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RESEARCH ARTICLE

A change in taste: the role of microRNAs in altering hedonic value

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ABSTRACT

The mechanisms associated with neophobia and anhedonia remain largely unknown. Neuropsychological disorders such as depression and schizophrenia are associated with excessive fear and anhedonia, and have been linked to microRNA 137. We hypothesized that microRNAs (miRNAs) in the snail *Lymnaea stagnalis* are important for regulating feeding behaviour through either preventing neophobia or establishing hedonic value. To test these hypotheses, we used an injection of poly-L-lysine (PLL) to inhibit miRNA biogenesis and observed its effects on feeding behaviour. We repeated these experiments with pre-exposure to novel stimuli capable of eliciting neophobia to disentangle the processes predicted to regulate feeding behaviour. Next, we exposed snails to food stimuli of high hedonic value after PLL injection to reset their hedonic value for that food. Finally, we consolidated our results with previous research by examining the effect of PLL injection on a one-trial appetitive classical conditioning procedure (1TT) to induce long-term memory (LTM). We found that miRNAs are likely not required for preventing neophobia. Moreover, we discovered that snails experienced anhedonia in response to inhibition of miRNA biogenesis, resulting in diminished feeding behaviour for food stimuli with a previously high hedonic value. Snails showed diminished feeding behaviour for multiple food stimuli of high hedonic value post-1TT with PLL injection. This finding suggests that PLL causes anhedonia rather than an impairment of LTM formation following the 1TT procedure. This is the first evidence suggesting that inhibiting the biogenesis of miRNAs contributes to anhedonia in *L. stagnalis*.

KEY WORDS: Poly-L-lysine, Memory, Learning, Novel, Anhedonia, Neuropsychiatric disorders

INTRODUCTION

Anxiety and depression are mood disorders that affect millions of people, causing disruptions in appetite, loss of interest (anhedonia), excessive worry or fear and feelings of extreme sadness, and guilt (WHO, 2012). Schizophrenia is another debilitating psychological disorder which results in positive, negative and cognitive symptoms (Tandon et al., 2009). Although research has been working towards increasing our understanding of the pathophysiology of these neuropsychiatric disorders, the mechanisms associated with their pathogenesis have yet to be completely understood. Most interestingly for us, genome-wide association studies (GWAS) indicate a common link between these disorders: microRNA 137 [Schizophrenia Psychiatric Genome-Wide Association Study (GWAS), 2011].

MicroRNAs (miRNAs) are short non-coding RNAs, 20–23 nucleotides in length. miRNAs play key roles in many biological processes and regulate protein synthesis and gene expression at the translational level through reversible translational repression or mRNA degradation (O'Brien et al., 2018). Like many known epigenetic processes that allow the nervous system to adapt to environmental signals, the miRNA system has been found to control the expression of genes (McNeill and Van Vactor, 2012). The miRNA system produces epigenetic changes by altering the neuronal compartments: axons, dendrites, synapses and growth cones of already synthesized RNAs (McNeill and Van Vactor, 2012).

miRNAs are prevalent in the central nervous system of both vertebrates and invertebrates and have been implicated in higher-order brain processing, including learning and memory, drug addiction and neurodegenerative disease (Cao et al., 2016). Problems with miRNA biogenesis and dysregulation of miRNA expression play a role in human neurological deficits and neuropathological disorders (Martino et al., 2009). One important biological process involving miRNAs is learning and memory formation. Long-term memory (LTM) formation requires activity-dependent gene transcription and translation in neurons, and recruits regulators of these processes, such as miRNAs (McNeill and Van Vactor, 2012). Thus, miRNAs play an essential role in the formation of LTM by regulating both transcriptional and translational processes in neurons.

Lymnaea stagnalis has been used as a model to study the role of non-coding RNAs in memory formation after single-trial classical conditioning of appetitive feeding behaviour (Korneev et al., 2018). In *L. stagnalis*, a one-trial training (1TT) procedure using amyl acetate as the conditional stimulus (CS) and sucrose as an unconditional stimulus (UCS) leads to LTM formation (Alexander et al., 1984). That is, after the 1TT procedure, amyl acetate elicits feeding behaviour, whereas before conditioning it did not. This training procedure allows learning-induced molecular changes underlying LTM to be connected. By blocking Dicer, an important ribonuclease for miRNA biogenesis, it was determined that Dicer was required for 1TT-induced LTM formation in *L. stagnalis* (Korneev et al., 2018). These authors used poly-L-lysine (PLL), a well-known inhibitor of Dicer (Watashi et al., 2010) for miRNA silencing experiments. A significant decrease of the conditioned feeding response was observed for animals injected with PLL 15 min after conditioning compared with control snails. Korneev et al. (2018) concluded that the inhibition of Dicer leads to impairment of LTM formation. Further, next-generation sequencing revealed that *Lym*-miR-137 was transiently upregulated 1 h after conditioning. They further showed that this miRNA targets mRNA encoding cAMP-response element binding protein 2 (CREB2), a transcription factor that is implicated in memory repression (Karpinski et al., 1992). Employing an *in vivo* loss-of-function approach, *Lym*-miR-137 was downregulated using a mirVANA

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miRNA inhibitor. Through this approach it was suggested that the injection of a specific *Lym*-miR-137 inhibitor before single-trial training increased the level of *Lym*-CREB2 mRNA and suppressed LTM in the same conditioned animals. Thus, Korneev et al. (2018) suggested that *Lym*-miR-137 is essential for single-trial training-induced LTM formation as it causes downregulation of *Lym*-CREB2 mRNA expression in learning ganglia. However, there are other interpretations of those data.

