Factors modulating dopamine receptor gene expression and calcium signalling in primary cell culture of honeybee Kenyon cells

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Abstract

Signaling via the neuroamine dopamine is known to be important in insects for controlling behaviours such as sleep, arousal, motivation, locomotion, learning and memory. In the honeybee *Apis mellifera* pheromones and hormones can further modulate behaviour, and how these factors mediate their effects on the honeybee is of particular interest. However, faced with such a complex environment as the honeybee brain, it can be difficult to assess the individual effects of single factors. The major goals of this thesis were to determine whether mushroom body Kenyon cells in primary cell culture could be used to investigate the individual effects of hormones on dopamine receptor gene transcript levels as well as to detect the presence of a dopamine-induced calcium response in Kenyon cells of the honeybee.

Using cell culture it was found that the hormones 20-hydroxyecdysone (20HE) and juvenile hormone (JH) had a significant effect on the transcript levels of *Amdop1*, *Amdop2*, *Amdop3*, and the putative ecdysone/dopamine receptor gene *Amgpcr19*. The effects of 20HE were also found to be age dependent, with Kenyon cells from newly-emerged adult workers and pollen foragers displaying distinct differences in the transcript levels of the dopamine receptor genes. These results indicate that both 20HE and JH could contribute to age-related changes in dopamine signaling in the honeybee brain.

Using the insight gained from the work on Kenyon cells *in vitro*, the potential effects of 20HE to modulate dopamine responses in the mushroom body calyces of the honeybee were investigated. This preliminary work showed that dopamine-induced changes in cAMP levels in mushroom body calyces of newly-emerged adult workers and in pollen foragers were increased in the presence of 10 nM of 20HE. In the absence of 20HE the response of newly-emerged adult worker and pollen forager mushroom body calyces to dopamine were different, with the application of dopamine reducing levels of cAMP in mushroom body...
Finally, Kenyon cells in primary cell culture were used to investigate the hypothesis that dopamine receptor activation (in particular AmDOP2) increases intracellular calcium. A dopamine-induced calcium influx was identified. Consistent with the hypothesis that these responses are mediated by AmDOP2 receptors, the responses could be blocked by the receptor antagonists cis-flupentixol and epinastine.

The work described in this thesis demonstrates the potential for using Kenyon cells in vitro to gain a clearer picture of how dopamine modulates the functioning of neurons in the mushroom bodies of the honeybee brain. Development of this technique provides an important bridging tool between the whole brain of the honeybee which due to the complex nature of its neural networks make the role of individual agents difficult to define, and transfected cell cultures, in which receptors are not in their native environment.
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<tr>
<td>Amdop1</td>
<td><em>Apis mellifera</em> dopamine receptor 1 gene</td>
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<tr>
<td>AmDOP1</td>
<td><em>Apis mellifera</em> dopamine receptor 1 protein</td>
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<td>Amdop2</td>
<td><em>Apis mellifera</em> dopamine receptor 2 gene</td>
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<td>AmDOP2</td>
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<td><em>Apis mellifera</em> octopamine receptor 1 gene</td>
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<tr>
<td>AmOA1</td>
<td><em>Apis mellifera</em> octopamine receptor 1 protein</td>
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<tr>
<td>Rpn2</td>
<td>ribophorin II gene</td>
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<tr>
<td>Rps8</td>
<td>ribosomal protein s8 gene</td>
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<td>dDA1</td>
<td><em>Drosophila</em> orthologue of AmDOP1 dopamine receptor</td>
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<tr>
<td>DAMB</td>
<td><em>Drosophila</em> orthologue of AmDOP2 dopamine receptor</td>
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<tr>
<td>DDR2</td>
<td><em>Drosophila</em> orthologue of AmDOP3 dopamine receptor</td>
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<tr>
<td>DmDopEcR</td>
<td><em>Drosophila</em> dopamine/ecdysteroid receptor</td>
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<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>OA</td>
<td>Octopamine</td>
</tr>
<tr>
<td>20HE</td>
<td>20 hydroxyecdysone</td>
</tr>
<tr>
<td>JH</td>
<td>Juvenile hormone</td>
</tr>
<tr>
<td>QMP</td>
<td>Queen mandibular pheromone</td>
</tr>
<tr>
<td>HVA</td>
<td>Homovanillyl alcohol</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine-3',5'-monophosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC(\beta)</td>
<td>Phospholipase C beta</td>
</tr>
<tr>
<td>MB</td>
<td>Mushroom body</td>
</tr>
<tr>
<td>ICC</td>
<td>inner-compact Kenyon cells</td>
</tr>
<tr>
<td>OCC</td>
<td>outer-compact Kenyon cells</td>
</tr>
<tr>
<td>NCC</td>
<td>non-compact Kenyon cells</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>GABA</td>
<td>(\gamma)-Aminobutyric acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacyl glycerol</td>
</tr>
<tr>
<td>CaMKII</td>
<td>(Ca_{2+})/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>IP(_3)</td>
<td>Inositol 1,4,5-triphosphate</td>
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<tr>
<td>PIP(_2)</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
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Chapter 1

Mushroom bodies of the honeybee
The honeybee *Apis mellifera* has proved to be a useful research tool in neuroethology, as honeybees exhibit a remarkable array of well characterised social and cognitive behaviours (Srinivasan, 2010, Menzel and Giurfa, 2006, Devaud et al., 2015, Galizia et al., 2012). In particular, their contribution to the understanding of memory and learning has been invaluable (Menzel, 1999, Menzel and Giurfa, 2006, Galizia et al., 2012). While other model organisms such as *Drosophila* have greater access to genetic tools, they lack the more complex behaviours and social interactions present throughout the life history of the honeybee. The honeybee’s rich repertoire of complex behaviours is necessarily accompanied by much larger and complex brain. Evidence of social modulation of brain function places the eusocial honeybee in a unique position as a research tool.

Studies seeking to understand the neural mechanisms that underlie honeybee behaviour, including learning and memory have focused on the mushroom bodies (Menzel, 1999, Menzel and Müller, 2001, Scheiner et al., 2006, Devaud et al., 2015, Lutz and Robinson, 2013, Raccuglia and Müller, 2013, Muenz et al., 2015, Scholl et al., 2015). The mushroom bodies are centres that receive multimodal sensory inputs from the primary olfactory centres (the antennal lobes) (Mobbs, 1984, Mauelshagen, 1993, Schröter and Malun, 2000, Gronenberg, 2001, Strausfeld, 2002, Kirschner et al., 2006, Galizia and Rossler, 2010), as well as from the primary visual centres (the optic lobes) of the brain (Gronenberg, 2001, Ehmer and Gronenberg, 2002). Mushroom bodies in insects play an important role in short and long-term appetitive and aversive associative learning and memory (Menzel, 2001, Müller, 2002, Heisenberg, 2003, Davis, 2005, Menzel and Giurfa, 2006, Himmelreich and Grunewald, 2012, Menzel, 2012). They have also recently been shown to be necessary for higher-order configural learning in the honeybee (Devaud et al., 2015).

In the honeybee the mushroom bodies are paired structures situated in the
The mushroom bodies of the honeybee protocerebrum of the brain, constituting a bilateral set of highly organised neuropils (Mobbs, 1984, Strausfeld, 2002). These neuropils are comprised of the vertical (α) and medial (β) lobes and the characteristic medial and lateral calycal cups (Figure 1.1 A). Strausfeld (2002) suggests that the α-lobe is further comprised of two distinct regions, the ‘true’ α-lobe, which constitutes the dorsal two-thirds of the lobe, and the γ-lobe, consisting of the ventral third of the lobe. The somata of the cells that constitute the mushroom bodies, known as the Kenyon cells, sit within and around the calycal cups (Figure 1.1 B). In the honeybee the Kenyon cells themselves make up a significant proportion of the total number of neurons in the brain. There are approximately 360,000 (two hemispheres containing 180,000) Kenyon cells out of a total of 960,000 neurons in the honeybee brain (Witthöft, 1967). The Kenyon cells comprise nearly 40% of the entire honeybee brain, compared to that of 5000 (two hemispheres containing 2500) Kenyon cells in Drosophila, out of total of about 200,000 neurons, or only about 4% (Hinke, 1961). Not only the increased number of Kenyon cells but also the greater proportion of total neurons in the brain indicates the increased complexity of the mushroom bodies in the honeybee.

The Kenyon cells within the mushroom bodies can be divided into distinct sub-populations (Farris et al., 1999, Strausfeld, 2002) which include the inner-compact cell (ICC) situated in the middle of the calycal cups, the non-compact cells (NCC) surrounding the ICC, and the outer-compact cell (OCC) which lie around the outside of each calyx (Farris et al., 1999). Recent studies have identified a fourth potential sub-group of Kenyon cells lying adjacent to the ICCs within the mushroom body calyces (Kaneko et al., 2013, Kaneko et al., 2016). Each of the ICC and NCC Kenyon cells extends a dendrite into the calycal neuropil and sends an axon-like projection through the pedunculus where it bifurcates sending one branch into the α-lobe and one branch into the β-lobe (Strausfeld, 2002). The OCCs, which are the first-born of the Kenyon
Figure 1.1: **Inputs and structure of the honeybee mushroom body**
A) The collar (COL) of the mushroom body (MB) medial (MC) and lateral (LC) calyces receives input via the anterior superior optical tract (ASOT) from the medulla (MED) and lobular (LOB) neuropils of the optic lobe (OL). The lip (LP) receives inputs from the antennal lobes (AL). Projections from the antennal lobes arrive via the medial (m-APT) and lateral (l-APT) antennal lobe protocerebral tracts. The basal ring (BR) receives both antennal and visual inputs. B) The Kenyon cells within the mushroom bodies are divided into distinct sub-populations; OCC outer compact cell, ICC inner compact cells, NCC non-compact cells. Dopaminergic neurons C1, C2, C3 extensively innervate the mushroom bodies. The mushroom body lobes (α), (β) and (γ) colourised to show the relative distribution of projections in the calyces and lobes. Diagram modified from Mobbs 1982, Schäfer and Rehder, 1989, Groenenberg, 2001, and Strausfeld 2002.
Mushroom bodies of the honeybee

cells and sit outside the calycal cups, do not bifurcate and send a single axon-like projection into the γ-lobe (Figure 1.1 B) (Farris et al., 1999, Schröter and Malun, 2000, Strausfeld, 2002).

Each calyx can be divided into distinct sub-regions based on morphology as well as sensory input. Those sub-regions are the lip, collar, and basal rings (Mobbs, 1982). The lip receives extrinsic inputs from the antennal lobes. Projections from the antennal lobes arrive via the medial and lateral antennal lobe protocerebral tracts (m-APT and l-APT respectively). The m-APT and l-APT travel in opposite directions through the honeybee brain, with the lateral tract traveling via the lateral horn before continuing on to the mushroom bodies, whereas the medial tract innervates the mushroom bodies before terminating in the lateral horn (Gronenberg, 2001, Kirschner et al., 2006, Roessler and Brill, 2013). The collar of the mushroom body calyces receives input from the optic lobes, and specifically the medulla and lobular neuropils which send projections to the mushroom bodies via the anterior superior optic tract (Figure 1.1 A). The basal ring receives both antennal and visual inputs (Gronenberg, 2001, Ehmer and Gronenberg, 2002, Roessler and Brill, 2013). The α/γ-lobe of the mushroom bodies show distinct strata of Kenyon cell inputs from the sub-regions of the calycal cup (Strausfeld, 2002) (Figure 1.1 B). For example, Kenyon cells with dendritic arbourisation in the basal ring project to the dorsal most layer of the α-lobe. Ventral to the layer consisting of the basal ring projections, projections from the Kenyon cells with dendritic arbours in the lip and collar of the mushroom body calyces also form distinct strata in the α-lobe (Strausfeld, 2002). The γ-lobe, which appears to receive inputs solely from the OCC, has strata in the reverse dorso-ventral order to that of the α-lobe; lip, collar, and basal ring (Strausfeld, 2002) Figure 1.1 B).

The mushroom bodies are structurally plastic and undergo physical changes during the lifetime of the honeybee (Fahrbach et al., 1998, Schulz and Robin-
Mushroom bodies of the honeybee

son, 1999, Farris et al., 2001, Maleszka et al., 2009, Lutz and Robinson, 2013). For example, the volume of the collar increases with age and foraging experience, as does arbourisation of the dendrites in the calyces (Farris et al., 2001, Muenz et al., 2015). The formation of appetitive olfactory long-term memories increases the density of microglomeruli in the lip of conditioned honeybees, while the neuropil volume of the lip remains constant (Hourcade et al., 2010). This region-dependent plasticity highlights the complex nature of the honeybee mushroom body. It has been proposed that there are two components to mushroom body neuropil enlargement in the honeybee; an experience-dependent component that involves activity-dependent growth within the mushroom body neuropil, and an activity-independent component (Fahrbach et al., 1998, Farris et al., 2001, Fahrbach et al., 2003, Maleszka et al., 2009). The activity-dependent component most likely relies on the input from other brain regions such as the antennal and optic lobes.

The role of dopamine signaling in the insect brain and its role in controlling behaviour is well studied. Dopamine has been shown to influence a wide array of behaviours such as sleep (Seugnet et al., 2008, Seugnet et al., 2009, Riemensperger et al., 2011, Ueno et al., 2012), arousal (Andretic et al., 2005, Kume et al., 2005, Riemensperger et al., 2011), locomotion (Mustard et al., 2010, Riemensperger et al., 2011), and learning and memory (Schwaerzel et al., 2003, Kim et al., 2007, Vergoz et al., 2007, Seugnet et al., 2008, ). In the honeybee brain dopamine titres increase between newly-emerged and in-colony tasked honeybees, and again between in-colony and forager honeybees. Dopamine titres show significant seasonal effects as well as significant variation between colonies (Harris and Woodring, 1992, Wagener-Hulme et al., 1999). Further, within an individual honeybee brain not all regions exhibit the same dopamine titres (Taylor et al., 1992, Schulz and Robinson, 1999). In the mushroom bodies, the dopamine titres are significantly lower in honeybees.
performing in-colony tasks compared to pollen foragers (Schulz and Robinson, 1999). Within the central brain (minus optic lobes) of foragers, dopamine titres are significantly higher in pollen foragers compared to nectar foragers (Taylor et al., 1992). The dopamine titres in the mushroom bodies appear to be age related, as reversion of pollen foragers to in-colony tasks such as nursing does not result in a drop in mushroom body dopamine to nurse-like titres (Taylor et al., 1992, Schulz and Robinson, 1999).

