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Histone deacetylase inhibitor treatment restores memory-related gene expression and learning ability in neonicotinoid-treated *Apis mellifera*

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Abstract

Apis mellifera **plays crucial roles in maintaining the balance of global ecosystems and stability of agricultural systems by helping pollination of flowering plants, including many crops. In recent years, this balance has been disrupted greatly by some pesticides, which results in great losses of honeybees worldwide. Previous studies have found that pesticide-caused memory loss might be one of the major reasons for colony loss. Histone deacetylase inhibitors (HDACis) are chemical compounds that inhibit the activity of histone deacetylases and are known to cause hyperacetylation of histone cores and influence gene expression. In our study, the HDACi sodium butyrate was applied to honeybees as a dietary supplement. The effect of sodium butyrate on the expression profiles of memory-related genes was analysed by quantitative reverse transcription PCR. The results revealed that this HDACi had up-regulation effects on most of the memory-related genes in bees, even in bees treated with imidacloprid. In addition, using the proboscis extension reflex to evaluate olfactory learning in bees, we found that this HDACi boosted the memory formation of bees after impairment owing to imidacloprid exposure. This study investigated the association between gene expression and memory formation from an epigenetic perspective. Additionally, we further demonstrate the possibility of enhancing bee learning using HDACis and provide initial data for future research.**

Keywords: honeybee, imidacloprid, histone deacetylase inhibitor, sodium butyrate, memory gene.

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Introduction

The honeybee is one of the most important model systems in studying learning and memory (Giurfa & Sandoz, 2012). The classification of honeybee memory consolidation depends on both time and various memory pathways. Time-based classification includes three stages: short-term memory (STM, in the second range), mid-term memory (MTM, in the minute and hour range) and long-term memory (LTM, 24 h postconditioning) (Giurfa & Sandoz, 2012; Matsumoto *et al.*, 2014). There is no known molecular pathway involved in STM, whereas studies on invertebrates have shown that LTM formation requires an up-regulation of the nitric oxide (NO)–cyclic guanosine monophosphate pathway, and of specific molecules, such as cyclic nucleotide-gated channels, calmodulin, adenylyl cyclase and Ca^{2+}/calmodulin-dependent protein kinase (Matsumoto *et al.*, 2014). Despite much behavioural research on learning and memory in bees, knowledge gaps in our understanding of these processes remain and it is crucial to identify these. It was previously reported that the formation of LTM in the honeybee is induced via temporally regulated demethylation and methylation of certain genes during the first 24 h after initial exposure to training (Zovkic *et al.*, 2013). Most of these genes are involved in the regulation of neural activities such as dendrite formation, neuron excitability and synapse morphology. Amongst these genes, three genes involved in DNA methylation and demethylation machinery, *DNA methyltransferase 1b* (*Dnmt1b*), *Dnmt3* and *ten-eleven translocation methylcytosine dioxygenase*, are initially up-regulated and then down-regulated. It was previously

shown that the Dnmt3 promoter is differentially methylated in response to learning (Biergans *et al*., 2015).

Apis mellifera, also known as the western honeybee, is a vital economic resource because these bees pollinate most flowering plants (Blank *et al*., 2015). In recent years, Europe and North America have suffered from severe bee loss owing to unknown reasons, called colony collapse disorder (CCD). It has been theorized that pesticides, such as imidacloprid (a novel type of neonicotinoid pesticide), might be responsible for CCD (Mullin *et al*., 2010; Henry *et al*., 2012). Neonicotinoid pesticides are novel analogues of nicotine that act as neurotoxins in insects by binding to the nicotinic acetylcholine receptor, causing neural hyperexcitation and leading to eventual death (Matsuda *et al*., 2001). Previous studies have found that a sublethal dose of imidacloprid affects the foraging behaviour of honeybees and impairs their olfactory associative learning ability (Peng & Yang, 2016).

In eukaryotic cells, DNA wraps around eight histone cores to form the nucleosome, which is the basic repeated unit of chromatin. The structure of chromatin depends on histone modifications and affects the gene expression level (Wang *et al*., 2013; Yan *et al*., 2014). Several chemical reactions, including acetylation, methylation and phosphorylation, occur at the histone tails and slightly modify the distance between histone and DNA, thereby changing the chromatin structure (Grant, 2001). Acetylation is related to the activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Gershey *et al*., 1968). These enzymes regulate the presence or absence of acetyl groups on the amino-terminus (N-terminus) of histone tails; when present, the negative charge of the acetyl group affects the histone framework (Dokmanovic *et al*., 2007). The activity of HDACs is interrupted by a class of compounds called HDAC inhibitors (HDACis; Bouchecareilh *et al*., 2012). HDACis have been reported in various studies. Sodium butyrate, one of the HDACis, targets HDAC classes 1 and 2a (Dokmanovic *et al*., 2007), and has been revealed to have the ability to selectively modify all histones present in the nucleosome (Gui *et al*., 2004). Butyrate is structurally a short chain fatty acid and it can function to inhibit the activities of histone deacetylase, resulting in altered gene expression in humans and mice (Davie, 2003). Recently, the inhibition of HDACs has been reported to be associated with memory formation (Merschbaecher *et al*., 2012). In this study, Merschbaecher *et al*. showed that inhibition of HDACs after conditioning resulted in an increased level of acetylation and an improved memory. HDACis regulate cyclic adenosine 3′,5′-monophosphate (cAMP) response element binding protein (CREB), which is involved in the formation of long-term memory (Felsenberg *et al*., 2015). Moreover, the role of sodium butyrate in memory enhancement in aged rats has also been investigated (Blank *et al*., 2015). In this study, we used sodium butyrate to investigate memory development in honeybees.

Recent evidence has suggested that the abuse of pesticides impairs honeybee learning and memory and is highly responsible for the massive disappearance of bees (Mullin *et al*., 2010). Our data suggest that sodium butyrate increases protein acetylation and memory gene expression in bees whose memory-related genes were originally suppressed by imidacloprid. We then conducted behavioural studies on these sodium butyrate-treated bees and found that the increased gene expression led to memory improvement. However, the underlying mechanism has not yet been fully elicited. In this study, this phenomenon was investigated epigenetically by treating bees with the HDACi sodium butyrate and the common pesticide imidacloprid.