While miR-137 has an established importance in memory formation, it has also been implicated in many other functions. miR-137 is a brain-enriched miRNA that plays an important role in determining the fate of neural stem cells, neuronal proliferation and differentiation, and synaptic maturation. Recently, miR-137 has been demonstrated to regulate several forms of synaptic plasticity and signalling cascades involved in schizophrenia (Thomas et al., 2018). miR-137 loss of function has been found to increase the probability of schizophrenia as the risk alleles for schizophrenia are associated with reduced miR-137 levels relative to the protective allele. Additionally, a reduction in miR-137 has been implicated in the etiology of major depressive disorder (Smalheiser et al., 2012) and anxiety (Yan et al., 2019). Likewise, partial loss of miR-137 in mice was found to cause repetitive behaviour and impaired sociability and learning in mice (Cheng et al., 2018). The involvement of miR-137 in all of these functions and pathologies highlights the ability of this miRNA as well as miRNAs in general to influence neuronal communication and thus brain function. The symptoms of anxiety and depression include disruptions in appetite, excessive worrying and fear as well as anhedonia, a loss of interest/pleasure (American Psychiatric Association, 2013). A common negative symptom of schizophrenia is also anhedonia (American Psychiatric Association, 2013). Considering the involvement of miR-137 in neuropsychiatric disorders such as anxiety, depression and schizophrenia, as well as their symptoms, we predicted that this miRNA may play an important role in preventing neophobia or establishing hedonic value, possibilities that were not considered by the Korneev et al. (2018) study.

Hedonic value refers to the degree of pleasantness or unpleasantness associated with a stimulus (as defined by the Oxford English Dictionary). When snails show a high feeding response to a certain stimulus (i.e. greater than or equal to 10 rasps per minute), we consider this to mean that the snails perceive this stimulus to be pleasant and thus that this stimulus is of high hedonic value. As such, alterations in hedonic value may interfere with an animal's normal feeding response, possibly confounding the conclusion of an obstruction to LTM formation. That is, rather than blocking LTM formation following the 1TT procedure by inhibiting the biogenesis of miR-137, the injection of PLL may have negatively affected feeding by impairing the hedonic value of food an animal experiences.

Neophobia, an aversion to novelty, is demonstrated by many animals (Greenberg, 1990; Candler and Bernal, 2014; McCormick et al., 2017). Neophobia benefits animals by protecting them from potentially toxic novel foods that they encounter. Avoiding novel foods, objects, predators and locations influences how animals react to new environments and their overall fitness (Ferrari et al., 2015). We chose to study neophobic foraging patterns, specifically avoidance of new foods, as this is an important behaviour for survival and does not rely on memory. The amount of exposure to a novel object can affect the neophobia an animal experiences towards that object (Siegel, 1974). For example, when confronted with a novel environment a second time, *Parus major* birds were quicker to explore the environment (Carere and van Oers, 2004). Moreover, neophobia has been found to diminish when the intake of a novel food is not followed by aversive consequences, leading to an increase in intake with subsequent encounters with the food (Siegel, 1974).

Thus, the objective of our study was to explore how inhibiting Dicer-mediated biogenesis of miRNAs using an injection of PLL in *L. stagnalis* affects levels of neophobia and the hedonic value of certain food stimuli, measured as a decrease in rasping behaviour to a food stimulus with a previously established high hedonic value. We hypothesized that inhibition of miRNA biogenesis would result in a decreased feeding response, which could be due to either neophobia or a decrease in the hedonic value of certain food stimuli. If a decreased feeding response persists after novel stimuli are removed, then we would consider inhibition of miRNA biogenesis to have decreased the hedonic value of certain food stimuli.

MATERIALS AND METHODS

Snails and animal maintenance

The animals used in this study were an inbred laboratory strain (W-strain) of *Lymnaea stagnalis* (Linnaeus 1758) maintained at the University of Calgary Biology Department. These W-strain *L. stagnalis* originated from an inbred stock maintained at the Vrije University of Amsterdam and were originally bred from animals collected in the 1950s in polders near Utrecht, The Netherlands. The snails were housed in artificial pond water (0.25 g l^{-1} of Instant Ocean in deionized water, Spectrum Brands, Madison, WI, USA) supplemented with $CaCO_3$ to ensure calcium concentrations remained above 50 mg (Dalesman and Lukowiak, 2010). Depending on the experiment, snails were fed either romaine lettuce or carrot shavings *ad libitum*. All snails were maintained at 20±1°C on a 16 h:8 h light:dark cycle. A total of 132 snails were used and rasping behaviour was measured for every snail.

Carrot slurry

The carrot slurry (C) was made by combining two medium-sized (600 g) commercially obtained organic carrots (purchased from multiple grocery stores) in a blender along with approximately 450 ml of pond water. Carrots were peeled before blending. Following blending and repeated straining of the mixture, a liquid carrot–pond water slurry was obtained without any visible carrot pieces. Carrot slurry was made as needed.

Experiments performed

A summary of the experimental procedures is provided in Fig. 1 and a full description of each is given below.

Saline and PLL injection

Based on the results obtained by Korneev et al. (2018), which demonstrated that 25 µmol l^{-1} PLL injection did not supress feeding, 25 µmol l^{-1} PLL was used for all experiments. A 25 µmol l^{-1} solution of PLL (poly-L-lysine hydrobromide, lot no. SLBZ6281, Sigma-Aldrich) was prepared in *Lymnaea* saline (51.3 mmol l^{-1} NaCl, 1.7 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 4.0 mmol l^{-1} $CaCl_2$ and 10.0 mmol l^{-1} Hepes, pH 8.0). The haemolymph volume of the snail was assumed to be 1 ml (van Aardt, 1968) so 0.1 ml injections were given to achieve a 25 µmol l^{-1} concentration of PLL inside the snail body. For control experiments, snails were injected with 0.1 ml of *Lymnaea* saline. All injections were done using a 1 ml syringe with a 30G1/2 needle into the haemocoel of the snail. Prior to all injections, snails were anaesthetized in cold pond water (4°C) for 2 min. After injection, snails were returned to their home tank.