Similar to *Drosophila* (Kim et al., 2007, Blum et al., 2009, Aso et al., 2010, Aso et al., 2012, Berry et al., 2012, Boto et al., 2014), dopamine also appears to be critical for learning and memory in the honeybee. In the honeybee, acutely applied dopamine antagonists block the acquisition and recall of short term memory (Vergoz et al., 2007). Dopamine also inhibits appetitive recall, but does not appear to affect short-term appetitive memory acquisition (Macmillan and Mercer, 1987, Mercer and Menzel, 1982); however, it does impair long-term appetitive memory formation (Klappenbach et al., 2013).

The intrinsic mushroom body Kenyon somata show no dopamine immunoreactivity (Schäfer and Rehder, 1989, Blenau et al., 1999). However, the calyces and lobes of the mushroom bodies receive extrinsic dopaminergic inputs from populations of dopaminergic neurons. Three of the major dopaminergic populations that project into the mushroom bodies have been termed C1, C2, and C3 (Schäfer and Rehder, 1989, Schurmann et al., 1989) (Figure 1.1 B). The C1 and C2 dopaminergic clusters are situated ventral to the mushroom body lobes. The C3 cluster is situated ventral to the lateral calyx between the lateral horn and the medial and lobula of the optic lobes (Schäfer and Rehder, 1989, Schurmann et al., 1989). The C1 and C2 dopamine neuron clusters consist of approximately one hundred cell bodies that extensively innervate the γ-lobe (Figure 1.1 B). A small cluster of unnamed dopaminergic projections situated between the medial calyx and the α-lobe innervates the dorsal strata of the
Mushroom bodies of the honeybee

α-lobe in a distinctive ‘comb’-like pattern (Figure 1.1 B). The C3 cluster sends a dense fibre bundle around the dorsal edge of the α-lobe before innervating both the central brain and the α-lobe (Blenau et al., 1999). A separate branch from the C3 cluster projects along the dorsal rim of the β-lobe before it invades the pedunculus and the calyx of the mushroom bodies (Schurmann et al., 1989, Schäfer and Rehder, 1989).

Within the sub-regions of the calyx, the highest density of dopaminergic innervation is in the collar, followed by the lip of the mushroom bodies. The basal ring receives the lowest density of dopaminergic input (Blenau et al., 1999). The Kenyon cell somata of the honeybee themselves do not show any dopamine immunoreactivity. However, thin dopaminergic fibers invade the calycal cups from the peduncle and lip region of both calyces equally, and the boutons of these dopaminergic fibres appear to be in direct contact with the Kenyon cell somata (Blenau et al., 1999).

*Drosophila* show similar clustering of dopaminergic neurons. The protocerebral posteriolateral 1 (PPL1) dopaminergic cluster is situated ventrolaterally to the calyces similar to the honeybee C3 while the C1/C2 cluster correlate to the protocerebral anteriomedial (PAM) dopaminergic cluster in *Drosophila* (Budnik and White, 1988, Mao and Davis, 2009). While strict comparisons between *Drosophila* and the honeybee cannot be drawn, what is known about the PAM and PPL1 clusters in *Drosophila* may offer some insight into how the C1-3 clusters may function in the honeybee. Not all dopaminergic neurons from the PPL1 and PAM clusters innervate the same regions of the mushroom bodies (e.g. Aso et al., 2010, Aso et al., 2012, Liu et al., 2012, Boto et al., 2014, Hige et al., 2015, Masek et al., 2015, Yamagata et al., 2015). For example, Boto et al., (2014) showed that signaling from distinct dopaminergic neurons of the PPL1 cluster, which innervates the tips of the α/α’ lobes as well and the heel of the neuropil, leads to differential patterning in cAMP and Ca$^{2+}$
Mushroom bodies of the honeybee

response along the mushroom body neuropil. Also, dopaminergic inputs are required from a small subset of the larger PAM cluster, along with the PPL1 cluster to form aversive memory (Aso et al., 2010). This suggests that different neurons within a cluster have distinct roles in learning and memory. This may also be true for the honeybee; however, this has yet to be determined.

Distinct Kenyon cell sub-populations within the mushroom bodies display different levels of the dopamine receptor gene transcripts (Blenau et al., 1998, Kurshan et al., 2003, Mustard et al., 2003, McQuillan et al., 2012,). Transcript levels have also been shown to change through the adult lifetime of the honeybee (Blenau et al., 1998, Kurshan et al., 2003, Mustard et al., 2003, McQuillan et al., 2012,). This suggests that dopamine signaling in the mushroom bodies changes over time. Three honeybee dopamine receptor genes have been cloned and their proteins characterised; AmDOP1 (Blenau et al., 1998, Mustard et al., 2003), AmDOP2 (Humphries et al., 2003, Beggs et al., 2011) and AmDOP3 (Beggs et al., 2005). The levels of Amdop1 transcript are similar between Kenyon cell sub-populations in both newly-emerged adult workers and pollen foragers (Kurshan et al., 2003, McQuillan et al., 2012). Amdop2 shows higher transcript levels in the ICCs than in the NCCs in both newly-emerged adult workers and pollen foragers, and transcript levels in the OCC also appear to increase slightly with age (McQuillan et al., 2012). Conversely, Amdop3 transcript levels show higher levels of expression in the NCC compared to the ICC, and Amdop3 increases in the whole brain within the first 6 days from adult emergence then appear to remain constant into pollen foraging (Beggs et al., 2005, McQuillan et al., 2012).

Dopamine receptors are G-protein-coupled receptors (GPCRs) which can be broadly characterised into two functional groups (Figure 1.2). AmDOP1 (Blenau et al., 1998) and AmDOP2 (Beggs et al., 2005, Beggs and Mercer, 2011) display ‘D1-like’ signaling properties, increasing intracellular cAMP in
Honeybee dopamine receptors

Representation of the three honeybee dopamine receptors. Binding of dopamine to AmDOP1 leads to an increase in cAMP levels. Binding of dopamine to AmDOP2 also increases cAMP levels in the cell, but has also been shown to increase calcium levels through PLC. AmDOP3 activation reduces intracellular cAMP levels. DA dopamine, AC adenylyl cyclase, PLC phospholipase C, Ca$^{2+}$ calcium.
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response to dopamine. The AmDOP1 and AmDOP2 receptors show approximately a 10-fold difference in their EC\textsubscript{50} for dopamine. In transfected cells dopamine-induced increase in cAMP mediated by AmDOP1 at equivalent dopamine concentrations is generally greater than that of AmDOP2 (Mustard et al., 2003). AmDOP1 also displays agonist-independent cAMP activity, a property that AmDOP2 appears to lack; however, the functional significance of the agonist-independent activity of AmDOP1 is unknown (Mustard et al., 2003, Beggs et al., 2005). There is some evidence that AmDOP2 may also signal through calcium (Beggs et al., 2011). In contrast to AmDOP1 and AmDOP2, the functional properties of the AmDOP3 receptor mediated cAMP signalling appear more complex. While AmDOP3 can be broadly characterised as a ‘D2-like’ receptor, and activation of the AmDOP3 receptor can reduce cAMP levels, it can also display agonist-independent activity that increases cAMP levels (Beggs et al., 2005). The cellular milieu in which the receptor is expressed can also affect its function, as it can couple to both the G\textsubscript{bg} and G\textsubscript{i/o} proteins and coupling to G\textsubscript{bg} leads to a dopamine-mediated increase in cAMP levels (Clark and Baro, 2007).

The orthologues of these receptors in Drosophila have been shown to have similar signaling properties. The AmDOP1 orthologue, dDA1/DmDOP1, elevates cAMP, and dumb mutant flies, lacking a functional dDA1/DmDOP1 gene, have impaired learning (Gotzes et al., 1994, Sugamori et al., 1995, Kim et al., 2003, Kim et al., 2007). The AmDOP2 orthologue, DAMB/DopR99B, also signals through both cAMP and Ca\textsuperscript{2+} and has been shown to be important for forgetting (Han et al., 1996, Feng et al., 1996, Berry et al., 2012, Berry and Davis, 2014, ). The AmDOP3 orthologue, DDR2, has been shown to down regulate cAMP, and is important in aversive and appetitive leaning in Drosophila larvae (Hearn et al., 2002, Qi and Lee, 2014). There is also evidence that DDR2 may act as an auto receptor in Drosophila larvae, with the
D2 agonist bromocriptine, and quinpirole decreasing a stimulated dopamine release (Vickrey and Venton, 2011).

Intra-cellular signaling mediated by GPCRs, such as changes in intracellular cAMP levels, play an important role in learning and memory both in vertebrates and invertebrates (Bernabeu et al., 1997, McGuire et al., 2005, Blum et al., 2009). In *Drosophila* for example, mutant flies which lack important genes in the cAMP-signaling pathway such as *rutabaga*, a Ca\(^{2+}\)/calmodulin dependent adenylyl cyclase; *dumb*, the gene encoding dDA1/DmDOP1, and *dunce*, a cAMP phosphodiesterase, whose mutants are unable to break down cAMP, all show impaired learning and memory (Bellen et al., 1984, Walter et al., 1984, Levin et al., 1992, Kim et al., 2007). Increase in cAMP leads to the activation of cAMP-dependent pathways such as cAMP-dependent protein kinase A (PKA). PKA can phosphorylate a range of target proteins within the cells, such as calcium dependent potassium channels (Zhou et al., 2002), and transcription factors such as the cAMP-response element binding protein (CREB) (Mayr and Montminy, 2001, Ishimoto et al., 2009). Increase in intracellular cAMP and activation of cAMP-dependent pathways are also important to behaviour and learning in the honeybee (Hammer and Menzel, 1995, Menzel, 1999, Müller, 2002, Eisenhardt, 2006, Eisenhardt, 2014). Müller (2000) found that inducing prolonged activation of cAMP-dependent PKA in the antennal lobes of the brain can induce long-term memory formation (Müller, 2000). Also, activation of the CREB has been shown to be critical in the transition between short-term memory and transcription-dependent long-term memory (Müller, 2000).

Activation of the AmDOP2 receptor, in addition to increasing cAMP, has also been shown to increase intracellular calcium (Beggs et al., 2011). Calcium signaling is known to be integral to insect behaviour, especially learning and memory (Müller, 2002, Perisse and Waddell, 2011), and has been shown to
be important in the honeybee (Müller, 2002, Perisse et al., 2009). Calcium dependent kinases such as calcium/calmodulin dependent kinase II (CaMKII) are differentially distributed within the honeybee mushroom bodies, and show increased activity in the NCCs compared to the ICCs, and also exhibit higher activity in the mushroom bodies of workers compared to the rest of their brain (Kamikouchi et al., 2000). RNAi of CaMKII disrupts both the early and late phases of long-term memory formation, whilst leaving short and mid-term memory intact (Scholl et al., 2015). Whether AmDOP2 signals through Ca\(^{2+}\) in the mushroom body Kenyon cells of the honeybee is unknown. Calcium signaling via AmDOP2 could potentially play an important role in honeybee behaviour, and in particular learning and memory. Existing literature indicates that calcium signals play an important role in the transition from short and mid-term learning to long-term memory (Müller, 1999, Perisse et al., 2009, Placais et al., 2012, Scholl et al., 2015).

A fourth receptor, *Amgpcr19* may also play a role in dopamine signaling (Geddes et al., 2013). While there is currently no published data on the activity of this receptor, *Amgpcr19* is highly expressed in the mushroom bodies of the honeybee (McQuillan et al., 2012, Geddes et al., 2013). *Amgpcr19* transcript levels also increase with age and are higher in the NCC of pollen foragers than in the ICC. The *Drosophila* orthologue of the AmGPCR19 receptor, DmDopEcR, has been shown to be activated by ecdysteroids such as 20-hydroxyecdysone (20HE) as well as by dopamine (Srivastava et al., 2005). More recently DmDopEcR has been shown to regulate ethanol-induced sedation and potentially interact with DmDOP1 (Petruccelli et al., 2016). The AmGPCR19 receptor in the honeybee could potentially provide a link between dopamine signaling and hormonal signals in the honeybee brain.

Growing evidence indicates that dopamine receptors are influenced both directly and indirectly by pheromone exposure and hormone titres (Jassim et
Mushroom bodies of the honeybee

al., 2000, Sasaki and Nagao, 2001, Sasaki and Nagao, 2002, Paul et al., 2005, Paul et al., 2006, Vergoz et al., 2007, Sasaki and Nagao, 2013, Geddes et al., 2013, McQuillan et al., 2014.). For example, the queen mandibular pheromone (QMP) has been shown to alter brain dopamine levels and levels of dopamine receptor gene transcript in the brain (Beggs, 2007). Young adult workers raised in the presence of QMP show significantly lower levels of *Amdop1* transcript than workers raised without exposure to QMP. Dopamine-induced changes in cAMP levels are reported to be significantly smaller in young adult workers exposed to QMP than those detected in honeybees maintained without exposure to the pheromone. The honeybee response to QMP changes with age and is accompanied by changes in dopamine titres in the brain (Beggs et al., 2007, Vergoz et al., 2009). Exposing young worker honeybees to QMP inhibits aversive learning (Vergoz et al., 2007), and the QMP component homovanillyl alcohol (HVA) alone has a similar effect. HVA has been shown to activate the D2-like receptor AmDOP3 which may, at least in part, mediate QMPs effects on aversive learning (Beggs et al., 2005). Whether HVA is having a direct effect on the honeybee brain, or is acting peripherally, remains unclear.