Results

Sodium butyrate treatment increases histone acetylation in honeybee heads

Sodium butyrate is an HDACi that inhibits the deacetylation of the histone core and results in increased gene expression (Hu *et al*., 2017). Here, we used western blotting to test whether this is true in the heads of the bees. Western blot analysis showed a significant increase in histone acetylation [acetyl-histone 3 (acetyl-H3) and acetyl-H4] with the addition of sodium butyrate ($P < 0.05$; Fig. 1A, B, Lane 3). These findings support our hypothesis that sodium butyrate decreases deacetylation, and therefore enhances gene expression. We also compared the effects of sodium butyrate and imidacloprid on histone acetylation. Western blotting showed no effect on histone acetylation in the imidacloprid-treated group (Fig. 1A, B, Lane 2). Previous studies demonstrated that sodium butyrate at 10 mM does not induce apoptosis (Hu *et al*., 2017; although there is evidence suggesting otherwise at higher concentrations). Based on these results, we exposed bees to 10 mM sodium butyrate for 7 days with the goal of increasing the acetylation of H3 and H4. Our PCR array analysis herein focused on the expression of genes related to memory.

Effects of sodium butyrate on memory gene signalling factors

Neonicotinoid pesticides such as imidacloprid have been demonstrated to have a long-term effect on the memory ability of the honeybee over a long period of time, even at low concentrations. Dopamine (DA) is an important neurotransmitter in invertebrate nervous systems and its expression is known to be affected by neonicotinoid pesticides, resulting in damage to the memory of honeybees

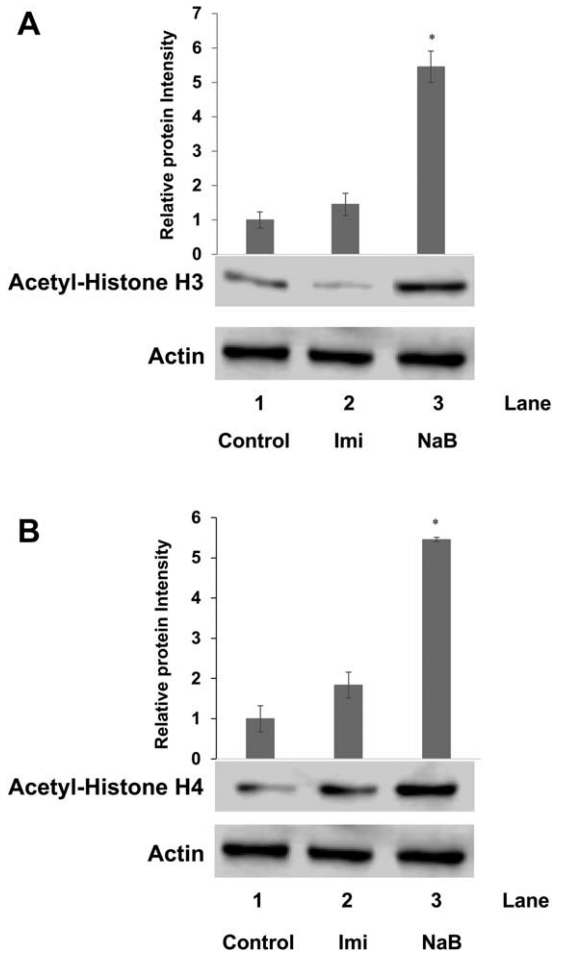

Figure 1. Regulation of acetyl-histone (acetyl-H) expression by sodium butyrate. Western blot of (A) acetyl-H3 and (B) acetyl-H4 in the feeding assay for 7 days with 10 mM sodium butyrate and imidacloprid with actin as the loading control. Proteins were extracted from the heads of the honeybees, and each well contained proteins extracted from the head of a single bee. The data are presented as the mean ± SD from three independent experiments. The value of the control group was set to 1, and all the other values were adjusted accordingly. Imi, imidacloprid treatment; NaB, sodium butyrate treatment. Asterisks indicate significant differences between test group and control group (*, $P < 0.05$).

(Yang *et al.*, 2008; Williamson & Wright, 2013). The expression level of DA has been shown to correlate with that of dopamine receptors. Dopamine receptors found in honeybees are classified into three types, *Apis mellifera* dopamine receptor 1 (*Amdop1*), Amdop2 and Amdop3, of which Amdop1 and Amdop2 have been shown to help bees in foraging and learning (Mustard *et al.*, 2003, 2005). Therefore, we examined the expression of Amdop1 and Amdop2 in imidacloprid-treated honeybees. Reverse transcription PCR (RT-PCR) revealed a significant decrease in Amdop1 and Amdop2 expression in the imidacloprid treatment group ($P < 0.05$; Fig. 2). However, when honeybees were cotreated with imidacloprid and sodium butyrate, expression of Amdop1 and Amdop2 was restored to normal levels (Fig. 2). To comprehensively investigate the effect of imidacloprid on memory-related genes, 32 memory-related genes were screened by quantitative PCR (qPCR) array (Table 1). As shown in Fig. 3 and Table 1, imidacloprid inhibited gene expression, as opposed to sodium butyrate, which has an up-regulation on most of the genes it influences. Imidacloprid down-regulated many memory-related genes [*Asparagine* (*Asn*) *synthetase*, *histone acetyltransferase P300* (*HAT P300*), *Dnmt3*, *histone 1B*, *histone 1.2* and *stress-sensitive B* (*sesB*)], including *HAT P300*, which is involved in long-term memory (Supporting Information Fig. S1A). Only three genes were down-regulated in the sodium butyrate-treated group: *histone 1.2*, *iron regulatory protein 1* (*Irp1*) and *synaptotagmin 1* (*synt1*) (Supporting Information Fig. S1B). Interestingly, gene expression levels were significantly increased in the sodium butyrate and imidacloprid cotreatment group (Supporting Information Fig. S1C, Table 1), suggesting that sodium butyrate could reverse the down-regulation of imidacloprid on gene expression. We therefore speculated as to whether the response to sodium butyrate at the gene expression level would be reflected in honeybee behaviour.

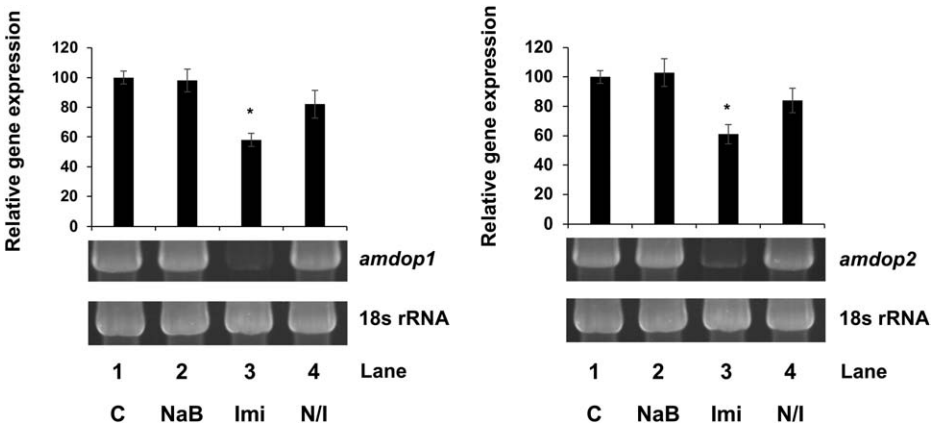

Figure 2. Relative expression of dopamine receptor genes. Reverse transcription PCR analysis of *Amdop1* and *Amdop2* expression levels after sodium butyrate, imidacloprid and sodium butyrate/imidacloprid treatments. The results were normalized to 18s ribosomal RNA. NaB, sodium butyrate treatment; Imi, imidacloprid treatment; N/I, sodium butyrate and imidacloprid cotreatment. Each group included three biological replicates. Asterisks indicate significant differences between test group and control group (*, $P < 0.05$). C = Control group (wild type).