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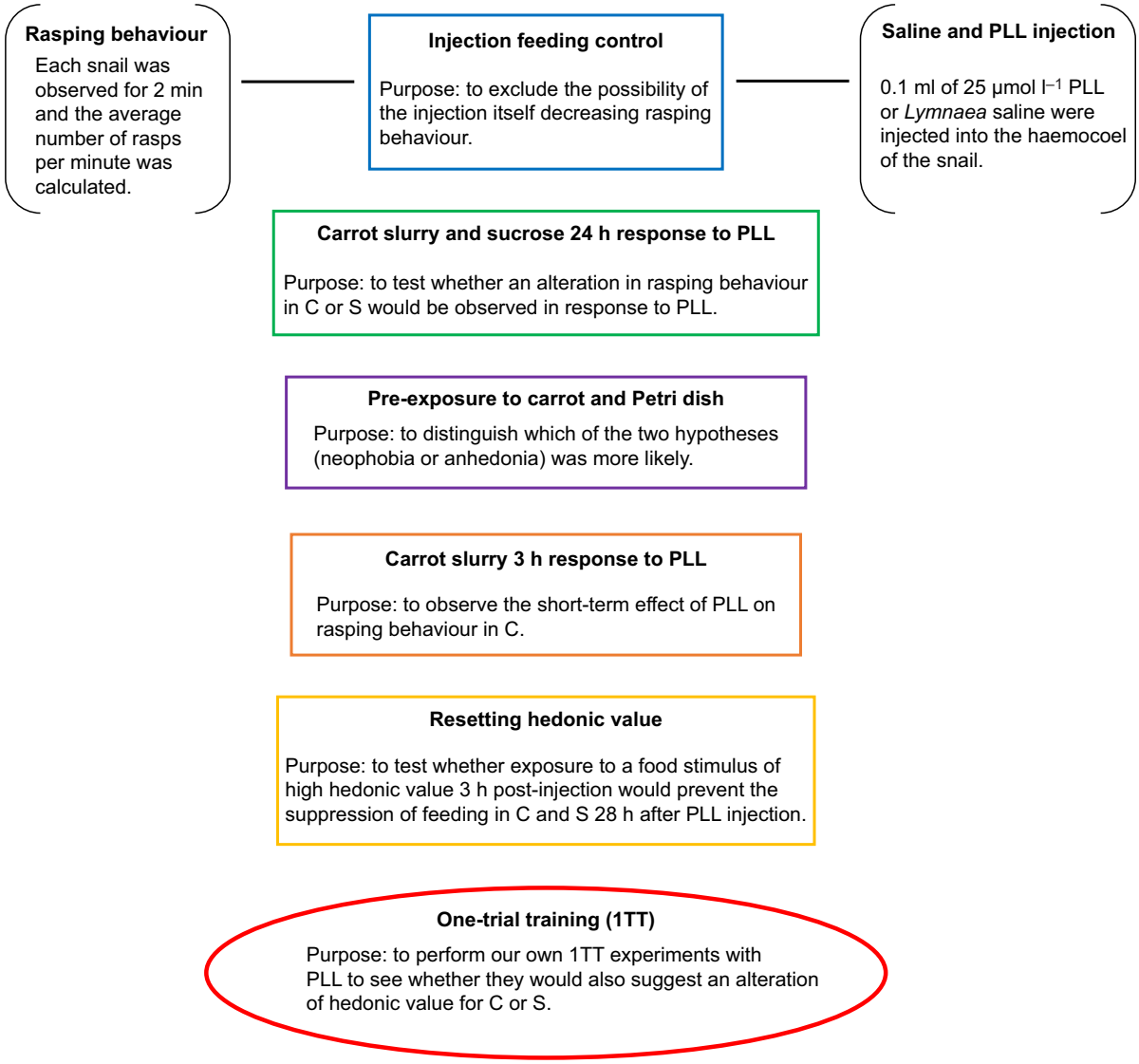

Fig. 1. Summary of experiments performed. A visual depiction of the experiments we performed along with the purpose of each experiment. Brief methodological notes have also been included for measurement of rasping behaviour as well as the saline and poly-L-lysine (PLL) injection procedure. C, carrot slurry; S, sucrose.

Rasping behaviour

Rasping in *L. stagnalis* is a rhythmic motor behaviour in which repeated movements of the radulae scrape the surface of a substrate, leading to the ingestion of food (Ito et al., 2013). In our experiments, snails were placed in a 14 cm diameter Petri dish with enough pond water or enough C for them to be partially submerged. The snails were given a 2–5 min acclimation period in each session before their rasping behaviour was observed. Each snail was then observed for 2 min and the number of rasps counted; the average number of rasps per minute was then calculated

Injection feeding control

To exclude the possibility of the injection itself decreasing rasping behaviour, we performed an injection feeding control. For this experiment, rasping behaviour was first observed in 0.67% sucrose (S) dissolved in pond water and then in C. Following this, the snails were injected with 0.1 ml of *Lymnaea* saline and returned to their home tank for 24 h. During this time, snails were fed with romaine lettuce *ad libitum*. Rasping behaviour was then tested in 0.67% S and in C 24 h after injection.

Carrot slurry and sucrose 24 h response to PLL

We hypothesized that if an alteration in rasping behaviour in C or S was observed in response to PLL, it would suggest a role for the miRNA pathway in hedonic value or neophobia. To test this hypothesis, rasping behaviour was observed initially in either C or 0.67% S. Three hours after this, snails were injected with 0.1 ml of 25 µmol l^{-1} PLL and returned to their home tank for 24 h. During this time, snails were fed with romaine lettuce *ad libitum*. Twenty-four hours after the injection, rasping behaviour was again observed in either C or S in response to PLL.

Pre-exposure to carrot and Petri dish

Inhibiting the biogenesis of miRNAs may elicit a neophobic response in snails, such that they develop a fear of new objects including the Petri dish and C, resulting in a suppression of rasping behaviour. Alternatively, inhibiting Dicer may alter the hedonic

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value of food, such that snails no longer find C or S pleasurable, resulting in a suppression of rasping behaviour (Smalheiser et al., 2012). To distinguish which of the two hypotheses was more likely, we performed experiments where we limited the new stimuli that could have elicited a neophobic response by pre-exposing snails to carrot and a Petri dish. In this experiment, snails were fed with only carrot shavings *ad libitum* for 1 week prior to observing rasping behaviour. Throughout the week, snails were placed in a Petri dish with C once every day for 5 min. After a week of exposure to carrot shavings *ad libitum* and C in a Petri dish, rasping behaviour was observed in C. Snails were then injected with 25 µmol l^{-1} PLL and returned to their home tank for 24 h. During this time, snails continued to feed on carrot shavings. Twenty-four hours after the PLL injection, rasping behaviour was observed in C.

Carrot slurry 3 h response to PLL

To observe the short-term effect of PLL on rasping behaviour in C, we decided to test rasping behaviour 3 h post-injection. Snails were injected with 0.1 ml of 25 µmol l^{-1} PLL and returned to their home tank for 3 h. During this time, snails were fed with romaine lettuce *ad libitum*. Three hours after the injection, rasping behaviour was observed in C. Following this, snails were returned to their home tank and fed with romaine lettuce *ad libitum.* Twenty-seven hours after the injection, rasping behaviour was again observed in C. A similar procedure was followed for a control group of snails with the only amendment being an injection with 0.1 ml of *Lymnaea* saline rather than PLL.