QMP also inhibits the synthesis of juvenile hormone (JH), which is a key regulator of honeybee caste development (Fahrbach and Robinson, 1996, Jas-sim et al., 2000, Sullivan et al., 2000, Sullivan et al., 2003). Changes in JH hormone titres may contribute to QMP-induced changes in dopamine signaling. The titres of JH over the life time of the honeybee show a small peak around the time of eclosion before increasing dramatically as the honeybee moves from in colony tasks to foraging (Jassim et al., 2000, Hartfelder et al., 2002, Amdam et al., 2010, Figure1.3). In the whole brain, as well as in the mushroom bodies, JH treatment of newly-emerged honeybees increases the levels of dopamine receptor gene transcript (Sasaki et al., 2012). Treating young honeybees with JH increases appetitive learning and acute JH treatment of 2-
The levels both juvenile hormone and 20-hydroxyecdysone change over the lifetime of the honeybee. The titres for these hormones previously reported in the hemolymph of the honeybee have been assayed at similar but not the same intervals. The timings of the hormone peaks and/or increases appear reasonably plastic within the ranges given above, but they do follow a generalised pattern. The figure is a generalised summary of the data presented in Jassim et al., 2000, Hartfelder et al., 2002, and Amdam et al., 2010.

day old honeybees enhances aversive learning performance (McQuillan et al., 2014, Maleszka and Helliwell, 2001). This supports the hypothesis that JH modulates dopamine signaling in the honeybee. It is interesting to note that a small peak in JH titres seen around the time of adult emergence is similarly timed with a rise in the levels of the ecdysteroid 20-hydroxyecdysone (20HE) (Hartfelder et al., 2002, Amdam et al., 2010).

Ecdysteroids have primarily been associated with insect development and reproduction, although in the honeybee 20HE’s role in reproduction is less
Mushroom bodies of the honeybee

clear (Hartfelder et al., 2002, Truman and Riddiford, 2002, Grunenko and Rauschenbach, 2008). While the pattern of 20HE titres in the honeybee are very different to that of JH, 20HE also follows a generally predictable age-related pattern in haemolymph titres over the lifetime of the honeybee. The highest peak in titres is seen just post-eclosion, before reducing to very low levels in the haemolymph (Figure 1.3). Recent evidence suggests that 20HE may be active within the adult insect brain (Ishimoto et al., 2008, Ishimoto et al., 2009, Ishimoto and Kitamoto, 2010, Yamazaki et al., 2011, Geddes et al., 2013, Ishimoto et al., 2013). For example, acute treatment with 20HE has been shown to modulate dopamine receptor transcript levels in the brain, and also disrupts short-term aversive learning (Geddes et al., 2013). Although circulating ecdysteroid titres in the haemolymph of pollen foragers are at significantly lower levels than measured in pupae and young honeybees (Hartfelder et al., 2002, Amdam et al., 2010), many ecdysteroid related genes are elevated in the adult honeybee (Paul et al., 2005, Paul et al., 2006). This suggests that 20HE may remain an important influence on honeybee behaviour throughout the lifetime of the honeybee.

Despite the honeybee’s long history of domestication, there is still a great deal of variability between hives, and also individual honeybees within a hive (Harris and Woodring, 1992, Wagener-Hulme et al., 1999). This can confound investigations into the actions of individual factors in the honeybee brain, as many of the agents that have been investigated have been found to be age dependent and even then, only a subset of honeybees of that age may respond (Vergoz et al., 2007, Geddes et al., 2013, Urlacher et al., 2014). With the very complex nature of the mushroom bodies in vivo it can be difficult to assess the role of individual pheromone components or hormones such as HVA, 20HE or JH. The study presented here attempts to reduce some of these variables by using Kenyon cells in primary cell culture. This thesis
examines whether Kenyon cells in vitro can be used to advance understanding of dopamine’s actions on mushroom body neurons, and whether dopamine signaling is affected by either the pheromone component HVA, or the hormones 20HE and JH. The possibility that dopamine signaling may be influenced by environmental factors such as pheromone signals and changes in circulating hormone levels in the honeybee is also examined using isolated mushroom body calyces. Finally, using primary cell culture, the presence of a dopamine-induced calcium response in the Kenyon cells is confirmed.
Chapter 2

Effect of homovanillyl alcohol, 20-hydroxyecdysone, and juvenile hormone on dopamine receptor genes transcript levels in primary cell culture of mushroom body Kenyon cells
2.1 Introduction

The behaviour of adult worker honeybees changes dramatically over their lifetime (Free, 1965, Robinson, 1992, Schulz and Robinson, 1999, Wagener-Hulme et al., 1999, Beshers and Fewell, 2001, Schulz et al., 2003, Leoncini et al., 2004), and there is evidence that expression of the dopamine receptor genes \textit{Amdop1}, \textit{Amdop2}, \textit{Amdop3}, and of the putative ecdysone/dopamine receptor gene \textit{Amgpcr19}, also change with age (Kurshan et al., 2003, Beggs et al., 2007, McQuillan et al., 2012a, McQuillan et al., 2012b). These dopamine receptor genes are widely expressed in the brain, including in Kenyon cells of the mushroom bodies where their levels of expression appear to be sub-population specific (Humphries et al., 2003, Kurshan et al., 2003, McQuillan et al., 2012b). The signal(s) that lead to developmental changes in levels of dopamine receptor gene transcripts are currently unclear. In this Chapter, \textit{in vitro} techniques are used to determine whether changes in dopamine receptor gene transcript levels can be detected in Kenyon cells maintained \textit{in vitro} and if so, whether the levels can be manipulated.

Primary cell culture techniques have been used extensively to investigate the neurophysiology of honeybee central neurons (Ellen and Mercer 2012). Neurons from the antennal lobes and mushroom bodies of the brain, particularly those from honeybees at the mid-pupal stages of development, survive well in primary cell culture (Gascuel and Masson, 1991, Kreissl and Bicker, 1992, Bicker and Kreissl, 1994). Primary cell culture has been used to provide detailed descriptions of the electrophysiological properties of honeybee Kenyon cells (Schäfer et al., 1994, Goldberg et al., 1999, Pelz et al., 1999, Wustenberg et al., 2004, Wustenberg and Grünewald, 2004, Barbara et al., 2005) and their responsiveness to neuroactive compounds such as acetylcholine, \(\gamma\)-aminobutyric acid (GABA), and glutamate (Goldberg et al., 1999, Barbara et al., 2005, Raccuglia and Müller, 2014). While dopamine has been shown to modulate
Expression of dopamine receptor genes

ion currents in antennal lobe neurons in vitro (Perk and Mercer, 2006), less is known about the effects of this amine on honeybee Kenyon cells (Müller, 1997). Potential modulators of Kenyon cell function are investigated in this Chapter and specifically their effects on levels of gene transcript in Kenyon cells in vitro. Effects of the queen pheromone component homovanillyl alcohol (HVA) on dopamine receptor gene transcript levels, as well as the hormone 20-hydroxyecdysone (20HE) and juvenile hormone (JH) were examined.

Queen mandibular pheromone (QMP) is one of the most well-documented pheromones modulating honeybee behaviour (Slessor et al., 1988a, Slessor et al., 1988b). This multi-component pheromone produced by the queen honeybee (Slessor et al., 1988b, Slessor et al., 2005, Jarriault and Mercer, 2012) inhibits the development of worker ovaries (Hoover et al., 2003) and influences a variety of honeybee behaviours including retinue behaviour (Slessor et al., 1988a), comb building (Ledoux et al., 2001), and foraging (Pankiw et al., 1996). QMP has distinct individual and age-related effects, with only about a quarter of young honeybee workers being attracted to it, while pollen foragers appear to show no attraction to the pheromone (Vergoz et al., 2009). QMP has also been shown to impair aversive learning in young adult workers (Vergoz et al., 2007) and reduce stress reactivity (Urlacher et al., 2014). Effects of QMP on aversive learning can be reproduced by exposing young honeybees to HVA (Vergoz et al., 2007), one of the key components of QMP (Slessor et al., 2005). HVA bears some structural resemblance to dopamine and has been shown to activate the honeybee dopamine receptor AmDOP3 expressed in vitro in heterologous cell lines (Beggs and Mercer, 2009). HVA does not appear to either activate or block AmDOP1- or AmDOP2-receptors, at least not when receptors are expressed and tested in vitro (Beggs and Mercer, 2009).

One central question relating to the actions of HVA in young worker honeybees is whether this compound acts peripherally, for example on AmDOP3 or on
other dopamine receptors expressed at the level of the antennae (Vergoz et al., 2009), or centrally, targeting the intrinsic mushroom body neurons (Kenyon cells). Kenyon cells in vitro are used in this study to determine whether HVA influences the expression of dopamine receptor gene transcripts in these cells and if so, whether Kenyon cells from newly-emerged adult workers respond to HVA in the same way as cells derived from the mushroom bodies of pollen foragers.

In the work described in this Chapter, cell culture techniques are also used to examine whether dopamine receptor gene expression in Kenyon cells is modulated by JH. JH is a key regulator of honeybee development (Fahrbach and Robinson, 1996, Jassim et al., 2000, Sullivan et al., 2000, Sullivan et al., 2003, Harano et al., 2008) and could be responsible for triggering changes in dopamine receptor gene expression in the brains of young worker honeybees (Robinson, 1987, Fahrbach and Robinson, 1996). Studies have shown that precocious foraging behaviour is accompanied by a dramatic increase in JH titres in the haemolymph of young worker honeybees (Jassim et al., 2000) and that application of JH, or its analogue methoprene, induces precocious foraging (Jaycox et al., 1974, Robinson et al., 1987, Sigg et al., 1997, Sullivan et al., 2000, Schulz et al., 2002). JH titres in forager honeybees are normally 10 to 20 times higher than those found in young workers (Jassim et al., 2000). However, young honeybees experience a small surge in JH levels which begins just prior to adult emergence and peaks around 1 day after emergence (Hartfelder et al., 2002, Amdam et al., 2010). This early JH peak has also been observed to occur as late as 2 to 4 days after emergence, a difference in timing that may be due to different developmental rates of individual honeybees and colonies (Jassim et al., 2000). Honeybees that emerge earlier than their colony mates have JH titres in the late pupal stage that are significantly higher than pupae that emerge later (Amdam et al., 2010).
Recently, JH has been found to modulate dopamine receptor gene transcript levels in the mushroom bodies of newly-emerged honeybees (McQuillan et al., 2014), and also to regulate levels of dopamine receptor gene transcript in the whole brain of drones (Sasaki et al., 2012). Interestingly, JH treatment of 2-day old honeybees is reported to enhance their aversive learning performance, suggesting a potential link between this hormone and dopamine signaling in the honeybee brain (McQuillan et al., 2014). In this Chapter, cell culture techniques are used to examine further the potential interaction between this hormone and levels of dopamine receptor gene expression in Kenyon cells.

Effects of the ecdysteroid 20HE on Kenyon cells in vitro are also examined as this hormone has been shown to have a range of effects on neurons, some of which are cell-population specific (Bennett and Truman, 1985, Truman and Reiss, 1988, Levine, 1989, Streichert et al., 1997, Oland and Hayashi, 1993, Grünewald and Levine, 1998). For example, 20HE has been shown to inhibit the death of pupal motor neurons in vitro from Manduca sexta (Bennett and Truman, 1985), but the same hormone triggers segment-specific death of larval accessory planta retractor neurons in this moth (Streichert et al., 1997). Oland and Hayashi (1993) found neuron type-specific effects of 20HE on the morphology of Manduca antennal lobe neurons. They further showed that 20HE at high concentrations negatively impacted the survival of these neurons in vitro, whereas low concentrations of 20HE increased the thickness of neuronal processes (Oland and Hayashi, 1993). 20HE was identified as a likely trigger for increased arbourisation of Manduca lateral neurosecretory neurons during development (McGraw et al., 1998), and as a switch that alters neurotransmitter expression in these peptidergic neurons (Tublitz and Loi, 1993).

While the majority of the effects of 20HE reported refer to developmental changes observed primarily in the moth Manduca sexta, there is evidence in honeybees that 20HE may play a role in the central nervous system of
adult workers. Yamazaki and colleagues (2011) found in honeybees that transcript level of the hydroxylase gene CYP314A1, known as Shade in *Drosophila* and required for the conversion of ecdysone into 20HE (Petryk et al., 2003), is up regulated in the brain of nurses and pollen foragers (Yamazaki et al., 2011). This result suggests that ecdysone may be converted into 20HE in the brain, and consistent with this hypothesis Yamazaki et al., (2011) showed that honeybee brain tissue maintained in culture produces 20HE. Yamazaki and colleagues concluded from their experiments that 20HE may play a role as a neurohormone in the honeybee brain (Yamazaki et al., 2011). More recently, 20HE treatment has been shown to inhibit aversive learning in pollen foragers but leave appetitive learning intact (Geddes 2013). This suggests that 20HE may affect dopamine receptor activity directly in the honeybee brain and evidence from *Drosophila* may support this, as feeding 20HE before aversive conditioning was found to increase long-term memory in male flies, as well as increase levels of cAMP in the fly brain (Ishimoto et al., 2009).