Table 1. Relative expression of memory-related genes in honeybees

Gene name/function	References	NaB		Imidacloprid		NaB/Imi	
Memory		Up (%)	Dn (%)	Up (%)	Dn (%)	Up (%)	Dn (%)
Actin	Ganeshina *et al*. (2012)	7	–	–	31	29*	–
Aldoreductase	Cristino *et al*. (2014)	–	23	–	40*	–	17
Asparagine synthetase	Ganeshina *et al*. (2012)	–	23	–	51*	–	11*
Cueball	Biergans *et al*. (2015)	–	11	–	37	–	6
DNA methyltransferase 1a (*Dnmt1a*)	Biergans *et al*. (2015)	6	–	8	–	27*	–
Dnmt1b	Biergans *et al*. (2015)	–	17	–	31	–	2
Dnmt2	Biergans *et al*. (2015)	–	12	–	27	–	9
Dnmt3	Biergans *et al*. (2015)	–	36	–	43	–	9*
Glyceraldehyde 3-phosphate dehydrogenase	Biergans *et al*. (2015)	50	–	–	29	54*	–
GB18684	Biergans *et al*. (2015)	–	–	–	20	17*	–
Histone 1B	Biergans *et al*. (2015)	–	26	–	47	5*	–
Histone 1.2	Biergans *et al*. (2015)	–	24	–	32	–	15
Headcase	Biergans *et al*. (2015)	–	17	–	34	–	4
Heat shock protein 90	Naeger *et al*. (2011)	–	16	–	24	–	15
Iron regulatory protein 1	Mandilaras *et al*. (2013)	–	89	–	72	–	18*
Myelin and lymphocyte protein, T cell differentiation protein	Biergans *et al*. (2015)	–	10	–	28	–	2
Molybdenum Cofactor sulphurase C-terminal domain containing 1	Biergans *et al*. (2015)	–	8	–	17	–	18
Mitochondrial ribosomal protein L35	Biergans *et al*. (2015)	–	23	–	27	2	–
N-acetylneuraminate pyruvate lyase	Biergans *et al*. (2015)	–	10	–	19	14	–
Phenol oxidase	Farooqui (2014)	–	12	–	40*	4*	–
Subunit 8 of RNA polymerase II. (RPB8)	Biergans *et al*. (2015)	–	20	–	31	–	11*
Regulatory particle non-ATPase 9	Biergans *et al*. (2015)	–	14	–	32	–	2
Sec61 β subunit (CG10130, FBgn0010638) Drosophila melanogaster	Uno *et al*. (2013)	–	–	–	20	12*	–
Stress-sensitive B	Biergans *et al*. (2015)	–	30	–	40*	79*	–
Synaptotagmin 1	Biergans *et al*. (2015)	–	60	105	–	–	10
Ten-eleven translocation methylcytosine dioxygenase	Biergans *et al*. (2015)	76	–	187	–	104	–
Long-term memory							
Apis mellifera cyclic adenosine 3′,5′-monophosphate response element binding protein	Felsenberg *et al*. (2015)	–	12	–	35	31*	–
Histone acetyltransferase P300	Merschbaecher *et al*. (2016)	–	33	–	44*	6*	–
Learning							
Ether a go-go	Biergans *et al*. (2015)	170	–	–	5	327*	–
Neurexin	Biswas *et al*. (2010)	–	13	–	23	2	–

NaB, sodium butyrate; Dn, down.
Asterisks indicate *P*-values < 0.05.

Sodium butyrate restored the olfactory learning of honeybees

Previous studies found that imidacloprid impairs the ability of honeybees to learn (Williamson & Wright, 2013). Our data are consistent with previous reports on the negative effect of imidacloprid and show that sodium butyrate could reverse the negative impact and raise the level of gene expression. Here, we investigated whether sodium butyrate could reverse the memory impairment triggered by imidacloprid from a behavioural perspective. Honeybees were treated with or without sodium butyrate for 7 days, followed by 1 day of imidacloprid treatment. After imidacloprid treatment, honeybees were subjected to 20 cycles of training (Fig. 4B). After training was completed, the proboscis extension reflex (PER) of the honeybees was measured daily for 7 days. The results of the PER experiment are shown in Fig. 4C. The memory ability of the bees started to decrease after 3 days of treatment with imidacloprid and decreased by 30% after 7 days of treatment with imidacloprid compared with the control ($P < 0.05$). However, the cotreatment of bees with imidacloprid and sodium butyrate significantly improved their memory ability. The results suggest that sodium butyrate regulates the expression of genes associated with memory and consequently improves memory in bees (see Discussion).

Discussion

As an insect with worldwide economic importance, the widespread loss of honeybees has attracted much attention (Potts *et al*., 2010). However, much remains unknown regarding the cause, mechanism and potential treatment of CCD and regarding appropriate preventive measures. Despite many detailed studies performed in mammals, many of the mechanisms by which insects cope with external stress as well as the consequences on their nervous systems remain unknown (Even *et al*., 2012). This study focused on gene expression in the memory system and the