Resetting hedonic value

We hypothesized that exposure to a food stimulus of high hedonic value 3 h post-injection would prevent the suppression of feeding in C and S 27 h after PLL injection. To test this hypothesis, snails were injected with 25 µmol l^{-1} PLL and returned to their home tank. Three hours after PLL injection, snails were placed in a beaker with either C or 0.67% S, or 0.004% amyl acetate for 1 h and then returned to their home tank for 24 h. During this time, snails were food deprived. Twenty-seven hours after the injection, rasping behaviour was observed in C and 0.67% S. Rasping behaviour in C and S 27 h post-injection was compared with average C and S rasping data which had been collected from different snails that had not been injected with either PLL or saline. We used typical C and S rasping data, which were taken from a different group of snails, as C and S typical pre-injection data avoid any chance of food aversion learning.

1TT protocol

Korneev et al. (2018) reported a significant decrease of the conditioned feeding response in snails injected with PLL 15 min after 1TT, suggesting that miRNAs are required for 1TT-induced LTM formation in *L. stagnalis.* We wanted to perform our own 1TT experiments to see whether they would also suggest an alteration of hedonic value for C or S. The initial experiment was a 1TT control. Rasping behaviour was first observed in 0.004% amyl acetate (AA). Snails were then returned to their home tank. Three hours later, snails underwent pairing of AA (the conditional stimulus, CS) with 0.67% S (unconditional stimulus, UCS), using the procedure of Alexander et al. (1984). Twenty-four hours after pairing, rasping behaviour was again observed in 0.004% AA (i.e. the CS). The next experiment was completed 1 week later during which the same snails underwent the 1TT procedure again. However, in this experiment snails were injected with 0.1 ml of 25 µmol l^{-1} PLL 15 min after pairing of the CS (i.e. AA) and the UCS (i.e. S). Twenty-four hours after the injection, rasping behaviour was observed in 0.004% AA. Rasping behaviour in these same snails was also observed in C or 0.67% S and then compared with C and S typical data.

Statistical analyses

Prism 9 for Mac was used to analyse the data in this study. We first determined whether the data were normally distributed by performing an Anderson–Darling test with a significance level of 0.05. When the data were normally distributed, we performed a parametric repeated-measures ANOVA (RM ANOVA). When the data were not normally distributed, we performed either a non-parametric Wilcoxon matched pairs signed rank test or a non-parametric Kruskal–Wallis ANOVA with follow up Dunn's multiple comparisons tests. Differences were considered significant if $P<0.05$.

RESULTS

Blocking the biogenesis of miRNAs alters rasping behaviour

We hypothesized that if PLL was found to alter rasping behaviour, it would suggest a role for the miRNA pathway in hedonic value or neophobia. Rasping behaviour of the saline-injected snails was not significantly suppressed in S or in C 24 h post-injection (RM ANOVA, $F_{15.72,113.7}=1.31$, $P=0.297$, $R^2=0.14$, $n=9$, data not shown). In contrast, snails injected with 25 µmol l^{-1} PLL demonstrated significant suppression of rasping behaviour 24 h post-injection in both C (Fig. 2A; Wilcoxon matched pairs signed rank test, $W=-66.00$, $P=0.001$, $n=11$) and S (Fig. 2B; Wilcoxon matched pairs signed rank test, $W=-76.00$, $P=0.001$, $n=12$).

miRNAs may establish hedonic value

Even after novel stimuli (i.e. C and a Petri dish) were limited, rasping behaviour was still significantly supressed in C 24 h after snails were injected with PLL (Fig. 3; Wilcoxon matched pairs signed rank test, $W=-66.00$, $P=0.001$, $n=11$).

Presentation of a novel food after PLL injection resets hedonic value

To test whether PLL supressed feeding before the 24 h test mark, we observed rasping behaviour 3 h after PLL injection (Fig. 4). We found a complete suppression of rasping behaviour in C 3 h after PLL injection, such that the snails did not rasp at all (Fig. 4A; Kruskal–Wallis test statistic=17.70, $P<0.001$, Dunn's multiple comparisons tests, $z=4.05$, $P<0.001$, C typical data $n=15$, C 3 h $n=8$). However, 24 h after injection, these snails no longer showed a significant decrease in feeding in C compared with feeding in C prior to injection (Dunn's multiple comparisons tests, $z=0.34$, $P>0.999$, C typical data $n=15$, C 24 h $n=8$). In contrast, when snails were injected with saline, they did not show a change in rasping behaviour in response to C at either 3 or 24 h post-injection (Fig. 4B; Kruskal–Wallis test statistic=1.41, $P=0.495$, C typical data $n=15$, C 3 h and C 24 h $n=10$).

Twenty-four hours after exposure to C in a beaker during the resetting hedonic value procedure, there was no significant suppression of feeding in C (Kruskal–Wallis test statistic=0.37, $P=0.985$, C typical data $n=15$, C post-C $n=12$; Fig. 5A). Similarly, 24 h after exposure to S in a beaker, there was no significant suppression of feeding in S or in C (Kruskal–Wallis test statistic=0.37, $P=0.985$, C and S typical data $n=15$, C post-S and S post-S $n=12$; Fig. 5B).

However, when snails were placed in a beaker with AA 3 h after injection, their feeding response to C and S was significantly