To investigate the expression of dopamine receptor genes in mushroom body neurons and factors that affect levels of expression of these genes, the work outlined in this Chapter aims:

1. to determine whether Kenyon cells in primary culture express dopamine receptor genes,
2. to investigate whether gene transcript levels are affected by the length of time Kenyon cells are maintained in culture,
3. to compare gene transcript levels in Kenyon cells obtained from newly-emerged adult workers with those from pollen foragers, and
4. to determine whether dopamine receptor gene transcript levels in Kenyon cells *in vitro* can be altered experimentally by exposure to HVA, or to the hormones 20HE and/or JH.
2.2 Materials and methods

2.2.1 Collection of honeybees for experimental manipulation

Newly-emerged Newly-emerged adult workers were sourced from brood frames from multiple colonies. Colony choice was dependent on available brood stocks. All honeybees were sourced from colonies housed at either the Department of Zoology or the Department of Biochemistry, University of Otago, New Zealand.

Pollen foragers Pollen foragers were collected using forceps as they returned to the entrance of the hive. Captured pollen foragers were placed in a glass container and cooled immediately on crushed ice until immobilised.

2.2.2 Honeybee Kenyon cells in primary cell culture

Establishment of Kenyon cells in culture Cell culture was used to examine gene expression levels \textit{in vitro} over time and the effects of pheromone and hormone treatments on levels of gene expression. Mushroom body calyces were dissected from either newly-emerged adult workers or returning pollen foragers. Mushroom bodies were placed into 500 μL of supplemented Liebovitz’s L-15 culture medium in an Eppendorf tube as described by Kreissl and Bicker (1992). L-15 medium (Gibco) was supplemented with 22 mM glucose, 14 mM fructose, 117 mM sucrose, and 29 mM proline (pH 7.2, 500 mOsM.). The dissection medium was removed and the calyces were incubated for 10 min in 200 μL calcium-free Ringer’s solution (130 mM NaCl, 5 mM KCl, 10 mM MgCl, 25 mM glucose, 180 mM sucrose, 10 mM HEPES, pH 7.2). A further 800 μL of calcium-free Ringer’s was then added and the calyces were gently triturated to dissociate the Kenyon cells that make up each calyx. The disso-
Expression of dopamine receptor genes

associated cells were left at room temperature for a further 5 mins, allowing the cell debris to settle to the bottom of the Eppendorf tube. The top 900 μL of the calcium-free Ringer’s solution containing the suspended Kenyon cells was decanted into a new 1.5 mL Eppendorf tube. These cells were then centrifuged for 3 mins at 2400 rpm. The supernatant was removed and the Kenyon cells were re-suspended in the required volume of cell culture medium which was supplemented with 13 % heat-inactivated fetal calf serum and 10 mM HEPES exchanged for 2.1 mM PIPES (pH 6.7, 500 mOsm). Kenyon cells from four honeybees (two pairs of calyces per brain, 16 calyces in total) were pooled in 1200 μL of cell culture medium. Aliquots of 400 μL were plated in 24-well cell culture plates (Costar 3526, Corning Incorporated).

**Extraction of total RNA and production of cDNA from mushroom body calyces and from Kenyon cells in cell culture**  The extraction of total RNA and production of cDNA from the honeybee mushroombodies are similar to those previously described (McQuillan et al., 2012a, McQuillan et al., 2012b, McQuillan 2013). For cell culture, the media was removed from the wells and replaced with 500 μL Trizol (LifeTechnologies). Samples were then, homogenised, and centrifuged using a Hermle cryofuge. Samples were then isolated using Micro or Midi purification columns as per the manufacturer’s instructions (Invitrogen). RNA yields were quantified using a Ribo-Green® RNA quantification assay kit (Invitrogen, Carlsbad, CA, USA) on a FLUOstar Omega (BMG Labtech) as previously described (McQuillan et al., 2012a, McQuillan et al., 2012b, McQuillan 2013). Typically, 150 ng of total RNA per sample was reverse transcribed using VILO Supercrypt (Invitrogen, Carlsbad, CA). Gene-specific amplification products from the target genes were generated using ExpressSYBR® GreenER qPCR SuperMix (Invitrogen, Carlsbad, CA) and gene-specific primer pairs (Table 2.1). Primers
Table 2.1: Gene-specific real time qPCR primer pairs

were designed to span intron-exon boundaries; therefore reverse transcription minus control reactions were not required. Single product amplification was confirmed by melting curve analysis. Primer efficiencies were calculated from standard curves generated using cDNA reverse transcribed from a pool of the experimental RNA. Primer pairs, average primer efficiencies and melting point temperatures of product are provided in Table 2.1.

Assessment of transcript abundance via RT-qPCR Real-time quantitative PCR was used to investigate changes in expression of amine receptor genes Amdop1, Amdop2, Amdop3, Amgpcr19, and the potential reference genes rpn2 and rps8 in Kenyon cells maintained in cell culture. Receptor transcript abundance was determined using the Δct method with assay efficiencies incorporated into the following formula:

**Algorithm 2.1 Normalisation of qPCR**

\[
N = \frac{(1 + E_{Target}^{\Delta ct})}{(1 + E_{Reference}^{\Delta ct})}
\]

**Table 2.1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>RE (%)</th>
<th>MP (°C)</th>
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<td>84</td>
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<tr>
<td></td>
<td>Reverse 5’ - ACCCAACGACCCTATCTGAG</td>
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</tr>
<tr>
<td>Amdop2</td>
<td>Forward 5’ - GGATCAACAGCGGAATGAAT</td>
<td>97</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ - GCGATTCTTTGACTCGGTTT</td>
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<td></td>
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<tr>
<td>Amdop3</td>
<td>Forward 5’ - CCACTTTCAATCTCGTAGCAATC</td>
<td>94</td>
<td>80</td>
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<td></td>
<td>Reverse 5’ - AGACCAACAGTATCGTCAACCAC</td>
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<tr>
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<tr>
<td></td>
<td>Reverse 5’ - CACGTTCTTTGGCTACG</td>
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RE = Relative efficiency, MP = Melting point (°C)
Expression of dopamine receptor genes

$E_T$ is the target gene primer efficiency; $E_R$ is the reference gene primer efficiency, and $\Delta \text{ct}$ is the crossing point. Reference genes were found to vary in their stability depending on experimental treatments. All samples were assayed in triplicate.

**Effect of time in culture on transcript levels in Kenyon cells**  To assess the transcript abundance of dopamine receptor genes over time, cells were plated as described above (Section 2.2.2). Cells were maintained in culture for either 1 or 4 days before being harvested and transcript abundances were measured. Levels of gene expression were examined in dissociated Kenyon cells sourced from newly-emerged adult workers and from returning pollen foragers. Changes in levels of mRNA over time *in vitro* were examined using Kenyon cells prepared as described above (Section 2.2.2). Plates were then incubated in a humidified incubator at 26 °C for either 24 hours or 4 days. All treatment groups were independent biological replicates.

**Effect of pheromone and hormone treatment on dopamine receptor gene expression**  To assess the effect of HVA on dopamine receptor transcript levels *in vitro*, cells from either newly-emerged adult workers or returning pollen foragers were aliquoted as above in Section 2.2.2 and incubated for 24 hours. Cells were then treated with HVA at a final concentration of 1 μM and controls received vehicle alone (cell culture medium). Cells were then incubated for a further 3 days before being harvested.

The effect of 20HE on dopamine receptor gene expression was assessed after both short-term (24 hours) and long-term (4 days) exposure. In all cases the cells were exposed to a final concentration of 30 nM 20HE. Controls received 1 μL volume of vehicle alone (cell culture medium plus $10^{-8}$ mL ethanol). To assess short-term exposure, cells from newly-emerged adult workers were aliquoted and left to settle for 3 hours before being treated with 20HE and
harvested at 24 hours. Long-term exposure was performed on cells from newly-emerged adult workers or returning pollen foragers. Cells were aliquoted and allowed to settle for 24 hours before being treated. Cells were harvested 3 days after treatment.

The effect of JH alone and the combined effects of 20HE and JH on gene transcript levels were assessed on cells from newly-emerged adult workers treated with 30 nM 20HE and/or 75 nM JH. Cells received 20HE at 3 hours post-plating and cells receiving JH were treated 24 hours post-plating. All cells were harvested at 4 days post-plating. Controls received 1 μL volume of vehicle alone (cell culture medium plus ethanol (10⁻⁸ mL) and acetone (10⁻⁸ mL)).

2.2.2.1 Statistical analysis

Differences between receptor gene expression levels in control and either HVA or 20HE treated groups were analysed using Students two-tailed unpaired t-tests. Analysis of gene expression changes in response to 20HE and JH were analysed by Two-Way ANOVA followed by Tukey’s multiple comparison tests. All analyses were conducted in PRISM® (7.0a GraphPad Software Inc, San Diego, CA).
2.3 Results

2.3.1 Expression of dopamine receptor gene transcripts by mushroom body Kenyon cells

2.3.1.1 Effect of time in culture

To examine the effect of time spent in culture on dopamine receptor gene transcript abundance, Kenyon cells were harvested from newly-emerged adult workers and maintained in culture for either 24 hours or 4 days. The two groups of cells were assessed for the presence and relative abundance of dopamine receptor gene transcripts (Figure 2.1 A-D). Transcript levels of two potential reference genes, rpn2 and rps8, were also examined (Figure 2.1 E, F). Amdop1 transcript levels in cells maintained 24 hours in vitro were similar to levels detected in cells maintained in culture for 4 days (Figure 2.1 A, p = 0.8413). In contrast, levels of Amdop2 transcript were significantly higher in cells maintained 4 days in vitro than in cells maintained in culture for only 24 hours (Figure 2.1 B, p = 0.0079). A pattern similar to that observed for Amdop2 was also apparent for Amdop3 (Figure 2.1 C, p = 0.0112), Amygpcr19 (Figure 2.1 D, p = 0.0195) and for the two potential reference genes, rpn2 (Figure 2.1 E, p=0.0079) and rps8 (Figure 2.1 F, p= 0.0362). Amdop3 showed the greatest relative increase in transcript abundance of the dopamine receptor genes. After 4 days in vitro transcript abundance was 2.9 ± 0.33 times higher than that detected at 24 hours. The reference gene rpn2 showed the greatest of all increase in transcript levels at 4 days in vitro with 3.82 ± 0.33 times the abundance of that seen at 24 hours.
Figure 2.1: Effect of time in culture on gene transcript levels in mushroom body Kenyon cells *in vitro*
Gene transcript levels in Kenyon cells collected from newly-emerged adult workers and maintained in culture for either 24 hours (white bars) or 4 days (grey bars). Relative transcript abundances for *Amdop1* (A), *Amdop2* (B), *Amdop3* (C), *Amgpcr19* (D), and potential reference genes, *rpn2* (E) and *rps8* (F). Data are normalised to the levels detected after 24 hours. Pairwise comparisons were performed using unpaired Student’s t-tests. Mean±SEM, n = 5 independent samples per group.
2.3.1.2 Comparison of levels of dopamine receptor gene transcripts in Kenyon cells in culture from newly-emerged adult workers versus pollen foragers

Relative levels of expression of the three dopamine receptor genes ($Amdop_1$, $Amdop_2$, and $Amdop_3$) in Kenyon cells taken from newly-emerged adult workers were compared to relative transcript abundances in Kenyon cells harvested from returning pollen foragers (Figure 2.2). The transcript levels were measured after cells had been in culture for 4 days. To examine relative levels of expression of the three dopamine receptor genes, data were normalised to the transcript abundances detected for $Amdop_1$. In Kenyon cells from newly-emerged adult workers (Figure 2.2 A), $Amdop_1$ and $Amdop_2$ transcript levels were not significantly different from one another. However, Tukey’s post hoc test showed that $Amdop_3$ transcripts were expressed at a significantly lower level than either of the other two genes (ANOVA $f_{(2, 12)} = 10.14$, $p = 0.0026$, $n = 15$). In contrast, $Amdop_3$ in pollen forager Kenyon cells had the highest transcript abundance (Figure 2.2 B). The mean transcript abundance of $Amdop_2$ was also significantly higher than that of $Amdop_1$ (ANOVA $f_{(2, 9)} = 21.17$, $p = 0.0004$, $n = 12$). Transcript levels were normalised to the average transcript abundance recorded for $Amdop_1$. 
Figure 2.2: Effect of age of Kenyon cells in culture on gene transcript levels
Relative transcript levels of the three dopamine receptor genes detected in Kenyon cells from newly-emerged adult workers (A) and pollen foragers (B). Kenyon cells were assessed after 4 days in vitro. The transcript levels are displayed relative to transcript abundances detected for Amdop1. ANOVA were performed with a Tukey’s post hoc test. Mean±SEM, n = 5 independent samples per group.

2.3.2 Modulation of dopamine receptor gene transcript levels in Kenyon cells in cell culture

With the presence of dopamine receptor transcripts established in the primary cell culture of both newly-emerged adult workers and pollen foragers, the effects of the pheromone component HVA and hormone treatments were investigated. Due to changes in gene transcript levels over time in vitro (see Figure 2.1) all cells were maintained in vitro for the same length of time and assayed at a single time point of either 24 hours or 4 days.

2.3.2.1 Effect of HVA on dopamine receptor transcript levels in Kenyon cells in cell culture

Kenyon cells from newly-emerged adult workers To elucidate if HVA has a direct effect on dopamine gene transcript abundance, cell culture of Kenyon cells from newly-emerged adult workers was established (Section 2.2.2).
At 24 hours after plating, 1 μM of HVA was added to the culture medium and the cells were left for a further 3 days (Figure 2.3 A-E). Control cells were maintained in culture medium without HVA. *Amdop1* transcript abundance was significantly higher in cells exposed to HVA than in control cells (Figure 2.3 A, p = 0.0352). On the other hand, expression levels of *Amdop2* (Figure 2.3 B, p = 0.0847), *Amdop3* (Figure 2.3 C, p = 0.2152), and *rpn2* (Figure 2.3 D, p = 0.0847) were not significantly affected by HVA treatment. See appendix 1 for dopamine receptor gene transcript levels normalised to house keepers *rpn2* (Section 5.1).