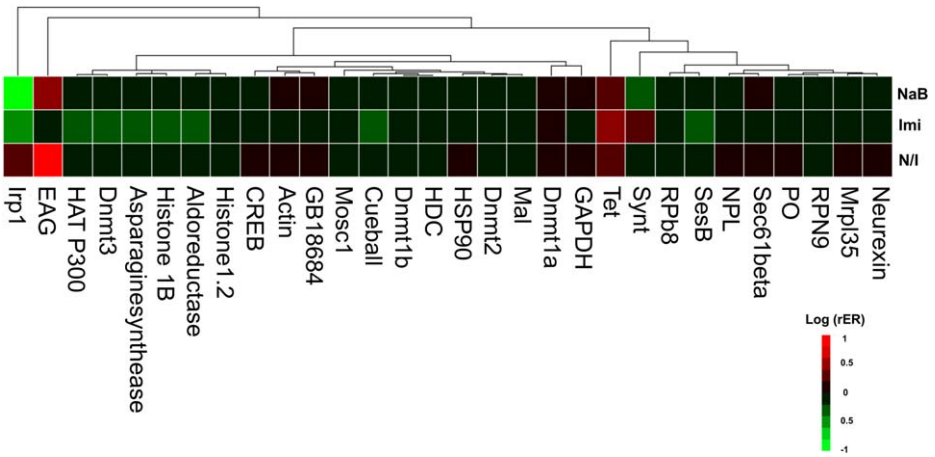

Figure 3. Relative expression (rER) of memory genes. PCR array analysis of memory gene expression levels after imidacloprid or sodium butyrate treatment. The scale is the logarithm of the fold change (control group = 1). The fold changes indicate comparisons with the data from the control groups. NaB, sodium butyrate; Imi, imidacloprid treatment; N/I, imidacloprid and sodium butyrate cotreatment. Expression of 18s ribosomal RNA was used as the control. All experiments were performed with at least three biological replicates, and the data were assessed using Mann–Whitney *U*-tests. Each treatment group in every biological replicate contained 20 bees. Mrpl35, mitochondrial ribosomal protein L35; RPN9, Regulatory particle non-ATPase 9; PO, phenol oxidase; Sec61Beta, Sec61 β subunit (CG10130, FBgn0010638) Drosophila melanogaster; NPL, N-acetylneuraminate pyruvate lyase; SesB, stress-sensitive B; Rpb8, subunit 8 of RNA polymerase II. (RPB8); Synt, Syntaxin 1A; Tet, tetracycline resistance protein; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; Dnmt1a, DNA methyltransferase 1a; Mal, myelin and lymphocyte protein, T cell differentiation protein; Dnmt2, DNA methyltransferase 2; HSP90, heat shock protein 90; HDC, Histidine Decarboxylase; Dnmt1b, DNA methyltransferase 1b; Mosc1, mitochondrial mosc domain-containing protein 1; CREB, cAMP response element binding protein; Dnmt3, DNA methyltransferase 3; HAT P300, Histone acetyltransferase P300; EAG, ether a go-go; Irp 1, Iron regulatory protein 1. [Colour figure can be viewed at wileyonlinelibrary.com]

outcome resulted from interactions between a pesticide and an HDACi. Specifically, we evaluated the effects of imidacloprid and sodium butyrate on memory-related genes in honeybees.

Imidacloprid has been demonstrated to damage honeybee memory and learning ability (Peng & Yang, 2016). It was reported to cause abnormal behaviours in forager bees, including an inability to find their own hive, in a behavioural study (Yang *et al.*, 2008). In addition, PER in honeybees is impaired after feeding imidacloprid for 30 min (Decourtye *et al.*, 2004). Imidacloprid has been suspected to be responsible for CCD for decades, since the first occurrence of bee loss (van der Sluijs *et al.*, 2013). Memory formation is a very complicated process in which epigenetic modifications play a huge role. Amongst the 32 memory-related genes analysed in this study, only six genes (*Asn synthetase*, *HAT P300*, *Dnmt3*, *histone 1B*, *histone 1.2* and *sesB*) were significantly down-regulated by imidacloprid treatment (Supporting Information Fig. S1A). The stabilization of memory formation requires a reduction of synaptic remodelling (Hourcade *et al.*, 2010). SesB is involved in energy metabolism during neurotransmission (Rikhy *et al.*, 2003), which might affect memory formation. The DNA-binding protein CREB has been revealed to be involved in long-term memory (Felsenberg *et al.*, 2015). As a coactivator of CREB, down-regulation of HAT P300 might impede memory formation. Interestingly, previous studies found that memory-associated genes are up-regulated after Dnmt inhibition (Biergans *et al.*, 2015). However, our data showed that the down-regulation of *dnmt3* did not result in *sesB* up-regulation (Fig. 3). Memory impairment by imidacloprid must be investigated in further detail at the gene level (Stanley *et al.*, 2015; Hu *et al.*, 2017).

Sodium butyrate reversed inhibited expression of several memory genes by imidacloprid (Fig. 3). The three genes significantly affected by sodium butyrate were *histone 1.2*, *irp1* and *synt1* (Supporting Information Fig. S1B). Several studies have reported that synt1 is responsible for neurotransmitter release involved in synaptic remodelling (Adolfsen & Littleton, 2001; Adolfsen *et al.*, 2004). A study of hippocampal LTM showed that facilitation of LTM could be achieved by the injection of butyrate, owing to acetylation of certain regions of histone 3 (Levenson *et al.*, 2004). This finding echoed Biergans and Levenson's theory that the formation of memory entails changes in chromatin structure (Levenson *et al.*, 2004; Biergans *et al.*, 2015). It has been shown in both vertebrate and invertebrate systems that histone acetylation enhances memory formation by acetylation of memory-related genes. In our PER experiment, the performance of imidacloprid-treated bees improved significantly after treatment with sodium butyrate, implying that gene expression plays an important role in memory formation (Fig. 4). Although the PER experiment was devised to study the learning ability of bees, it can also provide information on their memory capacity. PER

