecretory) activity of the LGCs.

LL extirpation improves LTM formation

Our main objective was to determine whether LL extirpation alters appetitive LTM formation. This learning model uses sucrose as a UCS and therefore relies on the ability of an animal to detect sucrose and respond with feeding movements to its application (Hermann et al., 2007). To exclude the possibility that surgery affected UCS efficacy, we first examined whether extirpation of the LL altered the unconditioned response (UR) to sucrose application. We found that the behavioural response characteristics of the animals were not affected by surgery (Fig. 3A); UCS application induced robust, statistically indistinguishable, URs in all three test groups (factorial ANOVA $F_{2,91}=0.6559$, $P=0.52$), whereas DS application did not elicit a significant behavioural response in either of the groups (factorial ANOVA $F_{2,91}=0.1341$, $P=0.87$). These findings lead us to conclude that neither surgery alone nor LL extirpation impeded or

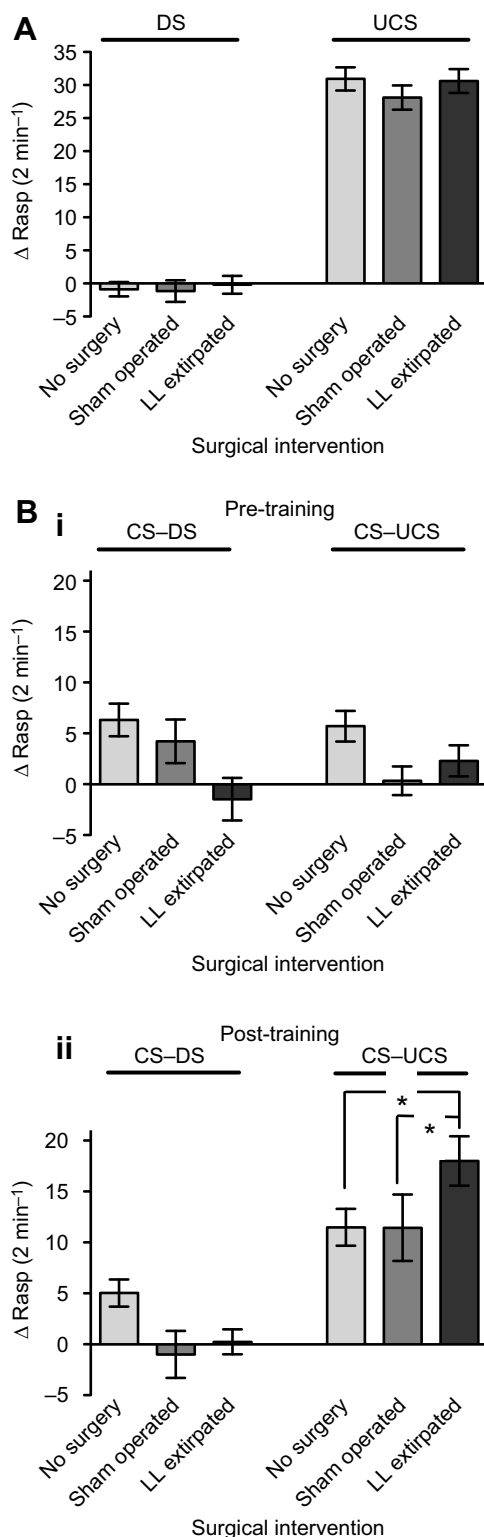


Fig. 3. Effect of LL extirpation on sensory and motor aspects of the feeding response and LTM formation. (A) Average change in the number of rasps evoked by application of a DS or UCS. Only the UCS induced a robust but similar response in animals that underwent no surgery, sham operation or LL extirpation. (B) LTM assessment in no surgery, sham-operated and LL-extirpated animals. None of the animals responded with significant feeding movements in the pre-test (i). Similarly, none of the non-conditioned animals (CS–DS) responded with substantial feeding movements in the post-training test (ii). In contrast, animals of all three treatment groups showed a robust feeding response upon conditioning (CS–UCS) in the post-training test (ii). Of the conditioned animals, LL-extirpated animals rasped significantly more in reaction to CS application in the post-test compared with the no-surgery and sham-operated animals. * $P < 0.05$.

displayed a conditioned response (CR; ANOVA, $F_{2,130}=2.138$, $P=0.122$; Fig. 3B). Training induced a significant increase in CS-induced rasping in the CS–UCS group of animals but not in the CS–DS control group (ANOVA, training \times treatment $F_{2,130}=4.265$, $P=0.016$). Furthermore, the CR of conditioned, LL-extirpated animals was potentiated relative to that of both the conditioned, sham-operated animals (planned comparison, $F_{1,130}=4.512$, $P=0.035$) and conditioned, no-surgery animals (planned comparison, $F_{1,130}=5.795$, $P=0.017$).