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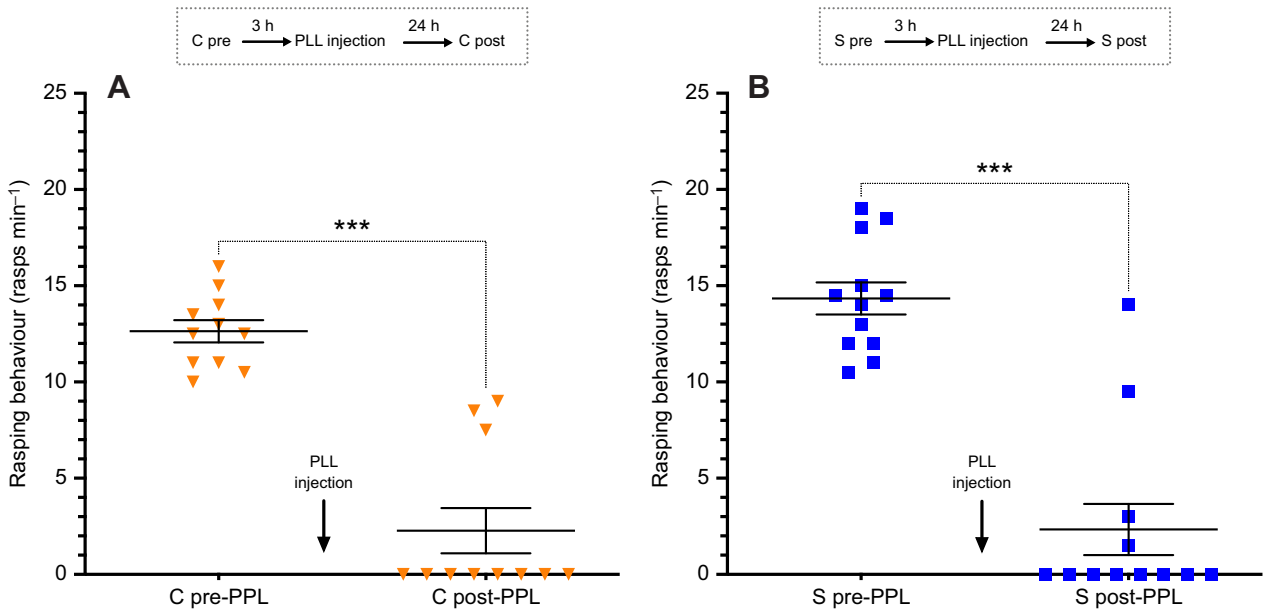

Fig. 2. Blocking biogenesis of microRNAs (miRNAs) leads to suppression of rasping behaviour. Rasping behaviour of *Lymnaea stagnalis* in (A) carrot slurry and (B) 0.67% sucrose (C and S, respectively) pre-injection and 24 h post-injection with 25 µmol l^{-1} PLL. PLL injection caused significant suppression of rasping behaviour in both C and S [Wilcoxon matched pairs signed rank test, (A) W=−66.00, ***P=0.001, n=11 and (B) W=−76.00, ***P=0.001, n=12]. Bars represent means±s.e.m.

supressed 28 h post-injection (Fig. 5B; S: Kruskal–Wallis test statistic=33.73, P<0.001) in both C (Dunn's multiple comparisons tests z=3.80, P<0.001, C typical data n=15, C post-AA n=12) and S (Dunn's multiple comparisons tests z=4.34, P<0.001, S typical data n=15, S post-AA n=12).

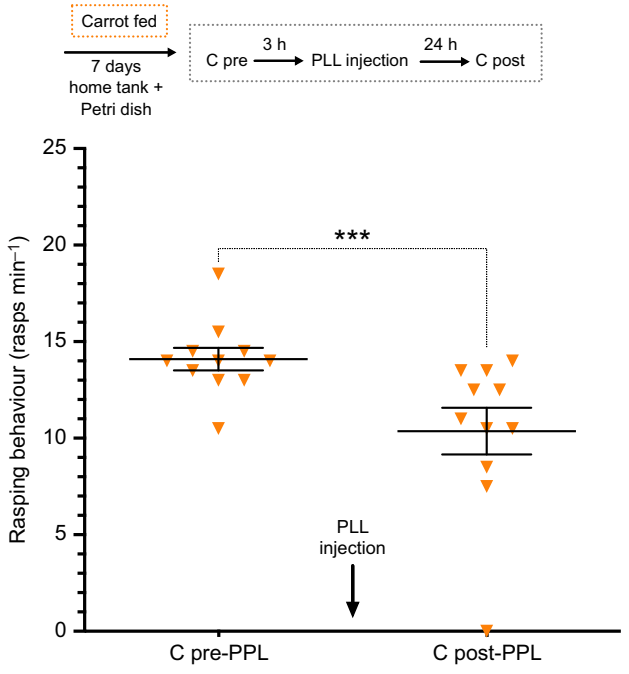

Fig. 3. Pre-exposure to carrot shavings, C and a Petri dish does not eliminate suppression of rasping behaviour following PLL injection. Rasping behaviour in C pre- and 24 h post-injection with 25 µmol l^{-1} PLL. PLL injection caused significant suppression of rasping behaviour in C 24 h post-injection (Wilcoxon matched pairs signed rank test, W=−66.00, ***P=0.001, n=11). Bars represent means±s.e.m.

Consolidating our results with 1TT

Using a similar 1TT procedure to that described by Korneev et al. (2018), we observed a significant increase in rasping behaviour in AA 24 h after pairing of AA with S (Fig. 6A; Kruskal–Wallis test statistic=37.78, P<0.001, Dunn's multiple comparisons test z=4.27, P<0.001, n=15; Fig. 6A). In these same snails, as predicted, the unconditioned feeding behaviour in C was not significantly altered by 1TT (Dunn's multiple comparisons test z=0.22, P>0.999, C typical data n=15, C post n=15). Similarly, unconditioned feeding behaviour in S (which was used as the UCS) was not significantly altered by 1TT (Dunn's multiple comparisons test z=0.53, P>0.999, S typical data n=15, S post n=15).

When we repeated this experiment including a PLL injection 15 min after pairing of AA and S, the response to AA post-1TT and PLL injection was not different from its pre-training response (Kruskal–Wallis test statistic=55.81, P<0.001, Dunn's multiple comparisons test z=1.16, P>0.999, n=13). However, the unconditioned feeding behaviour in both C and in S was significantly suppressed 24 h following PLL injection (Dunn's multiple comparisons test z=4.79, P<0.001, C typical data n=15, C post n=13; and z=4.72, P<0.001, S typical data n=15, S post n=13, respectively). Our control experiment, which utilized *Lymnaea*-saline in place of PLL, produced the same results as normal 1TT. We observed a significant increase in rasping behaviour in AA 24 h after pairing of AA with S (data not plotted; Wilcoxon matched pairs signed rank test, W=70.00, P=0.003).