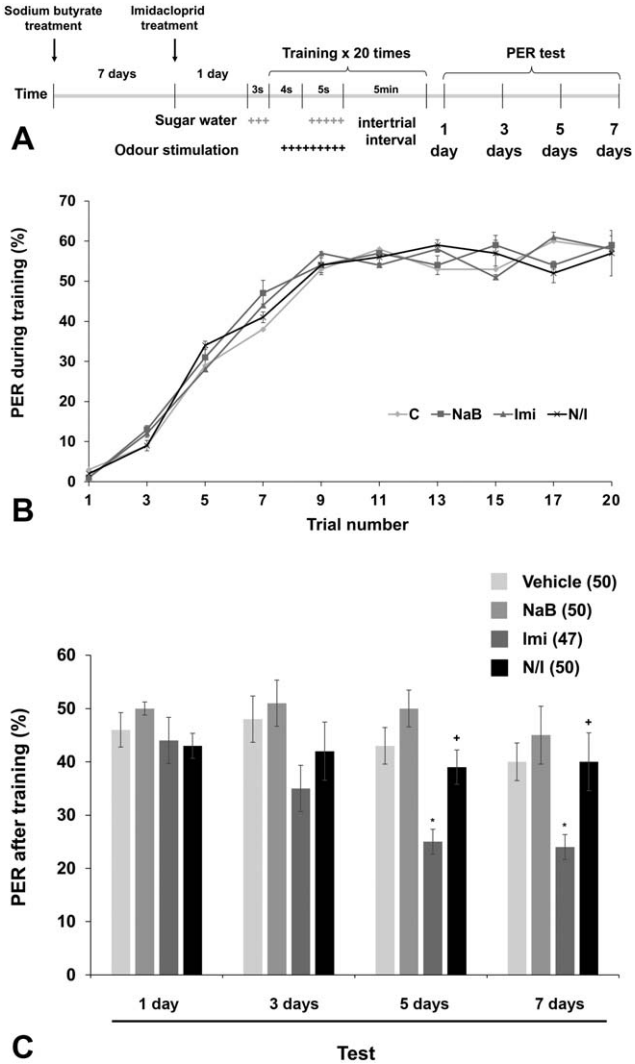

Figure 4. The histone deacetylase inhibitor sodium butyrate restores memory ability after imidacloprid treatment as indicated by the proboscis extension response (PER) test. (A) Schematic drawing of trials. (B) Learning curves for 20 odour-conditioning trials with limonene, showing percentage of correct PERs. (C) PER assay result at 1, 3, 5 and 7 days after imidacloprid treatment with or without sodium butyrate (10 mM). The values in parentheses indicate the number of bees per treatment group. The percentage of PER responding from antennal stimulation with limonene stood for the level of memory formation. C, control; Imi, imidacloprid treatment; NaB, sodium butyrate treatment; N/I, sodium butyrate and imidacloprid cotreatment. Each group represents $N > 45$ bees. The data are presented as mean ± SD from three independent experiments. '*' indicate significant differences between imidacloprid treatment group and control group (*, $P < 0.05$). '+' indicate significant differences between imidacloprid treatment group and sodium butyrate and imidacloprid cotreatment group (+, $P < 0.05$).

provides information linked to learning, whereas memory is required to retain the information learned. Therefore, sodium butyrate appears to be able to maintain or restore the memory capacity of bees affected by imidacloprid treatment. A previous report shows that fruit flies constantly reassess and reprocess learned information, and, in the long term, only the information that is able to optimize their behaviours is maintained (Felsenberg *et al.*, 2017). An earlier study reported that HDACi did not affect memory formation (Merschbaecher *et al.*, 2012). Our experimental results also showed that the HDACi tested (sodium butyrate) did not increase the expression of memory-related genes; nevertheless, it was shown to reverse the inhibited expression of these genes by imidacloprid (Fig. 3, Supporting Information Fig. S1) and to bring their expression level to approximately that of the control group. In our study imidacloprid-treated bees exhibited a significant decrease in PER during the later part of the experiment and a loss of LTM. This may be because the affected bees lost their ability to reassess and reprocess the learned information or because their memory capacity was affected. The modification of heterochromatin affecting biological processes is an interesting and complicated issue that requires further exploration.

Table 2. List of reverse transcription PCR primers and target genes

Gene name	Forward sequence	Reverse sequence
Actin	TTCCCATCTATCGTCGGAAG	CTCTCTTTGATTGGGCTTCG
Aldoreductase	TAGTCCCCTTGGATCACCTG	TTGGGTCATCTGGTTTAGCC
Asparagine synthetase	TGGAATTTGGGCTCTTTTTG	TTCTGGACCACGGTGTGTAA
Histone acetyltransferase P300	ACCAAGTGGAGGTCAACCTG	ATATTGTGGGTGGGCAAGAA
Cyclic adenosine 3′,5′-monophosphate response element binding protein	AATTGCAACCCAAGGTGAAG	TCAGTATGCACAAGGCCAAG
Cueball	CCAAAAGACGGGAAAAATGA	ACGCGTTAAAATCCCACTTG
DNA methyltransferase 1a (*Dnmt1a*)	TGATCCAAAAACAGATGAGGAA	TACAGCACCATTCGGATGAC
Dnmt1b	GAAATTACATGGGTGGGAGAA	GTCACTGCCTCTTCGAAACC
Dnmt2	TGAGTCCTCCATGTCAACCTT	GCCAAATTGACAAGGGCTTA
Dnmt3	CCTCCAACTGGACTTTGGAC	ACGTTCGGATTGTCCTTCAG
Ether a go-go	GATGACCAAGGGCCTAGACA	ATGCTCGTTGAACACCTTCC
Glyceraldehyde 3-phosphate dehydrogenase	GATGCACCCATGTTTGTTTG	TTTGCAGAAGGTGCATCAAC
GB18684	TATTGTTGCTTCGGATCGTG	CGGTGTTCTTTTTCCGATCT
Histone 1B	GCTAAGAAGCCAGCAACACC	TACCTTCGATGCGCTCTTTT
Histone 1.2	GCCAATCCAACAAAGAAAGC	ATTGGTGACCGTCGTGATTT
Histidine Decarboxylase	ATCCGGGAAGAGGAAGTGAT	TGTTCTCCATGGTGTCGTGT
Heat shock protein 90	GGCTGCCAAGAAGCATTTAG	AGCTTCAGCTTTTTGCCTCA
Iron regulatory protein 1	TATCGGAAAAGCTGGACCAC	TATCGGAAAAGCTGGACCAC
Myelin and lymphocyte protein, T cell differentiation protein	CATCCTTGCTAATGCTTCACC	GCAACAATTCCTCCTTGCAT
Mitochondrial mosc domain-containing protein 1	TTGTATGCCAGCAGAAGGAA	TTGGTTCACCACCAGTTAAGC
Mitochondrial ribosomal protein L35	AACTTTTGCTGGCCGTCAT	AACTTTTGCTGGCCGTCAT
Neurexin I	CGGAGAACGCTGCCTTAATA	TCAGAACAATGGCGATCAAC
N-acetylneuraminate pyruvate lyase	TAGTGAGGCAGCTCCAAACA	CGAGGAATTCTCCCATGTGT
Phenol oxidase	TGTGGAGGCAGAGCCTTAGT	TACACCGTCGCAAAAACTGA
Subunit 8 of RNA polymerase II. (RPB8)	TATCCTGATGGAGGGGAGTG	GGGTTCATTGCTTGCTTCAT
Ribosomal protein L32	CGTAACCTTGCACTGGCATT	TTGCTCATGGTGTGAGCAGT
Regulatory particle non-ATPase 9	CAAGCTTTCAATGCTGGTGA	CTTTGCTCCATTGTGGCTTT
Sec61 β subunit (CG10130, FBgn0010638) Drosophila melanogaster	CCCAGCAAAGCTATAGCACCT	TTCATCAGAACCAGCTCCTGT
Stress-sensitive *B*	TGATGTAGGCAAAGCTGGTG	GGCAGCACGATAGATGATGA
Synaptotagmin	CCAAACACGATCAGATCGGTG	CTTCAGATCCGTACGTGAAGG
Tetracycline resistance protein	TCACGAGCAAAAGACACCTGG	ACATGTTTTCCGGCTTATCG

In conclusion, this work examined the effects of sodium butyrate and imidacloprid on memory-related genes as well as on behaviour in honeybees. To date, limited studies have investigated gene expression and behavioural changes in honeybees in response to pesticides. The effects of HDACis and their interactions with target genes are sophisticated and are involved in a wide spectrum of biological processes. Our investigation provides valuable information on HDACi gene regulation associated with memory mechanisms at the epigenetic level. These findings provide insightful information to the future researches and may potentially benefit the beekeeping industry.