**Pollen forager Kenyon cells** The same experiment was repeated using Kenyon cells from returning pollen foragers (Figure 2.4 A-D). In these cells the abundance of the gene transcript *Amdop1* was unaffected by the treatment (Figure 2.4 A, p = 0.1753). The abundance of *Amdop2* showed a small relative increase in transcript levels (Figure 2.4 B, p = 0.0255) over the control cell, whereas both *Amdop3* (Figure 2.4 C, p = 0.777) and the reference gene *rpn2* (Figure 2.4 D, p = 0.8484) were unaffected by HVA. See appendix 1 for dopamine receptor gene transcript levels normalised to house keepers *rpn2* (Section 5.1).
Figure 2.3: Effect of homovanillyl alcohol on dopamine receptor transcript levels in newly-emerged adult worker Kenyon cells

Kenyon cells from newly-emerged adult workers were treated with 1 μM HVA and maintained in culture for 4 days. The gene transcript level of Amdop1 (A) was significantly increased ($p = 0.0352$). Amdop2 (B) showed no significant increase ($p = 0.0847$). Both Amdop3 (C) and the reference gene rpn2 (D) were unaffected ($p = 0.2152$, $p = 0.7041$ respectively). Unpaired Student’s t-tests, mean ± SEM of 5 independent samples per group.
Figure 2.4: Effect of homovanillyl alcohol on dopamine receptor transcript in pollen forager Kenyon cells
Pollen forager Kenyon cells maintained in vitro were treated with 1 μM HVA and maintained in culture for 4 days. The gene transcript level of Amdop1 (A) was unaffected by the treatment. Amdop2 (B) showed a small increase in transcript levels (p = 0.0255). Both Amdop3 (C) (p = 0.777) and the reference gene rpn2 (D) (p = 0.8484) were both unaffected. Unpaired Student’s t-tests, mean ± SEM of 5 independent samples per group.

2.3.2.2 Effect of 20HE on gene transcript levels in cell culture

Short-term exposure of Kenyon cells from newly-emerged adult workers The effects of 20HE on dopamine receptor gene transcript levels in Kenyon cells from newly-emerged adult workers were examined. Cells were maintained in vitro for 24 hours and exposed to 30 nM 20HE for a total of 21 hours (Section 2.2.2) and the relative abundances of gene transcripts were then measured (Figure 2.5 A-F). Transcript abundances of Amdop1 (Figure 2.5 A, p = 0.2932), Amdop2 (Figure 2.5 B, p = 0.0968), and Amdop3 (Figure 2.5 C, p = 0.777) were unaffected. Unpaired Student’s t-tests, mean ± SEM of 5 independent samples per group.
Expression of dopamine receptor genes

2.5 C, \( p = 0.2336 \) in cells treated with 30 nM 20HE did not differ significantly from transcript levels in control cells. However, there was a significant effect of 20HE on the relative abundance of the \( Amgpcr19 \) transcript (Figure 2.5 D, \( p = 0.0411 \)), which was significantly higher in 20HE-treated cells than in control cells. Transcript abundances of the reference genes \( rpn2 \) (Figure 2.5 E, \( p = 0.8549 \)) and \( rps8 \) (Figure 2.5 F, \( p = 0.4211 \)) were not affected by 20HE. See appendix 1 for dopamine receptor gene transcript levels normalised to house keepers \( rpn2 \) and \( rps8 \) (Section 5.1).

**Longer-term exposure of Kenyon cells from newly-emerged adult worker in cell culture to 20HE** The effect of exposing Kenyon cells to 30 nM 20HE for an extended period was also assessed. Kenyon cells from newly-emerged adult workers were maintained *in vitro* for 24 hours and then exposed to 30 nM 20HE for 3 days (Figure 2.6). \( Amdop1 \) transcript abundance was significantly higher in cells exposed to 20HE than in control cells (Figure 2.6 A, \( p = 0.0324 \)). Transcript abundance for \( Amdop2 \) (Figure 2.6 B, \( p = 0.0536 \)), whilst showing a strong trend, was not significantly affected by 20HE. The abundance of \( Amdop3 \) transcript (Figure 2.6 C, \( p = 0.5185 \)) was similar in treated and control cells. The largest effect on relative transcript abundance was on \( Amgpcr19 \) (Figure 2.6 D, \( p = 0.0035 \)). The expression of both reference genes, \( rpn2 \) (Figure 2.6 E, \( p = 0.794 \)) and \( rps8 \) (Figure 2.6 F, \( p = 0.0742 \)) showed no significant effect of 20HE. See appendix 1 for dopamine receptor gene transcript levels normalised to house keepers \( rpn2 \) and \( rps8 \) (Section 5.1).

**Longer-term exposure of Kenyon cells from pollen foragers in cell culture to 20HE** To determine whether 20HE has similar effects on Kenyon cells collected from older honeybees, the same experiment was repeated using cells harvested from pollen foragers. The cells were maintained *in vitro* for 24 hours and then exposed to 30 nM 20HE for 3 days (Figure 2.7 A-F). Consis-
Figure 2.5: **Effect of 20-hydroxyecdysone on gene transcript levels in Kenyon cells harvested from newly-emerged adult workers**

Kenyon cells collected from newly-emerged adult workers were maintained *in vitro* for 24 hours. Mean transcript abundances in cells treated with 30 nM 20HE were compared with those detected in untreated (control) cells harvested from the same animals. Data were normalised to the levels detected in controls. There were no significant differences in levels of *Amdop1* (A), *Amdop2* (B), or *Amdop3* (C) transcript between control cells and cells treated with 20HE. However, levels of *Amgpcr19* transcript (D) were significantly higher in 20HE-treated cells than in controls (p = 0.0411). Neither *rpn2* (E) nor *rps8* (F) transcript levels were affected by treatment with 20HE (p = 0.8549, p = 0.4211, respectively). Unpaired Student’s t-tests, mean ± SEM of 5 independent samples per group.
Expression of dopamine receptor genes

Figure 2.6: Effect of 20-hydroxyecdysone on gene transcript levels detected in Kenyon cells from newly-emerged adult workers

Gene transcripts of newly-emerged adult worker Kenyon cells maintained for a total of 4 days in vitro treated with 30 nM 20HE for 3 days. *Amdop1* and *Amgpcr19* transcript abundances were significantly higher in cells treated with 20HE than in controls (A, *p* = 0.0324; D, *p* = 0.0035). *Amdop2* transcript levels tended to be enhanced also in cells treated with this hormone, but the effect was not statistically significant (B, *p* = 0.0536). Treatment with 20HE had no effect on the levels of *Amdop3* transcript (C, *p* = 0.5185). The reference genes *rpn2* (E) and *rps8* (F) showed no significant response to 20HE (*p* = 0.794, *p* = 0.0742). Unpaired Student’s t-tests, mean ± SEM of 5 independent samples per group.
tent with effects of 20HE on cells from newly-emerged adult workers, transcript abundances for *Amdop1* (Figure 2.7 A, \( p = 0.0014 \)) and *Amgpcr19* (Figure 3.6 2.7 D, \( p = 0.0138 \)) were significantly higher in cells treated with 20HE than in untreated cells. However, in contrast to cells from newly-emerged adult workers, all of the dopamine receptor genes showed significant changes in transcript levels with *Amdop2* (Figure 2.7 B, \( p = 0.0213 \)) increasing significantly in abundance and *Amdop3* abundance showing a significant decrease (Figure 2.7 C, \( p = 0.0381 \)) relative to the controls. While *rps8* showed a strong downward trend, abundance levels of the reference genes, *rpn2* (Figure 2.7 E, \( p = 0.702 \)) and *rps8* (Figure 2.7 F, \( p = 0.0632 \)) were not significantly affected by 20HE. See appendix 1 for dopamine receptor gene transcript levels normalised to house keepers *rpn2* and *rps8* (Section 5.1).

### 2.3.2.3 Effect of JH and JH in combination with 20HE on dopamine receptor gene transcript levels

The effect of juvenile hormone (JH) on dopamine receptor gene transcripts in Kenyon cells *in vitro* was examined, as well as the possibility of interactions between the effects of JH and 20HE. The effect of 20HE on Kenyon cells harvested from newly-emerged adult workers were examined a second time in parallel with the JH experiments.

Transcript levels in untreated cells were compared with transcript levels detected in cells treated with one of the following: 30nM 20HE, 75 nM JH, or 30 nM 20HE together with 75 nM JH. Consistent with the experiments described in Figure 2.6, *Amdop1* transcript abundances were significantly higher in cells treated with 20HE than in control cells (Figure 2.8 A, ANOVA \( f_{(3,16)} = 11.34, p = 0.003 \), Tukey’s post hoc test). A similar increase was observed in cells treated with a combination of 20HE and JH (\( p = 0.0001 \)); however, JH alone did not induce a significant increase in transcript levels. There was also
Figure 2.7: Effect of 20-hydroxyecdysone on gene transcript levels detected in Kenyon cells from pollen foragers

Gene transcripts of pollen forager Kenyon cells maintained for a total of 4 days in vitro treated with 30 nM 20HE for 3 days post-treatment. Transcript abundances for Amdop1 (A), Amdop2 (B), Amdop3 (C) and Amgpcr19 (D) were significantly higher in cells treated with 20HE than in controls. rpn2 (E) transcript levels were not affected by 20HE (p = 0.702) but there was a trend for rps8 (F) to be expressed at lower levels in 20HE-treated cells than in controls (p = 0.0632). Transcript levels were normalised to the average transcript levels detected in control cells. Unpaired Student’s t-tests, mean ± SEM of 5 independent samples per group.
Expression of dopamine receptor genes

no significant difference between Am dop1 transcript levels in cells treated with 20HE alone, and in combination with JH (p = 0.4113).

The abundance of Am dop2 transcript showed JH (Figure 2.8 B, p = 0.0186) alone and JH+20HE (p = 0.0003) increased the relative abundance of Am dop2 transcript significantly relative to the control (ANOVA \( f (3,16) = 18.75, p = 0.000017 \), Tukey’s post hoc test). No significant change in the presence of 20HE (p = 0.6864). However, JH alone and JH+20HE increased the relative abundance of Am dop2 transcript significantly relative to the control (p = 0.0186, p = 0.0003). The presence of 20HE and JH together had a significantly greater effect on Am dop2 transcript levels than that of JH alone (p = 0.0103). Similarly, the abundance of Am dop3 transcript showed no significant change in response to 20HE (Figure 2.8 C, p = 0.7832, ANOVA \( f (3,16) = 11.16, p = 0.0003 \), Tukey’s post hoc test) but increased significantly in the presence of JH alone (p = 0.0062), and JH combined with 20HE (p = 0.0044). However, the effects of JH were not enhanced by the addition of 20HE (p = 0.7832).

Amgpcr19 showed a significant increase in relative abundance in the presence of 20HE (Figure 2.8 D, p = 0.0017, ANOVA \( f (3,16) = 24.96, p = 0.000003 \), Tukey’s post hoc test). JH significantly increased the abundance of this transcript to that seen in control cells (p = 0.000082). Levels of Amgpcr19 transcript in cells treated with a combination of JH+20HE were significantly higher than in control cells (p = 0.000053) and also had significantly higher abundance than in cells treated with either 20HE or JH alone (p = 0.0129, p = 0.017).

Transcript levels of the reference gene rpm2 was not significantly affected by 20HE or JH alone; however, JH in combination with 20HE increased rpm2 transcript abundance significantly relative to that detected in control cells (Figure 2.8 E, p = 0.037, ANOVA \( f (3,16) = 5.416, p = 0.0092 \), Tukey’s post hoc test). Transcript levels of the reference gene rps8 were not significantly affected by any of the hormone treatments (Figure 2.8 F, ANOVA \( f (3,16) = 1.509, p = 0.240 \)).
Expression of dopamine receptor genes

0.2503, Tukey’s post hoc test). See appendix 1 for dopamine receptor gene transcript levels normalised to house keepers $rpn2$ and $rps8$ (Section 5.1).
Figure 2.8: Effect of 20-hydroxyecdysone and juvenile hormone on gene transcript levels of newly-emerged adult worker Kenyon cells

Mean gene transcript levels detected in newly-emerged adult worker Kenyon cells that were treated with JH, 20HE or JH and 20HE in combination, and maintained in vitro for 4 days. Groups that share letters are not significantly different. Transcript levels are expressed relative to the average transcript levels of the control. ANOVA with Tukey’s post hoc, SEM, mean of 5 independent samples per group.
2.4 Discussion

The results of this Chapter provide evidence that Kenyon cells in vitro express the dopamine receptor genes $Amdop1$–$3$, and also the putative ecdysone/dopamine receptor gene, $Amgpcr19$. Levels of dopamine receptor gene expression were found to be dependent both on the time the cells spent in culture and on the age of the honeybee from which the Kenyon cells were sourced.

The increased expression of $Amdop2$ and $Amdop3$ receptor gene transcripts over time in culture may be linked with the growth of Kenyon cells in vitro. Dissociated neurons lose their axons during preparation for primary cell culture and once placed in culture undergo extensive neurite outgrowth (Kreissl and Bicker, 1992, Bicker and Kreissl, 1994, Kirchhof and Mercer, 1997). The large increase seen in ribosomal protein transcript $rps8$ and the ribophorin protein $rpn2$ transcript levels is also consistent with rapid growth of the cells in vitro. These two ribosomal protein genes are often used as house-keeping genes (McQuillan et al., 2012a, McQuillan et al., 2012b, Geddes et al., 2013). However, in this current study levels of expression of these genes in Kenyon cells in vitro were found to be unstable over time in culture. For this reason, and because $rps8$ also responded to JH exposure, the data were not normalised to an internal reference gene. Despite the absence of normalisation, effects of time in culture and treatment observed in this study were consistent between the independent experiments. The effect of 20HE on the transcript levels of the dopamine receptor genes in newly emerged Kenyon cells in 4 days in culture was not affected by normalisation to the reference genes $rps8$ and $rpn2$. This same result was also seen repeated in the 20HE and JH experiment, where once again normalisation to $rps8$ and $rpn2$ did not alter the relative change in gene transcript levels between control and 20HE treated cells. However, the presence of JH rendered $rpn2$ unsuitable for normalisation, and even though $rps8$ was not significantly affected by JH treatment, it did increase the vari-
ance seen within each treatment. Due to the consistency between independent experiments, it is felt that unnormalised results best represent the response of the Kenyon cells to treatment in culture. All of the subsequent results are interpreted in light of this decision.