DISCUSSION

Our study aimed to explore the role that miRNAs play in neophobia and the setting of hedonic value. Using a PLL injection, we showed that inhibition of Dicer caused a significant reduction of rasping behaviour in *L. stagnalis* which persisted after we eliminated novel stimuli. These findings are consistent with the idea that the miRNA pathway is involved in establishing or maintaining hedonic value for food stimuli. However, we believe that PLL may have more

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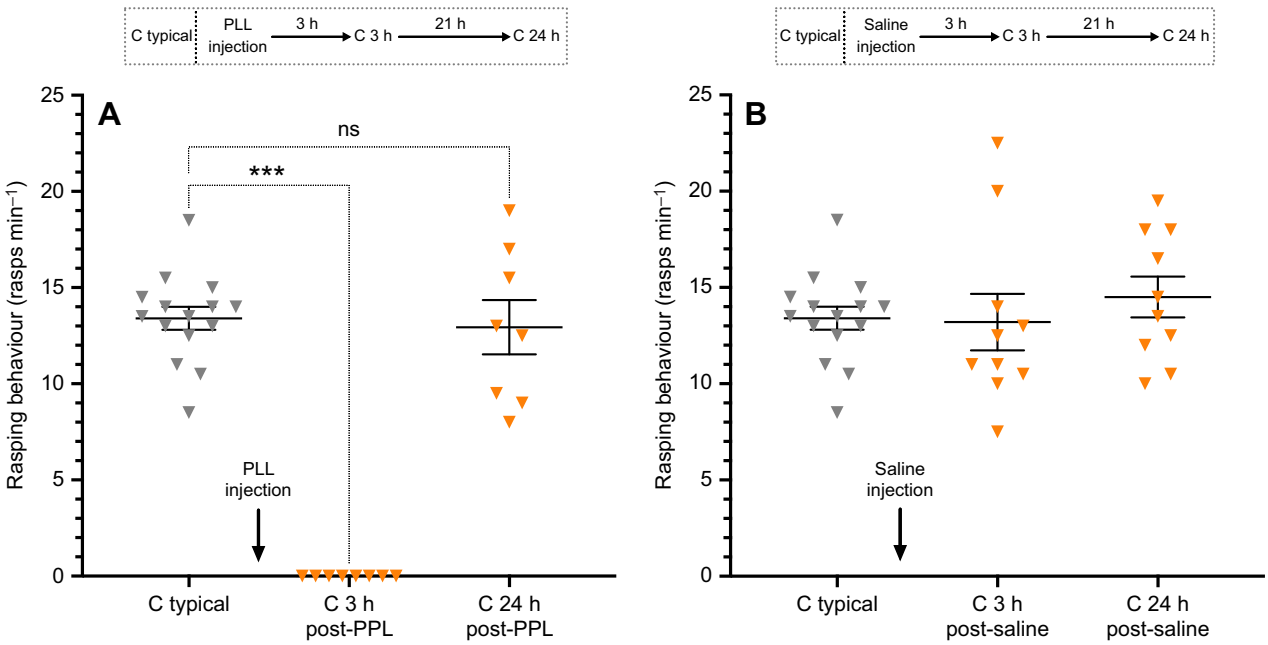

Fig. 4. Blocking the biogenesis of microRNAs (miRNAs) supresses feeding behaviour 3 h post-injection. The results of rasping behaviour in C 3 h and 24 h after snails were injected with either (A) 25 µmol l^{-1} PLL or (B) *Lymnaea* saline. Typical C rasping data (C typical, *n*=15), collected from different snails, were used to avoid any chance of food aversion learning. PLL injection caused significant suppression of rasping behaviour in C 3 h post-injection but not at 24 h post-injection (ns) [Kruskal–Wallis test statistic=17.70, *P*=0.0001; Dunn's multiple comparisons tests, *z*=4.05, ****P*=0.0002, C typical (*n*=15) versus C 3 h post-injection (*n*=8)]. Saline injection did not cause significant suppression of rasping behaviour (Kruskal–Wallis test statistic=1.41, *P*=0.495, *n*=10). Bars represent means±s.e.m.

widespread effects on *L. stagnalis* than just the blockade of Dicer, which may further contribute to an alteration in hedonic value. Further, our results suggest that the conclusions based on the previous experiments using a PLL injection following a 1TT procedure (Korneev et al., 2018) may not be entirely correct. Those authors concluded that the injection of PLL following a 1TT