The results described above support the conclusion that LL extirpation enhances appetitive LTM formation. Our results have various important implications. First, considering that extirpation of the LLs enhances MIP release by releasing the LGCs from inhibition (Geraerts, 1976a,b, 1992; Roubos et al., 1980; Geraerts et al., 1992; Smit et al., 1998), they argue for the existence of a causal link between learning and memory performance, somatotropic control and ILP neuroendocrinology in *L. stagnalis*. As such, our findings echo a growing literature linking somatotropic systems to cognition and other brain functions in both vertebrates and invertebrates (Creyghton et al., 2004; Isgaard et al., 2007; Studzinski et al., 2015; Chambers et al., 2015). Second, as the LLs of *L. stagnalis* arbitrate in the division of resources between growth and reproduction, which are two of the principal items of organismal resource budgets and two of the most important life history variables, our results implicitly corroborate the existence of trade-offs between reproduction and nervous system function, and, intriguingly, suggest that LLs may be one of the key loci that set the pace of neuronal and cognitive ageing in this model system.

The idea that ILPs play important and diverse roles in the fate of the nervous system is supported by various lines of evidence implicating ILPs in aspects of neuronal plasticity and cognitive function in a wide range of invertebrate and vertebrate species, including *L. stagnalis* (Creyghton et al., 2004; Zhao et al., 2004; Azami et al., 2006; Isgaard et al., 2007; Fernandez and Torres-Alemán, 2012; Murakami et al., 2013; Mita et al., 2014; Pirger et al., 2014; Chambers et al., 2015; Kojima et al., 2015; Studzinski et al., 2015; Akinola, 2016; Tanabe et al., 2017). Moreover, ILPs are also known to be important regulators of adult neurogenesis, angiogenesis and synaptogenesis, and insulin has been shown to affect synapse remodelling through downstream neurotrophic signalling cascades (Granata et al., 2007; Nelson et al., 2008; Åberg, 2010). On the flipside of the same coin, ILP signalling is frequently implicated in the nervous system's demise. For example, low levels of ILPs and/or insulin resistance are currently seen as a major factor in age-related memory impairment and neurodegenerative diseases (Williamson et al., 2012; Calvo-Ochoa and Arias, 2015; Akinola, 2016), but even normal and high levels of ILPs are increasingly implicated in cell damage and accelerated ageing in many animal model systems and tissues,

altered sensory or motor functions underlying the learning paradigm.

Next, we assessed the effect of LL extirpation on LTM formation. No-surgery ($n=59$), sham-operated ($n=34$) and LL-extirpated ($n=43$) animals were subjected to either a paired CS–UCS conditioning protocol or paired CS–DS protocol to control for unconditioned behavioural responses to water disturbance. Regardless of their surgical history, none of the naive animals

including the brain (Cohen and Dillin, 2008; Fernandez and Torres-Alemán, 2012). Likewise, the idea of ILP involvement in the regulation of aspects of ageing is, by itself, not a novel one (Sadagurski and White, 2013; Altintas et al., 2016). Yet, the present study adds to what we see as a fascinating novel perspective to this field: by associating the LLs, two tiny accessory CNS lobes that arguably constitute a metabolic control centre in *L. stagnalis*, with the associative learning abilities of an animal, we have may have identified the embodiment of a nexus of ultimate evolutionary causes of ageing and proximate mechanisms of brain ageing in this model system.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: P.M.H., W.C.W.; Methodology: L.d.W., P.M.H., W.C.W.; Formal analysis: L.d.W., P.M.H., W.C.W.; Investigation: L.d.W., P.M.H.; Writing - original draft: L.d.W., P.M.H., W.C.W.; Writing - review & editing: P.M.H., W.C.W.; Supervision: W.C.W.; Funding acquisition: W.C.W.