Not all genes examined in the present study displayed increases in transcription levels over time in vitro. In contrast to Amdop2 and Amdop3, for example, transcript levels of Amdop1 did not change over time in culture. This mirrors what is seen at the level of the whole mushroom body of newly-emerged adult workers in vivo (Kurshan et al., 2003, Beggs et al., 2005, McQuillan et al., 2012b). Amdop1 expression in the mushroom bodies of the honeybee is reported to be relatively stable, at least during the first week of adult life (McQuillan et al., 2012b). The results presented in this Chapter indicate this is also true in Kenyon cells in vitro.

In newly-emerged Kenyon cells in culture Amdop3 transcript levels were slightly but significantly lower than mRNA levels detected for either Amdop1 or Amdop2. In pollen forager Kenyon cells, considerably higher Amdop3 transcript levels were detected than levels of Amdop1 or Amdop2 transcript. These results reflect patterns previously described in vivo (Beggs et al., 2005, McQuillan et al., 2012b). For example, Amdop3 transcript levels at the whole brain level are reported to more than double within the first few days of adult life (McQuillan et al., 2012b). However, despite the large increase in Amdop3 transcript levels observed in the present study in Kenyon cells collected from newly-emerged adult workers, the levels overall in Kenyon cells collected from young bees were never as high as those recorded in cells collected from pollen foragers. This is consistent with earlier reports showing that Amdop3 transcript levels in Kenyon cells of young workers in vivo are significantly lower than those found in pollen foragers. Even then, Amdop3 remains at lower levels compared to that of Amdop1 and Amdop2 (Beggs et al., 2005, McQuillan
et al., 2012b).

The direct cause of the up regulation of *Amdop3 in vivo* is not known, but the timing of this increase correlates with early peaks in 20HE and JH titres in the haemolymph of the honeybee (Hartfelder et al., 2002, Amdam et al., 2010). Evidence from the experiments described in this Chapter is also consistent with the possibility that JH plays a role in increasing *Amdop3* expression, as treatment of Kenyon cells from newly-emerged honeybees with JH resulted in an increase in the levels of *Amdop3* transcript. This JH-induced increase in *Amdop3* mRNA levels is supported by *in vivo* experiments reported elsewhere, in which injection of JH into the haemolymph of newly-emerged adult workers was found to increase *Amdop3* transcript levels in mushroom bodies examined 18 hours after treatment (McQuillan 2013). Taken together these findings suggest that changes in *Amdop3* expression *in vivo* may be driven by changes in JH titres and potentially contribute to shifts in the behaviour in adult worker honeybees.

Levels of *Amdop1* and *Amdop2* transcript in Kenyon cells *in vitro* were also found to be affected by treatment with the pheromone component HVA. Interestingly, the effects of HVA on Kenyon cells from newly-emerged adult workers were not the same as in Kenyon cells sourced from pollen foragers. In cells from newly-emerged honeybees, HVA increased levels of *Amdop1* transcript with some suggestion of an effect also on *Amdop2* expression. In Kenyon cells sourced from pollen foragers only *Amdop2* transcripts were significantly increased by treatment with HVA. Effects of HVA on aversive learning have also been found to be age dependent (Vergoz et al., 2007), as are effects of the full QMP mixture of compounds (Vergoz et al., 2007, Vergoz et al., 2009). If the *in vitro* effects of HVA seen in this study translate to different levels of functional dopamine receptor expression *in vivo* between treated and untreated honeybees, then the responses of young honeybee workers to dopamine
may also be affected by exposure to HVA. This possibility is examined in the next Chapter.

Dopamine receptor gene transcript levels in Kenyon cells in vitro were affected not only by JH and HVA, but also by treatments with 20HE, either alone or in combination with JH. This is consistent with evidence showing that changes in hormonal environment have a significant impact on the expression of these genes in vivo (Geddes et al., 2013, McQuillan et al., 2014). In the present investigation, treatment of Kenyon cells with 20HE increased the transcript levels of Amdop1 and Amgpcr19. This was true in Kenyon cells sourced from pollen foragers as well as in cells from newly-emerged adult workers. However, Kenyon cells from pollen foragers, unlike those from newly-emerged adult workers, also responded to 20HE with an increase in Amdop2 transcript levels and a reduction in levels of Amdop3 transcript. Thus, effects of this hormone, like the effects of HVA, appear to be age dependent and more complex in Kenyon cells from pollen foragers than in cells from young worker honeybees.

Effects of 20HE on levels of Amgpcr19 expression were enhanced by co-treatment with JH. As noted earlier, JH levels increase during the lifetime of the adult worker honeybee (Jassim et al., 2000, Amdam et al., 2010) and are significantly higher in pollen foragers than in honeybees performing in-hive tasks (Jassim et al., 2000). The age-related differences in the responsiveness of Kenyon cells to 20HE observed in the present study could potentially reflect differences in the exposure of cells to JH before they were harvested for experimentation. Effects of JH on levels of Amdop2 transcript were enhanced by treatment with 20HE even though 20HE alone did not alter Amdop2 transcript levels. Interestingly, higher levels of Amdop2 transcript have been observed in the whole brain of pollen foragers compared to that of newly-emerged honeybees (McQuillan et al., 2012b). This suggests that hormone titres of JH and
20HE together may play an important role in regulating the dopamine receptor gene transcript levels, and the effects of these hormones may be dopamine receptor gene specific.

On the basis of the results obtained in this study, three key findings are investigated further in the following Chapter:

1. *In vitro*, Kenyon cells sourced from newly-emerged adult worker honeybees have dopamine receptor gene transcripts that change significantly over time in culture. The following Chapter examines whether the same trends are apparent in mushroom body Kenyon cells *in situ*.

2. Results described in this Chapter also showed that treatment of Kenyon cells *in vitro* with HVA increases levels of *Amdop1* transcript in cells with some possible effect on levels of *Amdop2* transcript. In the following Chapter, young honeybees are fed with HVA to determine whether this treatment increases levels of *Amdop1* and/or *Amdop2* gene transcript in the mushroom bodies of young worker honeybees and if so, whether this alters the cAMP responses to exogenously applied dopamine.

3. Finally, based on the finding that levels of dopamine receptor gene transcript in mushroom body Kenyon cells can be affected by 20HE, the effects of acute exposure of mushroom body tissues to 20HE are examined in the following Chapter. Whether 20HE alters dopamine-induced changes in cAMP levels is investigated, and in addition, whether effects of 20HE on the mushroom bodies of young honeybees are similar or different to the effects of this hormone on mushroom body calyces sourced from pollen foragers.
Chapter 3

Effect of age, homovanillyl alcohol, and 20-hydroxyecdysone on the dopamine-induced cAMP response in mushroom body calyces
3.1 Introduction

The results presented in the previous Chapter identified several key questions that provide the focus for the work described in this Chapter.

In the previous Chapter there were significant differences in expression levels of the dopamine receptor gene transcripts between newly-emerged and pollen forager honeybee Kenyon cells in culture. Newly-emerged honeybee Kenyon cells also showed distinct changes in gene expression over time in culture. It is not known if changes in dopamine receptor gene transcripts are an artifact of cell culture or if these changes relate to changes seen in vivo. There is evidence that dopamine receptor gene transcripts change significantly over the first few days of life in newly-emerged honeybees (Kurshan et al., 2003, Beggs et al., 2005, McQuillan et al., 2012a, McQuillan et al., 2012b). If the change in dopamine receptor gene transcript levels in vivo is related to expression patterns seen in vitro, it would be expected that changes in transcript levels in the mushroom body calyces in the first few days post emergence would show similar patterns of expression to those seen in the previous Chapter.

Specifically, in vitro Kenyon cells sourced from newly-emerged adult workers were found to have levels of *Amdop*3 transcript that increased significantly over the time cells were held in culture but remained lower than the levels detected in Kenyon cells sourced from pollen foragers. This Chapter examines whether increases in levels of *Amdop*3 transcript can also be detected in mushroom body calyces taken from young worker honeybees and if so, whether up regulation of the ‘D2-like’ dopamine receptor, *Amdop*3, is reflected by a change in response to dopamine applied exogenously to the calyces. If this increase in *Amdop*3 transcript also correlates with an increase in functional AmDOP3 protein, it would be predicted that activation of this D2-like receptor would lead to a decrease in cAMP levels in the mushroom bodies calyces when treated with dopamine. However, while AmDOP3 has been shown to de-
crease cAMP levels (Beggs et al., 2005), it has also been shown that AmDOP3 can differentially couple to Gβγ and Gi/o, with Gβγ leading to an increase in cAMP in response to dopamine (Clark and Baro, 2007). Previous experiments have shown that mushroom body calyces of 2-day old honeybees respond to dopamine with an increase in cAMP levels (Beggs et al., 2007); however, the cAMP response to dopamine of mushroom body calyces from pollen foragers is not yet known, and their response may potentially be different.

The results of the previous Chapter also revealed that treatment of Kenyon cells in vitro with HVA increased levels of Amdop1 transcript with a similar but not significant effect on Amdop2. In this Chapter, young honeybees are fed with HVA from the time of adult emergence to determine whether changes in levels of dopamine receptor gene transcript can be detected in the mushroom bodies of young worker honeybees and if so, whether up regulation of the ‘D1-like’ dopamine receptors transcript leads to a detectable enhancement of the response to dopamine. The AmDOP1 receptor has been shown to be constitutively active and the activation of AmDOP1 receptors by dopamine has been shown to increase cAMP levels. AmDOP2 receptor does not display any constitutive activity (Mustard et al., 2003); however, activation of the receptor also leads to an increase in cAMP levels (Blenau et al., 1998, Mustard et al., 2003). The increase in cAMP generated from activation of AmDOP1 at equivalent dopamine concentrations appears to be significantly greater than from AmDOP2 activation. Therefore, if HVA produces similar effects on Amdop1 transcript in vivo as it does in vitro, it would be predicted that activation of this ‘D1-like’ receptor would be the major contributor leading to any potential increase in cAMP levels in mushroom body calyces when treated with dopamine.

The effects of 20HE on Kenyon cells may be age dependent. Treatment of cells from newly-emerged adult workers with 20HE increased levels of Amdop1
transcript and \textit{Amgpcr19} transcript levels. While these effects were also apparent in cells sourced from pollen foragers, Kenyon cells from pollen foragers responded to 20HE with an increase in levels of \textit{Amdop2} transcript and a down regulation of \textit{Amdop3} transcript levels. In this Chapter, effects of acute exposure of mushroom body calyces to 20HE are investigated to determine whether exposing Kenyon cells \textit{in situ} to 20HE alters their responses to exogenously applied dopamine. Dopamine-induced changes in cAMP levels are measured to examine whether effects of 20HE on the mushroom bodies of young honeybees are similar or different to effects of this hormone on mushroom body calyces sourced from pollen foragers. While it is not expected that transcript levels would change in the short exposure time to 20HE of these experiments, 20HE is predicted to affect the functioning of the putative ecdysone/dopamine receptor AMPCR19. For this reason, it is of interest to know whether dopamine-induced changes in cAMP levels are altered if calyces are treated with 20HE.

The goals of the work described in this Chapter are:

1. to investigate changes in dopamine receptor gene transcript levels in the mushroom bodies of the honeybee over the first 4 days after adult emergence,
2. to determine whether exposure to HVA via feeding alters dopamine receptor gene transcript levels,
3. to assess the effect of HVA on dopamine-induced changes in cAMP in the mushroom body calyces of young workers, and
4. to compare the effects of 20HE on dopamine-induced changes in cAMP levels in newly-emerged adult workers and in pollen foragers.
3.2 Materials and methods

3.2.1 Collection of honeybees for experimental manipulation

**Newly-emerged** Honeybees used in this Chapter were collected as in Chapter 2, Section 2.2.1. The brood frames were held in a humidified incubator at 34 °C and newly-emerged adult workers were collected within 2 hours of emergence.

**Pollen foragers** Returning pollen foragers were collected as described in Chapter 2, Section 2.2.1.

**Age-matched honeybees** In order to obtain bees of a known age, brood frames were sourced from multiple colonies. Colony choice was dependent on available brood stocks and brood frames were held in a humidified incubator at 34 °C. To maintain consistency and to avoid any undue variation caused by the time of day, newly-emerged adult workers were only collected between 9 am and 1 pm. If multiple sets of newly-emerged adult workers were required, brood frames were completely cleared of bees and the time period was reset. Honeybees that were used in experiments on age-matched bees were collected within a 1 hour window of emergence.

**Cage-raised honeybees** Collected honeybees were maintained in groups of 40-60 sister bees in acetate cages measuring 100x100x135 mm, as used previously by Geddes and McQuillan (Geddes et al., 2013, McQuillan et al., 2012) based on a design used by Pain (1966; Figure 3.1). Honeybees were fed *ad libitum* water and 30 % sucrose in distilled water (Vergoz et al., 2007, Geddes et al., 2013).
Figure 3.1: Cage for maintaining honeybees

Honeybee-rearing cage. A) Water feeder, used to supplement water access from B) Sucrose feeder, which was also used to deliver HVA treatment. C) Wax foundation, and D) pollen feeder troughs which were not used in these experiments.
3.2.2 Analysis of dopamine receptor gene transcript levels in the Kenyon cells of cage-raised honeybees

Adult honeybees were held in cages from the time of emergence and fed *ad libitum* with either 30 % sucrose or 30 % sucrose containing 100 μM HVA. Mushroom body calyces were dissected from newly-emerged, 2-day old, and 4-day old honeybees.