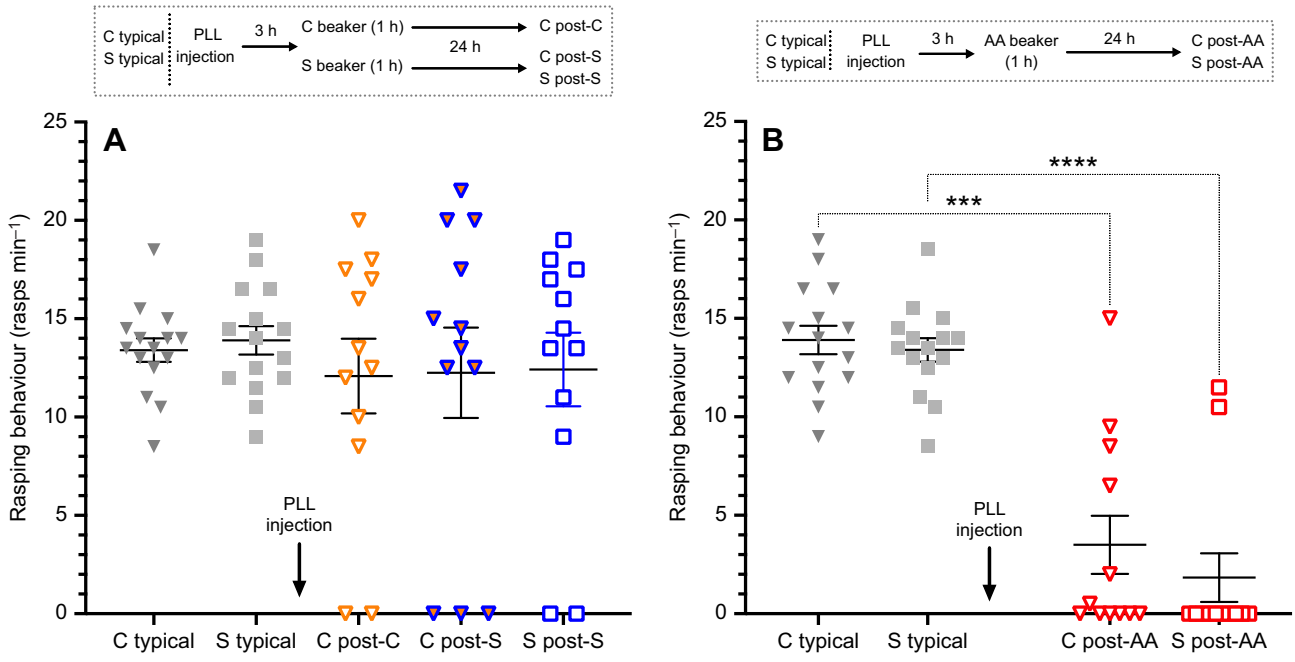

Fig. 5. Exposing snails to C or S attenuates neophobia and resets hedonic value, whereas exposing snails to amyl acetate (AA) does not. Rasping behaviour in C (triangles) and S (squares) 28 h after snails were injected with 25 µmol l^{-1} PLL and exposed (A) to a beaker of C (orange) or 0.67% S (blue) or (B) to a beaker of 0.004% AA (red), 3 h post-injection. Typical C and S rasping data (*n*=15), collected from different snails, were used to avoid any chance of food aversion learning. Exposure to C or S 3 h post-injection resulted in no significant suppression of rasping behaviour in C or in S 28 h after injection (Kruskal–Wallis test statistic=0.37, *P*=0.985, *n*=12). Exposure to AA 3 h post-injection resulted in a significant suppression of rasping behaviour in C and in S 24 h later (Kruskal–Wallis test statistic=33.73, *P*<0.0001, *n*=12, Dunn's multiple comparisons tests ***P*=0.0009, *****P*<0.0001). Bars represent means±s.e.m.

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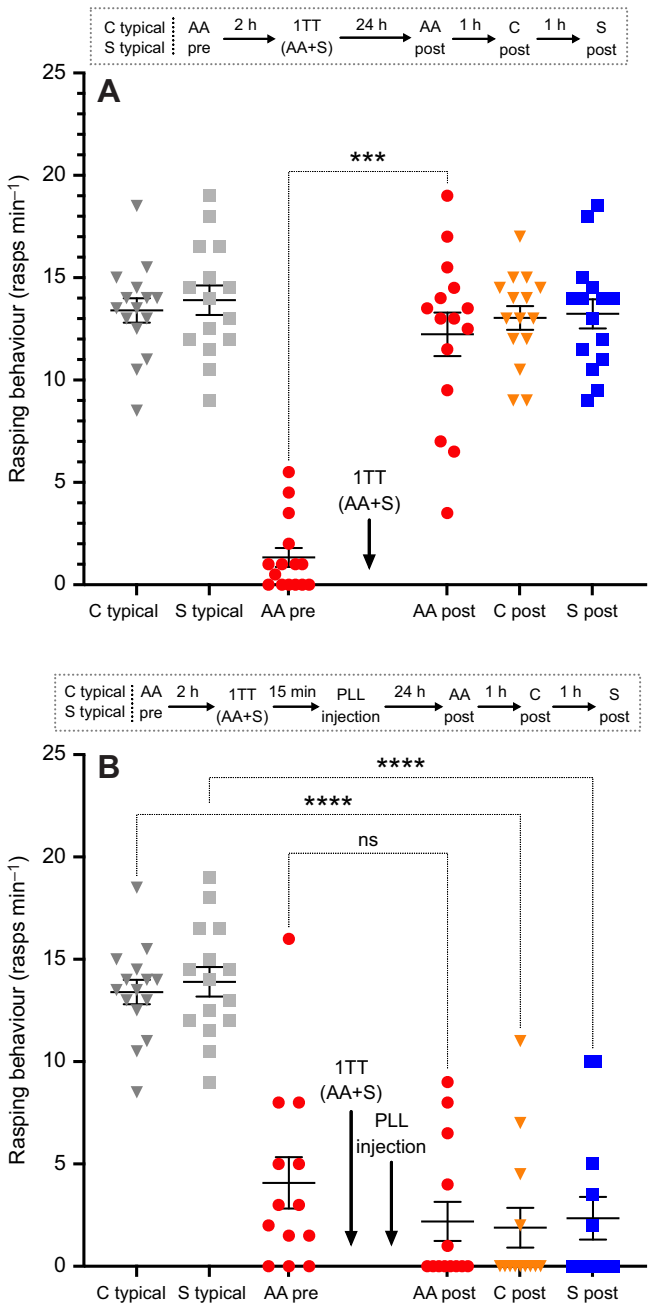

Fig. 6. PLL injection suppresses conditioned and unconditioned feeding behaviour following 1TT appetitive classical conditioning. Rasping behaviour in AA 24 h after snails (A) underwent pairing of AA with S and (B) were injected with 25 µmol l^{-1} PLL. PLL injections were given 15 min after 1TT conditioning. Typical C and S rasping data (n=15), collected from different snails, were used to avoid any chance of food aversion learning. 1TT caused a significant increase of conditioned feeding behaviour in AA (Kruskal–Wallis test statistic=37.78, P<0.0001, Dunn's multiple comparisons tests z=4.27, ***P=0.0003, n=15). The feeding response to C and S remained unaffected by 1TT. PLL injection following 1TT caused no significant (ns) change of conditioned feeding behaviour in AA and significant suppression of unconditioned feeding behaviour in C and S [Kruskal–Wallis test statistic=55.81, P<0.0001, Dunn's multiple comparisons tests ****P<0.0001, C and S typical (n=15) compared against C, and S post-injection (n=13), respectively]. Bars represent means±s.e.m.

procedure blocked LTM formation. That is, as the response of snails to AA following the 1TT procedure and the injection of PLL was not different from the response before conditioning, a logical conclusion was that the PLL injection blocked LTM formation. Our data were similar. However, we also found in the same snails that food stimuli (C and S) that were not used in the pairing of stimuli in the 1TT procedure were also suppressed following the injection of PLL. Thus, our data are consistent with the hypothesis that inhibiting the biogenesis of miRNAs reduces the hedonic value snails designate to food substances such as C and S. These results have important implications for our understanding of the functions of miRNAs.