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References

- Åberg, D. (2010). Role of the growth hormone/insulin-like growth factor 1 axis in neurogenesis. *Endocr. Dev.* **17**, 63–76.
- Akinola, O. B. (2016). Sweet old memories: a review of the experimental models of the association between diabetes, senility and dementia. *Metab. Brain Dis.* **31**, 1003–1010.
- Alexander, J. E., Jr, Audesirk, T. E. and Audesirk, G. J. (1982). Rapid, nonaversive conditioning in a freshwater gastropod. II. Effects of temporal relationships on learning. *Behav. Neural Biol.* **36**, 391–402.
- Alexander, J., Jr, Audesirk, T. E. and Audesirk, G. J. (1984). One-trial reward learning in the snail *Lymnaea stagnalis*. *J. Neurobiol.* **15**, 67–72.
- Altintas, O., Park, S. and Lee, S.-J. V. (2016). The role of insulin/IGF-1 signaling in the longevity of model invertebrates, *C. elegans* and *D. melanogaster*. *BMB Rep.* **48**, 81–92.
- Audesirk, T. E., Alexander, J. E., Jr, Audesirk, G. J. and Moyer, C. M. (1982). Rapid, nonaversive conditioning in a freshwater gastropod. I. Effects of age and motivation. *Behav. Neural Biol.* **36**, 379–390.
- Azami, S., Wagatsuma, A., Sadamoto, H., Hatakeyama, D., Usami, T., Fujie, M., Koyanagi, R., Azumi, K., Fujito, Y., Lukowiak, K. et al. (2006). Altered gene activity correlated with long-term memory formation of conditioned taste aversion in *Lymnaea*. *J. Neurosci. Res.* **84**, 1610–1620.
- Beaulieu, E., Ioffe, J., Watson, S. N., Hermann, P. M. and Wildering, W. C. (2014). Oxidative-stress induced increase in circulating fatty acids does not contribute to phospholipase A2-dependent appetitive long-term memory failure in the pond snail *Lymnaea stagnalis*. *BMC Neurosci.* **15**, 56.
- Benjamin, P. R., Slade, C. T. and Soffe, S. R. (1980). The morphology of neurosecretory neurones in the pond snail, *Lymnaea stagnalis*, by the injection of Procion Yellow and horseradish peroxidase. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **290**, 449–478.
- Calvo-Ochoa, E. and Arias, C. (2015). Cellular and metabolic alterations in the hippocampus caused by insulin signalling dysfunction and its association with cognitive impairment during aging and Alzheimer's disease: studies in animal models. *Diabetes Metab. Res. Rev.* **31**, 1–13.
- Chambers, D. B., Androschuk, A., Rosenfelt, C., Langer, S., Harding, M. and Bolduc, F. V. (2015). Insulin signaling is acutely required for long-term memory in *Drosophila*. *Front. Neural Circuits* **9**, 8.
- Cohen, E. and Dillin, A. (2008). The insulin paradox: aging, proteotoxicity and neurodegeneration. *Nat. Rev. Neurosci.* **9**, 759–767.
- Creyghton, W. M., van Dam, P. S. and Koppeschaar, H. P. (2004). The role of the somatotrophic system in cognition and other cerebral functions. *Semin. Vasc. Med.* **4**, 167–172.
- Fernandez, A. M. and Torres-Alemán, I. (2012). The many faces of insulin-like peptide signalling in the brain. *Nat. Rev. Neurosci.* **13**, 225–239.
- Flatt, T. and Heyland, A. (2011). *Mechanisms of Life History Evolution. The Genetics and Physiology of Life History Traits and Trade-Offs*. Oxford: Oxford University Press.
- Geraerts, W. P. M. (1976a). Control of growth by the neurosecretory hormone of the light green cells in the freshwater snail *Lymnaea stagnalis*. *Gen. Comp. Endocrinol.* **29**, 61–71.
- Geraerts, W. P. M. (1976b). The role of the lateral lobes in the control of growth and reproduction in the hermaphrodite freshwater snail *Lymnaea stagnalis*. *Gen. Comp. Endocrinol.* **29**, 97–108.
- Geraerts, W. P. M. (1992). Neurohormonal control of growth and carbohydrate metabolism by the Light Green cells in *Lymnaea stagnalis*. *Gen. Comp. Endocrinol.* **86**, 433–444.
- Geraerts, W. P. M., Smit, A. B., Li, K. W. and Hordijk, P. L. (1992). The Light Green cells of *Lymnaea*: a neuroendocrine model system for stimulus-induced expression of multiple peptide genes in a single cell type. *Experientia* **48**, 464–473.
- Gkikas, I., Petratou, D. and Tavernarakis, N. (2014). Longevity pathways and memory aging. *Front. Genet.* **5**, 155.
- Granata, R., Trovato, L., Lupia, E., Sala, G., Settanni, F., Camussi, G., Ghidoni, R. and Ghigo, E. (2007). Insulin-like growth factor binding protein-3 induces angiogenesis through IGF-I- and SphK1-dependent mechanisms. *J. Thromb. Haemost.* **5**, 835–845.
- Hatakeyama, D., Ito, I., Kojima, S., Fujito, Y. and Ito, E. (2000). Complement receptor 3-like immunoreactivity in the light green cells and the canopy cells of the pond snail, *Lymnaea stagnalis*. *Brain Res.* **865**, 102–106.