3.2.2.1 Extraction of total RNA from mushroom body calyces

Mushroom body calyces were dissected out and samples were placed in 500 μL Trizol (LifeTechnologies), homogenised, and centrifuged using a Hermle cryofuge. Samples were then treated as in Section 2.2.2.

**Assessment of transcript abundance via qPCR**  
Real-time quantitative PCR was used as described in Section 2.2.2 to investigate changes in expression of amine receptor genes, *Amdop1*, *Amdop2*, *Amdop3*, *Amgpcr19*, and the potential reference genes, *rpm2* and *rps8*, in mushroom body calyces.

3.2.3 Modulation of dopamine-induced changes in cAMP levels in mushroom body calyces

Dopamine-induced changes in intracellular cAMP levels in mushroom body calyces were assessed using the protocol described in (Beggs et al., 2007), which is outlined below.

Freshly dissected calyces were placed into dissection medium (Section 2.2.2) and kept on ice before adding 100 μM 3-isobutyl-1-methylxanthine (IBMX) either on its own or in conjunction with a treatment (detailed below) to give a final volume of 200 μL. Each sample of brain tissue was incubated with or without treatment for 20 min at 30 °C. The incubation medium was then carefully
removed. Each sample was measured in duplicate. The tissue cAMP concentration was determined by using a Biotrak cAMP immunoassay (Amersham Biosciences, Piscataway, NJ).

3.2.3.1 Effect of homovanillyl alcohol on dopamine-induced changes in intra-cellular cAMP levels

To examine the effect of the queen pheromone component HVA on dopamine-induced changes in cAMP, mushroom body calyces were dissected from 2-day old bees held in cages from the time of adult emergence and fed *ad libitum* with either 30 % sucrose or 30 % sucrose containing 100 μM HVA (Section 3.2.2). The calyces were assayed for their dopamine-induced cAMP response by acute treatment with dopamine at a final concentration of 10 μM. Each sample assayed for cAMP contained the complete set of calyces from two honeybees. Two measurements were taken from each sample, and six independent samples were measured for each treatment.

3.2.3.2 Effect of 20-hydroxyecdysone on dopamine-induced changes in intracellular cAMP levels

The effect of acute treatment with the hormone 20HE on dopamine-induced changes in cAMP was examined using freshly dissected mushroom body calyces. Mushroom body calyces were dissected either from newly-emerged adult workers or returning pollen foragers. Each sample was treated with 1 μL of one of the following, to a final concentration of 20HE (in ethanol) of: 1 nM, 10 nM, 100 nM, 1 μM, or 10 μM together with 10 μM dopamine or with vehicle alone (10⁻⁸ mL ethanol in culture medium). Each sample assayed for cAMP contained the complete set of calyces from two honeybees. Two measurements were taken from each sample and three independent samples were measured for each treatment. The entire experiment was repeated twice on tissues from
both newly-emerged adult workers and from pollen foragers.

3.2.4 Statisitical analysis

All statistical analyses were performed using PRISM® version 7.0a (GraphPad Software Inc, San Diego, CA). Differences in gene expression between honeybees raised with or without HVA, and the effect of HVA and 20HE on the dopamine-induced cAMP response were analysed by two-way ANOVA, followed by Tukey’s multiple comparison tests.
3.3 Results

3.3.1 Effect of HVA treatment on dopamine receptor transcript levels in the mushroom bodies of 2- and 4-day old honeybees

Honeybees were sampled at emergence, 2 days old, and 4 days old. Honeybees raised in cages for 2 or 4 days were fed \textit{ad libitum} with sucrose or sucrose containing 100 \( \mu \text{M} \) HVA to examine the effects on the expression of dopamine receptor genes (Figure 3.2). The relative levels of \textit{Amdop}1 transcript (A) did not change with age and were unaffected by HVA treatment (two-way ANOVA \( f_{(1,16)} = 1.369, p = 0.28, n = 5 \)). Levels of \textit{Amdop}2 transcript (B) in adult workers raised in the presence of HVA were not significantly lower in 4-day old compared to adult workers raised in the absence of HVA; however, transcript levels in honeybees raised in the presence of HVA were significantly lower than newly-emerged (two-way ANOVA \( f_{(1,16)} = 2.921, p = 0.047, n = 5 \)). The expression of \textit{Amdop}3 (C) shows an age dependent increase in transcript level between newly-emerged adult workers and both 2- and 4- day olds, but there is no significant difference between the treatment and control groups (two-way ANOVA \( f_{(1,16)} = 11.21, p = 0.0041, n = 5 \)). The level of expression of \textit{Amgpcr}19 (D) shows a similar trend in expression levels to \textit{Amdop}3, increasing significantly with age both in 2-day old HVA treated honeybees and 4-day old controls (two-way ANOVA \( f_{(1,16)} = 5.22, p = 0.005, n = 5 \)). The expression levels of \textit{rpn}2 transcript (E) over 4 days was unaffected by either age or the presence of HVA (two-way ANOVA \( f_{(1,16)} = 1.998, p = 0.134, n = 5 \)). The expression of \textit{rps}8 (F) over 4 days also showed no significant difference (two-way ANOVA \( f_{(1,16)} = 0.576, p = 0.683, n = 5 \)). The geometric mean was calculated for \textit{rpn}2 and \textit{rps}8 (G) and was not significantly different (2-way ANOVA \( f_{(1,16)} = 0.29, p = 0.881, n = 5 \)).
Figure 3.2: Effect of HVA treatment and age on the dopamine receptor gene transcripts in the mushroom body calyces of cage reared honeybees

Honeybees were maintained in cages from adult emergence and the effects of HVA on the levels of dopamine receptor gene expression were measured. The mean transcript level for Amdop1 (A), Amdop2 (B), Amdop3 (C), Amgpcr19 (D) and the two potential reference gene rpn2 (E) and rps8 (F) were measured. Mean ± SEM, n=5.
3.3.1.1 Effect of HVA on the dopamine-induced change in the cAMP levels in mushroom body calyces

Dopamine-induced changes in cAMP levels were measured in the mushroom bodies (Figure 3.3) of newly-emerged adult workers maintained in cages with or without HVA (Methods 3.2.3). Honeybees were assessed at 2 days of age when the calyces of both groups were treated with 10 μM dopamine and the cAMP levels measured. A two-way ANOVA indicated that the mean level of cAMP recorded in the mushroom body calyces of both HVA exposed and non-exposed were significantly increased with dopamine treatment \((f(1, 20) = 34.43, p = 0.00004)\). However prior exposure of honeybees to HVA did not affect the change in cAMP levels induced by dopamine \((f(1, 20) = 1.374, p = 0.25)\).

3.3.2 Effect of 20HE on the dopamine-induced cAMP response in mushroom body calyces

The effect of 20HE on dopamine-induced cAMP responses in mushroom body calyces from newly-emerged adult workers and returning pollen foragers was tested.

3.3.2.1 Effect of 20HE on dopamine-induced changes in cAMP levels in newly-emerged adult worker mushroom body calyces

Mushroom body calyces from newly-emerged adult workers were treated with increasing concentrations of 20HE and with or without 10 μM dopamine (Section 3.2.3). Two independent trials were carried out (Figure 3.4). The levels of cAMP in the mushroom body calyces of untreated newly-emerged adult workers were 146.6 ± 5.83 pmol/well. When calyces were exposed to 20HE alone, there was no significant change in the levels of cAMP recorded, indi-
Figure 3.3: Effect of HVA on dopamine-induced changes in cAMP in mushroom body calyces
Newly-emerged adult workers were raised in cages and assessed at 2 days to compare the effects of HVA on the dopamine-induced cAMP response in the mushroom bodies. Dopamine had a significant effect on the cAMP levels in the mushroom body calyces. Treatment with HVA did not affect the cAMP response to dopamine. N = 6.
cating that 20HE has no effect on the cAMP levels in mushroom body calyces from newly-emerged adult workers (Trial 1 $f_{(5, 29)} = 1.637, p = 0.18$; Trial 2 $f_{(5, 29)} = 1.269, p = 0.3$). When the calyces were treated with 10 μM dopamine there was considerably more variation in the cAMP levels (Trial 1 $f_{(5, 29)} = 2.724, p = 0.03$; Trial 2 $f_{(5, 29)} = 1.292, p = 0.33$). To increase the statistical power of this experiment, the two trials were combined by normalising to their respective untreated controls (Figure 3.4 C). In newly-emerged adult worker mushroom bodies dopamine did not significantly enhance cAMP levels ($p = 0.324$ Figure 3.4). In the presence of 10 nM 20HE; however, the change in cAMP levels in response to dopamine was significantly enhanced (two-way ANOVA $f_{(1,131)} = 3.433, p = 0.0461$) (Figure 3.4 C)). For 100 nM, 1μM, and 10 μM 20HE there was no significant variation in the level of cAMP in response to dopamine. The effect of 20HE on the dopamine-induced changes in cAMP levels for the 0 nM and 10 nM 20HE treated groups of both trials and the combined results are presented in Figure 3.5. This figure illustrates more clearly the absence of a significant change in cAMP levels of untreated newly-emerged adult workers when treated with dopamine, and the significant enhancement of the cAMP levels in the presence 10 nM 20HE when treated with dopamine.

3.3.2.2 Effect of 20HE on dopamine-induced changes in cAMP levels in returning pollen forager mushroom bodies

Mushroom body calyces from returning pollen foragers were treated with 10 μM dopamine and increasing concentrations of 20HE (Methods 3.2.3). Two independent trials were carried out (Figure 3.6 A, B, respectively). The levels of cAMP in the mushroom body calyces of untreated pollen foragers were 123.7 ± 4.24 pmol/well. In both trials the addition of 10 μM dopamine reduced the cAMP levels in the mushroom body calyces (Figure 3.6). In trial 1 there was
Figure 3.4: Effect of 20HE with or without 10 μM dopamine on the cAMP levels in the mushroom bodies of newly-emerged adult workers

Two independent trials using freshly plucked mushroom body calyces from newly-emerged adult workers treated with increasing concentrations of 20HE with or without 10 μM dopamine (A, B). Different letters indicate significant differences. Mean ± SEM, each trial n = 6. Trials 1 and 2 combined (C). Different letters indicate significant differences. Mean ± SEM, n = 12.
Figure 3.5: Selected trials showing the effect of 20HE with or without 10 μM dopamine on the cAMP levels in the mushroom bodies of newly-emerged adult workers
The 10 nM 20HE treated and untreated groups from the previous experiment are presented individually to illustrate the effect of 20HE on the dopamine-induced changes in cAMP levels in mushroom bodies of newly-emerged adult workers. Mean ± SEM, n = 12.
a significantly lower level of cAMP in the presence of 1 nM and 10 μM 20HE (ANOVA $f_{(4, 25)} = 28.37, p = 0.002, p = 0.0098$); however, this significant drop was only seen in the presence of 10 μM 20HE in trial 2 (ANOVA $f_{(4, 25)} = 10.24, p = 0.0013$) Figure 3.6). In the presence of 10 μM dopamine and 10 nM 20HE in both trials the level of cAMP was higher than with dopamine alone. The two trials were combined to increase the statistical power and each trial was normalised to the untreated control (Figure 3.6 C). Dopamine alone significantly reduced the cAMP levels in mushroom body calyces of returning pollen foragers (two-way ANOVA $f_{(1, 131)} = 5.564, p = 0.019$). Conversely, in the presence of 10 nM 20HE the cAMP response to dopamine was reversed and significantly enhanced the cAMP levels (two-way ANOVA $f_{(1, 131)} = 2.891, p = 0.0091$). The effect of 20HE on the dopamine-induced changes in cAMP levels for the 0 nM and 10 nM 20HE treated groups of both trials and the combined results are presented in Figure 3.7. This figure illustrates more clearly the significant decrease in the cAMP levels of untreated pollen foragers when treated with dopamine, and the significant enhancement of the cAMP levels in the presence 10 nM 20HE when treated with dopamine.
Effect of age, HVA, and 20HE on MB neurons

Figure 3.6: Effect of 20HE with or without 10 µM dopamine on the cAMP levels in the mushroom bodies of pollen foragers

Two independent trials using freshly plucked mushroom body calyces from pollen foragers treated with increasing concentrations of 20HE with or without 10 µM dopamine. Different letters indicate significant differences. Mean ± SEM, each trial n = 6. Trials 1 and 2 combined (C). Different letters indicate significant differences. Mean ± SEM, n = 12.
Figure 3.7: Selected trials showing the effect of 20HE with or without 10 μM dopamine on the cAMP levels in the mushroom bodies of pollen foragers

The 10 nM 20HE treated and untreated groups from the previous experiment are presented individually to illustrate the effect of 20HE on the dopamine-induced changes on the cAMP levels in mushroom bodies calyces of pollen foragers. Mean ± SEM, n = 12.
3.4 Discussion

This study revealed age-related changes in dopamine receptor gene transcript levels similar to those described previously in the whole brain of young honeybees (Kurshan et al., 2003, McQuillan et al., 2012). Both *Amdop*3 and *Amgper*19 showed a significant increase in transcript levels during the first two days of adult life. However, despite this increase, *Amdop*3 transcript levels remained significantly lower than those of either *Amdop*1 or *Amdop*2. The increase in *Amdop*3 and *Amgper*19 transcript levels correlates with early peaks of 20HE and JH in young honeybees described *in vivo* (Amdam et al., 2010).

In the previous Chapter increases in *Amdop*3 and *Amgper*19 transcripts were also seen in Kenyon cells over time in culture. Further, JH was shown to increase *Amdop*3 transcript levels *in vitro*. 20HE, as well as the combined treatment of JH+20HE, increased *Amgper*19 transcript levels. This suggests that the increases in *Amdop*3 and *Amgper*19 transcript levels seen in young honeybees may be triggered by changes in the titres of these two hormones.