Experimental procedures

Bee rearing

Western honeybees (*A. mellifera*) were obtained from a bee farm in Taoyuan County, Taiwan. Workers were collected for qPCR and PER analysis. Workers (at 12 days of age) were caged in a BugDorm (30 × 30 × 30 cm) (Nature Store, Taichung, Taiwan) and maintained in an incubator at 37 °C with access to 50% sucrose solution (w/v) or sucrose solution containing 10 mM sodium butyrate (Hu *et al.*, 2017). The feeding solution was prepared from a stock solution of 500 mM sodium butyrate in double-distilled H_2O (ddH_2O) or imidacloprid commercial product (28.1%) (Great Victory Chemical Industry CO., LTD, Yunlin County, Taiwan) was dissolved in 100% acetone. Bees were treated with sodium butyrate and regular food for 7 days. At the end of the treatment, the bees were collected for gene analysis or PER assays.

Western blot analysis

The honeybees used for the gene expression analysis were divided into three groups: no treatment, sodium butyrate only and imidacloprid only. Sodium butyrate (10 mM) in ddH_2O was used in the feeding assay for 7 days. Protein was extracted from the heads of the honeybees for western blot analysis using a kit from Millipore (Billerica, MA, USA; Hu *et al.*, 2017). Each well of the gel contained proteins extracted from the head of a single bee. Total protein was suspended in sample buffer from Bio-Rad (Hercules, CA, USA). Ten micrograms of protein from each sample was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose filters (Schleicher & Schuell Bioscience, Inc., Keene, NH, USA) by electroblotting for 1 h in 200 mM glycine, 2.5 mM Tris/HCl and 20% methanol. The filters were blocked with phosphate-buffered saline containing 5% nonfat dried milk and 0.05% Tween-20 (AMRESCO, Inc., Solon, OH, USA) and incubated with primary antibodies against acetyl-H3, acetyl-H4 and actin (Millipore), followed by incubation with horseradish peroxidase-conjugated rabbit antimouse antibody

(Millipore). The proteins were detected via an enhanced chemiluminescence system (Immobilon Western, Millipore). Each group included three biological replicates.

Total RNA preparation

The honeybees used for the gene expression analysis were divided into four groups: no treatment (acetone only), sodium butyrate only, imidacloprid only and sodium butyrate/imidacloprid. Sodium butyrate (10 mM) in ddH_2O was used in the feeding assay for 7 days. After treatment with or without imidacloprid (10.447 ng/bee) for 24 h, RNA was extracted for RT-PCR or quantitative reverse transcription PCR (RT-qPCR) analysis. Honeybee RNA was extracted using an RNA extraction kit (GeneMark, GMbiolab Co., Ltd, Taichung, Taiwan). Four honeybee heads were pooled together for homogenization, and the RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

cDNA synthesis

First-strand cDNAs were obtained using SuperScript® III First-Strand Synthesis SuperMix following the manufacturer's protocol (Thermo Fisher Scientific Inc.). One μg of RNA harvested from each sample was used for the generation of first-strand cDNAs with the following conditions on a PCR machine (TAdvanced 96, Biometra GmbH, Göttingen, Germany): 50 °C for 50 min and 85 °C for 5 min.

Analysis of gene expression by RT-PCR and RT-qPCR

Sodium butyrate (10 mM) in ddH_2O was used in the feeding assay for 7 days. After treatment with imidacloprid for 24 h, four honeybee heads were pooled together for homogenization. RNA was extracted for RT-PCR or RT-qPCR analysis. The eluted RNAs were reverse transcribed using a PrimeScript™ RT-PCR Kit (Takara Bio Inc., Kusatsu, Shiga, Japan), and cDNA was reverse transcripted from 800 ng RNA in each sample. PCR was conducted using the following primers: *18s*-F: AGCCTGCTAAATAGACGTAACTTATGG; 18s-R: GATTTGTTTGTACGTTGGTAGTAAAAACC; *Amdop1*-F: TGAACGATCTCCTCGGCTAT; *Amdop1*-R: ACCCAACGACCGTATCTG AG; *Amdop2*-F: GGATCAACAGCGGAATGAAT; *Amdop2*-R: GCGATTCTTTGACTCGGTTT. For qPCR, honeybee-specific primers for memory-related genes (Table 2) were used as described in previous studies (Gregorc *et al*., 2012; Biergans *et al*., 2015; Hu *et al*., 2017). qPCR was performed using an ABI PlusOne real-time system (StepOnePlus™, Applied Biosystems Inc., Foster City, CA, USA) with SYBR Green enzyme (Bioline USA Inc., Boston, MA, USA). All samples were amplified simultaneously, and three independent experiments were performed. 18s ribosomal RNA (rRNA) was included in each reaction as an internal standard, and relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ (Delta Ct is that Ct value of target gene minus Ct value of housekeeping gene) method. Each group included three biological replicates.

PCR-array images and data analysis

PCR-array images were analysed with R statistical software (R Development Core Team, 2008). Fold changes were calculated by the relative quantification method ($2^{-\triangle\triangle Ct}$; Livak & Schmittgen, 2001). Each group of tested genes was normalized to a reference gene, *18s rRNA*, for memory-related genes; the fold change in the control group was used as a calibrator. Raw Ct values are listed in Supporting Information Table S1.

Statistical analysis

Statistical analysis was performed using SPSS statistical software (IBM SPSS Statistics for Windows, Version 22.0. IBM Corp., Armonk, NY). All data were analysed using Mann–Whitney *U*-tests. *P*-values < 0.05 between the sample group (imidacloprid, sodium butyrate, or sodium butyrate/imidacloprid treatment) and the control group were considered statistically significant. Each group included three biological replicates. Data (means $\pm$ SDs) were collected from triplicate assays of three independent experiments.