- Hermann, P. M., Wildering, W. C. and Bulloch, A. G. M. (2000). Functional recovery of respiratory behavior during axonal regeneration in snails (*Lymnaea stagnalis*) is experience dependent. *Behav. Neurosci.* **114**, 410–423.
- Hermann, P. M., Lee, A., Hulliger, S., Minvielle, M., Ma, B. and Wildering, W. C. (2007). Impairment of long-term associative memory in aging snails (*Lymnaea stagnalis*). *Behav. Neurosci.* **121**, 1400–1414.
- Hermann, P. M., Park, D., Beaulieu, E. and Wildering, W. C. (2013). Evidence for inflammation-mediated memory dysfunction in gastropods: putative PLA2 and COX inhibitors abolish long-term memory failure induced by systemic immune challenges. *BMC Neurosci.* **14**, 83.
- Hermann, P. M., Watson, S. N. and Wildering, W. C. (2014). Phospholipase A2 -nexus of aging, oxidative stress, neuronal excitability, and functional decline of the aging nervous system? Insights from a snail model system of neuronal aging and age-associated memory impairment. *Front. Genet.* **5**, 419.
- Isgaard, J., Åberg, D. and Nilsson, M. (2007). Protective and regenerative effects of the GH/IGF-I axis on the brain. *Minerva Endocrinol.* **32**, 103–113.
- Kemenes, I., O'Shea, M. and Benjamin, P. R. (2011). Different circuit and monoamine mechanisms consolidate long-term memory in aversive and reward classical conditioning. *Eur. J. Neurosci.* **33**, 143–152.
- Kirkwood, T. B. L. (1977). Evolution of ageing. *Nature* **270**, 301–304.
- Kirkwood, T. B. L. (2011). Systems biology of ageing and longevity. *Philos. Trans. R. Soc. B* **366**, 64–70.
- Kirkwood, T. B. L. and Austad, S. N. (2000). Why do we age? *Nature* **408**, 233–238.
- Kojima, S., Sunada, H., Mita, K., Sakakibara, M., Lukowiak, K. and Ito, E. (2015). Function of insulin in snail brain in associative learning. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* **201**, 969–981.
- Kooijman, S. A. L. M. (2010). *Dynamic Energy Budget Theory for Metabolic Organisation*, 3rd edn. Cambridge, UK: Cambridge University Press.
- Marra, V., O'Shea, M., Benjamin, P. R. and Kemenes, I. (2013). Susceptibility of memory consolidation during lapses in recall. *Nat. Commun.* **4**, 1578.
- Mita, K., Yamagishi, M., Fujito, Y., Lukowiak, K. and Ito, E. (2014). An increase in insulin is important for the acquisition conditioned taste aversion in *Lymnaea*. *Neurobiol. Learn. Memory* **116**, 132–138.
- Murakami, J., Okada, R., Sadamoto, H., Kobayashi, S., Mita, K., Sakamoto, Y., Yamagishi, M., Hatakeyama, D., Otsuka, E., Okuta, A. et al. (2013). Involvement of insulin-like peptide in long-term synaptic plasticity and long-term memory of the pond snail *Lymnaea stagnalis*. *J. Neurosci.* **33**, 371–383.
- Nelson, T. J., Sun, M.-K., Hongpaisan, J. and Alkon, D. L. (2008). Insulin, PKC signaling pathways and synaptic remodeling during memory storage and neuronal repair. *Eur. J. Pharm.* **585**, 76–87.
- Perlman, R. L. (2013). Life history tradeoffs and the evolutionary biology of aging. In *Evolution and Medicine* (ed. R. L. Perlman), pp. 51–64. Oxford: Oxford University Press.
- Pirger, Z., Naskar, S., László, Z., Kemenes, G., Reglődi, D. and Kemenes, I. (2014). Reversal of age-related learning deficiency by the vertebrate PACAP and IGF-1 in a novel invertebrate model of aging: the pond snail (*Lymnaea stagnalis*). *J. Gerontol. A Biol. Sci. Med. Sci.* **69**, 1331–1338.
- Roff, D. A. (2002). *Life History Evolution*. Sunderland: Sinauer Associates, Inc.
- Roubos, E. W., Geraerts, W. P. M., Boerrigter, G. H. and Van Kampen, G. P. J. (1980). Control of the activities of the neurosecretory Light Green and Caudodorsal cells and of the endocrine Dorsal Bodies by the Lateral Lobes in the freshwater snail *Lymnaea stagnalis* (L.). *Gen. Comp. Endocrinol.* **40**, 446–454.
- Sadagurski, M. and White, M. F. (2013). Integrating metabolism and longevity through insulin and IGF1 signaling. *Endocrinol. Metab. Clin. North Am.* **42**, 127–148.
- Scheibenstock, A., Krygier, D., Haque, Z., Syed, N. and Lukowiak, K. (2002). The soma of RPeD1 must be present for long-term memory formation of associative learning in *Lymnaea*. *J. Neurophysiol.* **88**, 1584–1591.
- Smit, A. B., Van Kesteren, R. E., Li, K. W., Van Minnen, J., Spijker, S., Van Heerikhuizen, H. and Geraerts, W. P. M. (1998). Towards understanding the role of insulin in the brain: Lessons from insulin-related signaling systems in the invertebrate brain. *Progr. Neurobiol.* **54**, 35–54.