Neophobia, an aversion to novelty, arises through a cognitive assessment of novel stimuli and is an ecologically relevant fear behaviour (Greggor et al., 2015). The cognition underlying fear behaviour is important to consider. Responding to something because of its novelty relies on the cognitive ability to classify an encountered stimulus as novel. Therefore, neophobia involves an additional cognitive process to other fear reactions and may not correlate with overall fearfulness (Villalba et al., 2009). We found that when snails were pre-exposed to novel stimuli capable of eliciting a neophobic response such as C and the Petri dish, a significant suppression of rasping behaviour was still observed. This result suggests that blocking the biogenesis of miRNAs did not significantly impact the cognition underlying neophobia, but rather resulted in anhedonia. Thus, our working hypothesis is that inhibiting the biogenesis of miRNAs by PLL injection caused snails to experience a decrease in the hedonic value of their food, resulting in a suppression of feeding behaviour in *L. stagnalis*.

Through our carrot slurry 3 h response to PLL and hedonic value resetting procedure, we were able to demonstrate that exposing snails to a food stimulus of high hedonic value 3 h post-PLL injection may reset hedonic value, resulting in normal rasping behaviour 24 h later. Consistent with our hypothesis, the results of the experiment where C was presented showed a significant suppression of rasping behaviour 3 h post-PLL injection, but no significant suppression 28 h after injection. Moreover, snails also showed normal feeding behaviour 24 h after they were presented with a beaker of C or S 3 h post-injection. However, exposing snails to a beaker of AA 3 h post-PLL injection did not rectify feeding behaviour 28 h after injection. AA is a neutral stimulus that snails can sense, but it does not elicit a feeding response above the spontaneous rate of rasping. Thus, contrary to C and S, which are of high hedonic value, AA is of low hedonic value. These results suggest that inhibiting the biogenesis of miRNAs decreased the hedonic value of food stimuli, which was rectified by presenting snails with food stimuli of a high hedonic value (i.e. C or S) 3 h post-PLL injection. As such, these data further support our hypothesis that miRNAs are important for establishing hedonic value.

A previous report by Korneev et al. (2018) concluded that miRNAs are required for 1TT-induced LTM formation in *L. stagnalis*. They reported a significant decrease of the conditioned feeding response in snails injected with PLL 15 min after 1TT and based on that result concluded that the inhibition of Dicer leads to impairment of LTM formation. However, our data showed a significant suppression of unconditioned feeding behaviour following a similar PLL injection. Thus, we hypothesized that the decreased feeding response observed by Korneev et al. (2018) may have been due to a decrease in hedonic value rather than an impairment of LTM formation. However, it should be noted that the difference in feeding response post-PLL injection that we observed compared with Korneev et al. (2018) could be a result of different environmental conditions (Rothwell and Lukowiak, 2019).

Using a similar 1TT procedure to Korneev et al. (2018), we showed that our snails successfully underwent 1TT and formed

LTM. When we repeated the experiment with administration of a PLL injection 15 min after pairing of AA and S, we found that the response to AA was similar to that reported by Korneev et al. (2018). That is, LTM of appetitive conditioning was not found. Unlike Korneev et al. (2018), we also compared the rasping behaviour in response to C and S (two unconditioned stimuli) before versus after 1TT and PLL injection. In both cases, the unconditioned feeding behaviour in C and in S was significantly suppressed 24 h following 1TT and PLL injection. Additionally, we performed a final control experiment demonstrating successful 1TT appetitive conditioning with a *Lymnaea*-saline injection 15 min after the AA and S pairing. Together, our data demonstrate that suppression of conditioned and unconditioned feeding behaviour was not due to the injection itself (i.e. saline injection). As we predicted, inhibiting Dicer had more widespread effects than just impairing LTM formation. These results suggest that miRNAs play a more complicated role than LTM formation, as was previously proposed. miRNAs may also be required to establish hedonic value.

Anhedonia is considered to be a significantly diminished interest or pleasure and is a symptom of major depression, schizophrenia and other neuropsychiatric disorders (American Psychiatric Association, 1994). The neural basis of anhedonia reflects deficits in hedonic capacity, which is linked to the neural circuits subserving reward-related processes (Der-Avakian and Markou, 2012). Our results demonstrate that blocking the biogenesis of miRNAs impairs hedonic capacity in *L. stagnalis*, causing snails to lose interest and possibly pleasure in food stimuli that previously had a high hedonic value, resulting in diminished feeding behaviour. Thus, our findings suggest that miRNAs may play important roles in the neural circuits responsible for reward-related behaviours.

While we predicted miR-137 to be principally involved in establishing hedonic value based on its involvement in neuropsychiatric disorders such as depression and schizophrenia, in which anhedonia is a key symptom, it should be noted that we did not perform sequencing analyses to study all the miRNAs involved. Thus, there are likely other miRNAs in *L. stagnalis* that play a role in neophobia and hedonic value. Future experiments could utilize a specific miR-137 inhibitor to isolate its specific contribution to the effects studied.

Finally, our data should not be taken to mean that the inhibition of Dicer by PLL injection does not also prevent LTM formation as concluded by Korneev et al. (2018). Our data shown here are consistent with their conclusion as PLL injection following pairing of AA with S resulted in AA not eliciting a feeding response. However, in those same snails, the feeding response to C and S was also suppressed, even though those food substances were not part of the 1TT training procedure. Thus, although we believe that PLL injection following the 1TT procedure might block LTM formation, because PLL injection also suppressed the response in those snails to C and S, which were not conditioned, we cannot know for certain whether this is the case. Thus, PLL injection may both prevent LTM formation and reduce hedonic value in snails.

The results of this study demonstrate a decrease in the hedonic value of food stimuli resulting from the inhibition of miRNA biogenesis, suggesting that miRNAs are important for establishing hedonic value. Our differing interpretation of results compared with Korneev et al. (2018) can be explained by our observations of rasping behaviour in multiple food stimuli following 1TT and PLL injection. Importantly, our results demonstrate a connection between miRNAs and anhedonia, which could contribute to research on neuropsychological disorders involving miRNAs. Future experiments, such as those we have suggested focusing on miR-137, could provide miRNA targets as a suitable approach for treatment of symptoms such as anhedonia.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: D.K., K.L.; Methodology: D.K., A.B., V.R.; Formal analysis: D.K., A.B., V.R., K.L.; Investigation: D.K.; Resources: K.L.; Writing - original draft: D.K.; Writing - review & editing: D.K., A.B., K.L.; Visualization: D.K., A.B., V.R., K.L.; Supervision: A.B., K.L.; Project administration: K.L.; Funding acquisition: D.K., K.L.

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