PER training

To test the olfactory associative behaviour of the honeybees, the principles of classical conditioning were applied with odour as the conditioned stimulus and sugar water as the unconditioned stimulus to observe their PER. Bees were first starved for 3 h before the test (Liang *et al*., 2016), followed by anaesthesia by placing them in a 4° C ice bucket for 5–10 min. After anaesthesia, they were fixed at the tips of 1000-μl pipettes with beeswax/resin mixture and left for 1 h for their physiological condition to recover. Cotton swabs were soaked in 50% (w/v) sucrose solution and then applied over the antennae of the honeybees. Honeybees with a normal PER were tested for their olfactory associative behaviour whereas those who could not produce a PER were eliminated. Honeybees were treated with or without sodium butyrate for 7 days, followed by imidacloprid treatment for 1 day. Approximately 45 honeybees per treatment group were tested for their olfactory associative behaviour after their respective treatment. When training the bees to associate, they were offered 50% sucrose solution for 3 s, followed by odour stimulation (4 μl/ml of limonene) for 4 s and then coexposure to odour stimulation and sugar water for 5 s. These steps accounted for one training trial. The training trial was repeated 20 times at 5-min intertrial intervals. The odour stimulation used in this experiment was 4 μl/ml of limonene. The test for PER by providing odour stimulation was to observe whether the honeybees had associated this stimulation with sugar water. The PER response rate was calculated according to Ray & Ferneyhough (1997) as:

$$\text{PER response rate} = (\text{Counts of honeybees with response} / \text{Counts of honeybees tested}) \times 100\% \quad (1)$$

Fig. 4A illustrates how the training trial and the PER test process were conducted.

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References

Adolfsen, B. and Littleton, J.T. (2001) Genetic and molecular analysis of the synaptotagmin family. *Cell Mol Life Sci* **58**: 393–402.

Adolfsen, B., Saraswati, S., Yoshihara, M. and Littleton, J.T. (2004) Synaptotagmins are trafficked to distinct subcellular domains including the postsynaptic compartment. *J Cell Biol* **166**: 249–260.

Biergans, S.D., Galizia, C.G., Reinhard, J. and Claudianos, C. (2015) Dnmts and Tet target memory-associated genes after appetitive olfactory training in honey bees. *Sci Rep* **5**: 16223.

Biswas, S., Reinhard, J., Oakeshott, J., Russell, R., Srinivasan, M. and Claudianos, C. (2010) Sensory regulation of neuroligins and neurexin I in the honeybee brain. *PLoS ONE* **5**: e9133.

Blank, M., Werenicz, A., Velho, L.A., Pinto, D.F., Fedi, A.C., Lopes, M.W. *et al*. (2015) Enhancement of memory consolidation by the histone deacetylase inhibitor sodium butyrate in aged rats. *Neurosci Lett* **594**: 76–81.

Bouchecareilh, M., Hutt, D.M., Szajner, P., Flotte, T.R. and Balch, W.E. (2012) Histone deacetylase inhibitor (HDACi) suberoylanilide hydroxamic acid (SAHA)-mediated correction of alpha1-antitrypsin deficiency. *J Biol Chem* **287**: 38265–38278.

Cristino, A.S., Barchuk, A.R., Freitas, F.C., Narayanan, R.K., Biergans, S.D., Zhao, Z. *et al*. (2014) Neuroligin-associated microRNA-932 targets actin and regulates memory in the honeybee. *Nat Commun* **5**: 5529.

Davie, J.R. (2003) Inhibition of histone deacetylase activity by butyrate. *J. Nutr* **133**: 2485S–2493S.

Decourtye, A., Armengaud, C., Renou, M., Devillers, J., Cluzeau, S., Gauthier, M. *et al*. (2004) Imidacloprid impairs memory and brain metabolism in the honeybee (*Apis mellifera* L.). *Pestic Biochem Phys* **78**: 83–92.

Dokmanovic, M., Clarke, C. and Marks, P.A. (2007) Histone deacetylase inhibitors: overview and perspectives. *Mol Cancer Res* **5**: 981–989.

Even, N., Devaud, J.M. and Barron, A.B. (2012) General stress responses in the honey bee. *Insects* **3**: 1271–1298.

Farooqui, T. (2014) Oxidative stress and age-related olfactory memory impairment in the honeybee Apis mellifera. *Front Genet* **5**: 60.

Felsenberg, J., Dyck, Y., Feige, J., Ludwig, J., Plath, J.A., Froese, A. *et al*. (2015) Differences in long-term memory stability and AmCREB level between forward and backward conditioned honeybees (*Apis mellifera*). *Front Behav Neurosci* **9**: 91.

Felsenberg, J., Barnstedt, O., Cognigni, P., Lin, S. and Waddell, S. (2017) Re-evaluation of learned information in *Drosophila*. *Nature* **544**: 240–244.

Ganeshina, O., Erdmann, J., Tiberi, S., Vorobyev M. and Menzel, R. (2012) Depolymerization of actin facilitates memory formation in an insect. *Biol Lett* **8**: 1023–1027.

Gershey, E.L., Vidali, G. and Allfrey, V.G. (1968) Chemical studies of histone acetylation. The occurrence of epsilon-N-acetyllysine in the f2a1 histone. *J Biol Chem* **243**: 5018–5022.

Giurfa, M. and Sandoz, J.C. (2012) Invertebrate learning and memory: fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learn Mem* **19**: 54–66.

Grant, P.A., 2001. A tale of histone modifications. *Genome Biol* **2**: REVIEWS0003.

Gregorc, A., Evans, J.D., Scharf, M. and Ellis, J.D. (2012) Gene expression in honey bee (*Apis mellifera*) larvae exposed to pesticides and *Varroa* mites (*Varroa destructor*). *J Insect Physiol* **58**: 1042–1049.

Gui, C.Y., Ngo, L., Xu, W.S., Richon, V.M. and Marks, P.A. (2004) Histone deacetylase (HDAC) inhibitor activation of p21(WAF1) involves changes in promoter-associated proteins, including HDAC1. *Proc Natl Acad Sci USA* **101**: 1241–1246.

Henry, M., Beguin, M., Requier, F., Rollin, O., Odoux, J.F., Aupinel, P. *et al*. (2012) Response to comment on "A common pesticide decreases foraging success and survival in honey bees". *Science* **337**: 1453.

Hourcade, B., Muenz, T.S., Sandoz, J.C., Rossler, W. and Devaud, J.M. (2010) Long-term memory leads to synaptic reorganization in the mushroom bodies: a memory trace in the insect brain? *J Neurosci* **30**: 6461–6465.

Hu, Y.T., Wu, T.C., Yang, E.C., Wu, P.C., Lin, P.T. and Wu, Y.L. (2017) Regulation of genes related to immune signaling and detoxification in *Apis mellifera* by an inhibitor of histone deacetylation. *Sci Rep* **7**: 41255.