- Stearns, S. C.** (1992). *The Evolution of Life Histories*. Oxford: Oxford University Press.
- Studzinski, A. L. M., Barros, D. M. and Marins, L. F.** (2015). Growth hormone (GH) increases cognition and expression of ionotropic glutamate receptors (AMPA and NMDA) in transgenic zebrafish (*Danio rerio*). *Behav. Brain Res.* **294**, 36–42.
- Tanabe, K., Itoh, M. and Tonoki, A.** (2017). Age-related changes in insulin-like signaling lead to intermediate-term memory impairment in *Drosophila*. *Cell Rep.* **18**, 1598–1605.
- Van Minnen, J., Reichelt, D. and Lodder, J. C.** (1979). An ultrastructural study of the neurosecretory canopy cell of the pond snail *Lymnaea stagnalis* (L.), with the use of the horseradish peroxidase tracer technique. *Cell Tissue Res.* **204**, 453–462.
- Watson, S. N., Nelson, M. A. and Wildering, W. C.** (2012a). Redox agents modulate neuronal activity and reproduce physiological aspects of neuronal aging. *Neurobiol. Aging* **33**, 149–161.
- Watson, S. N., Risling, T. E., Hermann, P. M. and Wildering, W. C.** (2012b). Failure of delayed nonsynaptic neuronal plasticity underlies age-associated long-term associative memory impairment. *BMC Neurosci.* **13**, 113.
- Williamson, R., McNeilly, A. and Sutherland, C.** (2012). Insulin resistance in the brain: an old-age or new-age problem? *Biochem. Pharmacol.* **84**, 737–745.
- Zhao, W.-Q., Chen, H., Quon, M. J. and Alkon, D. L.** (2004). Insulin and the insulin receptor in experimental models of learning and memory. *Eur. J. Pharmacol.* **490**, 71–81.