Levenson, J.M., O'Riordan, K.J., Brown, K.D., Trinh, M.A., Molfese, D.L. and Sweatt, J.D. (2004) Regulation of histone acetylation during memory formation in the hippocampus. *J Biol Chem* **279**: 40545–40559.

Liang, C.H., Chuang, C.L., Jiang, J.A. and Yang, E.C. (2016) Magnetic sensing through the abdomen of the honey bee. *Sci Rep* **6**: 23657.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* **25**: 402–408.

Mandilaras, K., Pathmanathan, T. and Missirlis, F. (2013) Iron absorption in Drosophila melanogaster. *Nutrients* **5**: 1622–1647.

Matsuda, K., Buckingham, S.D., Kleier, D., Rauh, J.J., Grauso, M. and Sattelle, D.B. (2001) Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends Pharmacol Sci* **22**: 573–580.

Matsumoto, Y., Sandoz, J.C., Devaud, J.M., Lormant, F., Mizunami, M. and Giurfa, M. (2014) Cyclic nucleotide-gated channels, calmodulin, adenylyl cyclase, and calcium/calmodulin-dependent protein kinase II are required for late, but not early, long-term memory formation in the honeybee. *Learn Mem* **21**: 272–286.

Merschbaecher, K., Haettig, J. and Mueller, U. (2012) Acetylation-mediated suppression of transcription-independent memory: bidirectional modulation of memory by acetylation. *PLoS ONE* **7**: e45131.

Merschbaecher, K., Hatko, L., Folz, J. and Mueller, U. (2016) Inhibition of different histone acetyltransferases (HATs) uncovers transcription-dependent and -independent acetylation-mediated mechanisms in memory formation. *Learn Mem* **23**: 83–89.

Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., vanEngelsdorp, D. *et al*. (2010) High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. *PLoS ONE* **5**: e9754.

Mustard, J.A., Blenau, W., Hamilton, I.S., Ward, V.K., Ebert, P.R. and Mercer, A.R. (2003) Analysis of two D1-like dopamine receptors from the honey bee *Apis mellifera* reveals agonist-independent activity. *Mol Brain Res* **113**: 67–77.

Mustard, J.A., Beggs, K.T. and Mercer, A.R. (2005) Molecular biology of the invertebrate dopamine receptors. *Arch Insect Biochem* **59**: 103–117.

Naeger, N.L., Van Nest, B.N., Johnson, J.N., Boyd, S.D., Southey, B., Rodriguez-Zas, S.D. *et al*. (2011) Neurogenomic signatures of spatiotemporal memories in time-trained forager honey bees. *J Exp Biol* **214**: 979–987.

Peng, Y.C. and Yang, E.C. (2016) Sublethal dosage of imidacloprid reduces the microglomerular density of honey bee mushroom bodies. *Sci Rep* **6**: 19298.

Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O. and Kunin, W.E. (2010) Global pollinator declines: trends, impacts and drivers. *Trends Ecol Evol* **25**: 345–353.

R Development Core Team (2008) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3–900051-07-0. http://www.R-project.org

Ray, S. and Ferneyhough, B. (1997) The effects of age on olfactory learning and memory in the honey bee *Apis mellifera*. *Neuroreport* **8**: 789–793.

Rikhy, R., Ramaswami, M. and Krishnan, K.S. (2003) A temperature-sensitive allele of *Drosophila* sesB reveals acute functions for the mitochondrial adenine nucleotide translocase in synaptic transmission and dynamin regulation. *Genetics* **165**: 1243–1253.

van der Sluijs, J.P., Simon-Delso, N., Goulson, D., Maxim, L., Bonmatin, J.M. and Belzunces, L.P. (2013) Neonicotinoids, bee disorders and the sustainability of pollinator services. *Curr Opin Environ Sustain* **5**: 293–305.

Stanley, D.A., Smith, K.E. and Raine, N.E. (2015) Bumblebee learning and memory is impaired by chronic exposure to a neonicotinoid pesticide. *Sci Rep* **5**: 16508.

Uno, Y., Fujiyuki, T., Morioka, M. and Kubo, T. (2013) Mushroom body-preferential expression of proteins/genes involved in endoplasmic reticulum Ca2+-transport in the worker honeybee (Apis mellifera L) brain. *Insect Mol Biol* **22**: 52–61.

Wang, X., Wheeler, D., Avery, A., Rago, A., Choi, J.H., Colbourne, J.K. *et al*. (2013) Function and evolution of DNA methylation in *Nasonia vitripennis*. *PLoS Genet* **9**: e1003872.

Williamson, S.M. and Wright, G.A. (2013) Exposure to multiple cholinergic pesticides impairs olfactory learning and memory in honeybees. *J Exp Biol* **216**: 1799–1807.

Yan, H., Simola, D.F., Bonasio, R., Liebig, J., Berger, S.L. and Reinberg, D. (2014) Eusocial insects as emerging models for behavioural epigenetics. *Nat Rev Genet* **15**: 677–688.

Yang, E.C., Chuang, Y.C., Chen, Y.L. and Chang, L.H. (2008) Abnormal foraging behavior induced by sublethal dosage of imidacloprid in the honey bee (Hymenoptera: Apidae). *J Econ Entomol* **101**: 1743–1748.

Zovkic, I.B., Guzman-Karlsson, M.C. and Sweatt, J.D. (2013) Epigenetic regulation of memory formation and maintenance. *Learn Mem* **20**: 61–74.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Relative expression of memory genes showing significant changes ($P < 0.05$). (A) Sodium butyrate treatment. (B) Imidacloprid treatment. The grey bar represents the control group; the black bar represents the treatment group. (C) Sodium butyrate/imidacloprid treatment. The grey bar represents the imidacloprid treatment group; the black bar represents the sodium butyrate/imidacloprid treatment group. The results from the control groups were set to 1. The fold changes indicate comparisons with the data from the control groups. Imi, imidacloprid treatment; NaB, sodium butyrate treatment. All experiments were performed with at least three biological replicates, and the data were assessed using Mann–Whitney *U*-tests. Each treatment group in every biological replicate contained 20 bees. '*' indicate significant differences between the imidacloprid or sodium butyrate treatment group and control group (*, $P < 0.05$). '+' indicate significant differences between the imidacloprid treatment group and sodium butyrate and imidacloprid cotreatment group (+, $P < 0.05$).

Table S1. Quantitative reverse transcription PCR threshold cycle values of memory genes in bees. Analyses of messenger RNA were determined with quantitative reverse transcription PCR and 18s ribosomal RNA was used as the reference gene.