Together, the results of this Chapter and of Chapter 2 suggest that Kenyon cells *in vitro* retain some of the properties displayed *in vivo*.

The results of the cAMP experiments suggest that dopamine signaling changes over the lifetime of the honeybee. Mushroom body calyces from newly-emerged adult workers, from 2- to 4-day olds, and from pollen foragers responded differently to exogenously applied dopamine. Dopamine (10 μM) had no significant effect on cAMP levels in the calyces of newly-emerged workers, whereas calyces taken from the mushroom bodies of 2- and 4-day old honeybees responded to 10 μM dopamine with an increase in cAMP levels. This result is consistent with previous work on 2-day old honeybees (Beggs et al., 2007). The lack of change in cAMP levels in the calyces of newly-emerged adult workers in response to dopamine is interesting as the levels of dopamine receptor gene transcripts, and specifically those of *Amdop*1 and *Amdop*2, were...
found to be similar between newly-emerged honeybees and 2-day olds. One possible explanation is that levels of expression of AmDOP1 receptor protein differ between newly-emerged adults and 2-day old honeybees. Kokay and Mercer (1997) have shown that there is a significant increase in the density of dopamine-receptor binding sites with age in the honeybee brain. The receptor sites were identified using radiolabelled SCH22390, a D1-like dopamine receptor ligand (Kokay and Mercer, 1996). Of the two D1-like dopamine receptors in honeybees, AmDOP1 is considered to be the major contributor to dopamine-induced increases in levels of cAMP (Blenau et al., 1998, Mustard et al., 2003, Beggs et al., 2011). Changes in cAMP levels in response to activation of AmDOP2 receptors in vitro are generally smaller in amplitude than the changes resulting from activation of AmDOP1 (Humphries et al., 2003, Mustard et al., 2003). In contrast to the mushroom bodies of 2-day olds, in the calyces of mushroom bodies of pollen foragers, dopamine treatment reduced the levels of cAMP. One likely explanation for this is the significant increase in expression of the D2-like dopamine receptor, AmDOP3, in the Kenyon cells of pollen foragers.

Dopamine titres in the honeybee brain also change significantly over the lifetime of the honeybee and are particularly high in the mushroom bodies of foragers (Taylor et al., 1992, Harris and Woodring, 1992, Schulz and Robinson, 1999, Sasaki and Nagao, 2001). Evidence suggests that changes in dopamine titres in the mushroom bodies are age-, rather than behaviour-related (Schulz and Robinson, 1999). For example, dopamine levels in the mushroom bodies of pollen foragers that revert to nursing behaviour remain high rather than dropping to levels normally found in bees performing nursing duties. As noted above, levels of Amdop3 transcript in particular rise significantly in the calyces of pollen foragers, both in vivo (Beggs et al., 2005, McQuillan et al., 2012) and in vitro (present investigation). It would be interesting to assess transcript
levels in reverted nurses and to investigate the effects of behavioural reversion on responses to dopamine in the calyces of the mushroom bodies.

Haemolymph titres of ecdysteroid in pollen foragers are reported to be low; however, recent evidence shows that 20HE may be produced in the brain (Yamazaki et al., 2011), and many ecdysteroid related genes are preferentially as well as differentially expressed in the mushroom bodies (Takeuchi et al., 2001, Paul et al., 2005, Paul et al., 2006, Takeuchi et al., 2007). For example ecdysteroid regulated genes such as Broad-Complex (BR-C) and the putative transcription factor Mushroom body large-type Kenyon cell-specific protein-1 (Mblk – 1) are preferentially expressed in non-compact Kenyon cells, while other ecdysteroid regulated genes such as AmE74 are preferentially expressed in inner-compact cells of the mushroom bodies (Takeuchi et al., 2001, Paul et al., 2005, Paul et al., 2006). It has been suggested that the different expression patterns as well as age-related changes in the levels of expression of genes in the mushroom bodies may play a role in behavioural changes observed in the honeybee (Yamazaki et al., 2006).

Interestingly, while levels of Amgper19 transcripts are generally higher in inner-compact cells than non-compact cells throughout the honeybee life, the levels of Amgper19 transcript in inner-compact cells remain stable between age groups. However, Amgper19 is preferentially expressed in non-compact Kenyon cells in the mushroom body calyces of pollen foragers than in newly-emerged adult workers (McQuillan et al., 2012). In the present investigation, acute treatment with 20HE had no effect on cAMP levels in the calyces of newly-emerged honeybees, but at a concentration of 10 μM, this hormone reduced cAMP levels in the calyces of pollen foragers. There is currently no published data on 20HE having a direct effect on dopamine receptors of the honeybee, but increases in cAMP levels resulting from activation of the Drosophila orthologue of AmGPCR19, DmDopEcR, have been shown to be
blocked by 20HE (Srivastava et al., 2005). It is possible that interaction of 20HE with AmGPCR19 might contribute to the reduced levels of cAMP observed in this study in the calyces of pollen foragers treated with the highest concentrations of 20HE.

The cAMP response to dopamine in 20HE treated mushroom body calyces varied depending on the concentration of 20HE applied. This variation could be due to the innate variability of the endogenous hormone levels already present in the honeybee (Hartfelder et al., 2002, Amdam et al., 2010). Even with strict control of the sampling time of the honeybees, with honeybees being collected within 2 hours of each other there is still potential for considerable variation in the endogenous titres of JH and 20HE. Given that very small changes in hormone titres could have significant effects on the cAMP response to dopamine, the limited consistency between samples is not necessarily unexpected. Nonetheless, both in newly-emerged honeybees and in pollen foragers, treatment of the mushroom body calyces with 10 nM 20HE enhanced dopamine-induced increases in cAMP levels. Interestingly, of the different concentrations of hormone applied, 10 nM 20HE is the closest to physiological levels (~30 nM) observed in the young adult honeybee (Hartfelder et al., 2002, Amdam et al., 2010). The effect of 20HE on cAMP levels in pollen forager mushroom body calyces is particularly interesting. If 20HE is released as a neurohormone as suggested by Yamazaki et al. (2006), the results of the present investigation suggest the hormone is likely to influence dopamine signaling in the brain of pollen foragers. While it is still unclear how 20HE is interacting with dopamine signaling in the honeybee, the results from this Chapter suggest that the AmGPCR19 receptor might be involved.

Results with the pheromone component HVA were less clear. HVA had no significant influence on dopamine receptor gene transcript levels in mushroom body Kenyon cells. From the results of the previous Chapter, as well as from
previous work (Beggs et al., 2005), two different effects of HVA treatment might have been predicted. In the previous chapter direct application of HVA on newly-emerged Kenyon cells in culture induced an increase in *Amdop1* transcript levels, but a similar change was not seen *in vivo* in honeybees fed with HVA. Conversely, treatment with 100 μM HVA may have been predicted to reduce the transcript levels of *Amdop1*, as QMP has been shown to have this effect (Beggs et al., 2005). In previous studies feeding HVA has been shown to reduce dopamine titres in the brain of 2-day old honeybees (Beggs et al., 2007), suggesting that HVA treatments of this kind can have an effect on the honeybee brain. However, whether the effects of HVA are direct or indirect remains unclear. The feeding regime used in the present study was similar to the regime used by Vergoz et al., (2007) who found that feeding HVA-laced sucrose was sufficient to affect aversive learning behaviour in 4-day old honeybees, but different to that of Beggs et al., (2007) who presented HVA to bees in pollen cakes and recorded the reduced dopamine titres.

It is possible that effects of HVA are time- and/or dose-dependent. In the present investigation only a single concentration of HVA (100 μM) was tested. Additional work is thus required to resolve whether HVA has a direct effect on dopamine signaling in the mushroom bodies of the honeybee, or whether its effects *in vivo* are indirect. It would be useful to test HVA at a range of concentrations and to examine the effects of injecting HVA directly into the haemolymph, as previously described in crickets (Rillich and Stevenson, 2014, Rillich and Stevenson, 2015). In the cricket, HVA has been found to reduce the length of suppressed aggressiveness after a loss to another cricket (Rillich and Stevenson, 2014, Rillich and Stevenson, 2015). Injecting young honeybees with HVA might be predicted to increase activation of AmDOP3, as well as reduce dopamine titres in the brain (Beggs et al., 2005).

Effects of HVA in honeybees, like those of QMP, are age-dependent (Phamdelegue
et al., 1991, Phamdelegue et al., 1993, Moritz et al., 2001, Vergoz et al., 2009). For example, some young honeybees are strongly attracted to QMP, whereas others respond neutrally to this pheromone (Vergoz et al., 2009). Young honeybees showing strong attraction to QMP have been found to have significantly higher transcript levels of AmDOP3 in their antennae than bees of the same age that are not attracted to QMP (Vergoz et al., 2009). As HVA has been shown to activate the honeybee AmDOP3 receptor (Beggs and Mercer, 2009), it is possible that the behavioural effects seen in HVA-treated young honeybees are mediated via receptors located in the antennae rather than centrally, for example, at the level of the mushroom body.

Currently, there are no specific dopamine receptor antibodies available that could be used to analyse the distribution of dopamine receptor proteins in the mushroom bodies of the honeybee. Hence, it is unclear whether all three dopamine receptors are located within the calyces of the mushroom bodies, or if they display differential expression patterns within the lobes. It is also unclear whether their distribution pattern in mushroom bodies changes throughout the lifetime of the honeybee. The correlation between transcript levels and protein levels can vary greatly, with significant differences between the level of the transcript detected and the level of protein being expressed (Abreu et al., 2009, Maier et al., 2009, Vogel and Marcotte, 2012, Hu et al., 2016). Thus, many questions remain to be answered. It is unclear, for example, to what extent increases in AmDOP3 or Amgpcr19 transcript, such as those observed in this study, are accompanied by increases in their respective proteins over the lifetime of the honeybee, and whether the density of receptors that show relatively uniform transcript levels, such as AmDOP1, change significantly over the lifetime of the honeybee. Being able to answer such questions and assess where in the calyces and lobes the dopamine receptors are expressed will give further insight into the role of dopamine in the mushroom body of the hon-
eybee. What is known in *Drosophila* is that subpopulations of dopaminergic neurons, for example those arising from discrete clusters in the anterior medial and posterior lateral regions of the protocerebrum, send projections to distinct regions within the lobes of the mushroom bodies, and are responsible for distinct aspects of learning and memory (Mao and Davis, 2009, Aso et al., 2010, Aso et al., 2012, Tomchik, 2013, Hige et al., 2015). Whether this is also true in the mushroom bodies of the honeybee has yet to be determined.

In summary, the experiments described in this Chapter suggest that responses of mushroom body neurons to dopamine change during the lifetime of the honeybee, and that dopamine signaling may be influenced by the hormonal state of the individual honeybee. Novel to this Chapter is the evidence that 20HE may play an important role in modulating dopamine receptor activity in the honeybee.
Chapter 4

Dopamine-induced calcium signaling of mushroom body Kenyon cells in primary cell culture
4.1 Introduction

The results from Chapter 2 show that honeybee Kenyon cells in vitro express all three of the honeybee dopamine receptor genes, *Amdop1*, *Amdop2*, and *Amdop3*, as well as the putative ecdysone/dopamine receptor gene, *Amgpcr19*. This Chapter investigates the potential for Kenyon cells in primary culture to be used as a tool to examine the functional properties of honeybee dopamine receptors and in particular, the invertebrate-specific receptor AmDOP2. The *Amdop2* gene has been cloned and the receptor protein characterised (Kurshan et al., 2003, Humphries et al., 2003, Mustard et al., 2003, Beggs et al., 2011). *Amdop2* has been shown to be highly expressed in the mushroom bodies of the brain and differentially expressed across major subpopulations of mushroom body neurons (Humphries et al., 2003, Kurshan et al., 2003, McQuillan et al., 2012). Using *in situ* hybridisation techniques, Kurshan et. al., (2003) showed that early in metamorphic adult development the *Amdop2* receptor gene is highly expressed also in glial cells, but around pupal stage four expression of this gene in glial cell populations is switched off.

In Kenyon cells of the mushroom bodies, expression of *Amdop2* continues throughout development (Kurshan et al., 2003). Laser capture microdissection techniques have been used to confirm that levels of *Amdop2* transcript differ significantly in different subpopulations of Kenyon cells, and have revealed that this gene is particularly highly expressed in the major subpopulation known as inner compact cells (ICC, McQuillan et al., 2012). Like its orthologue in *Drosophila* (DAMB, Feng et al., 1996, Han et al., 1996), which is also highly expressed in the mushroom bodies, AmDOP2 receptor activation increases intracellular cAMP levels and also levels of intracellular calcium (Beggs et al., 2011). While the role of AmDOP2 in honeybee learning and memory is unknown, the *Drosophila* orthologue, DAMB, has been linked with learning and memory in both larval flies (Selcho et al., 2009) and adults (Musso et al., 2015, 2015).
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Berry and Davis, 2014). In adult flies this receptor has been implicated in forgetting of both aversive and appetitive memories (Berry et al., 2012, Berry and Davis, 2014) and in a recent study the DAMB receptor has also been implicated in appetitive long term memory (Musso et al., 2015). The AmDOP2 receptor is invertebrate-specific and is more closely related to the invertebrate octopamine receptors than to the vertebrate-like dopamine receptors such as AmDOP1 (Humphries et al., 2003, Mustard et al., 2003, Beggs et al., 2011). The AmDOP2 receptor exhibits a unique and interesting pharmacological profile, sharing with the honeybee octopamine receptor, AmOA1, a sensitivity to the receptor antagonist, epinastine (Beggs et al., 2011).

The goals of the work described in this Chapter are:

1. to determine whether honeybee mushroom body Kenyon cells in primary culture display a calcium signal in response to exogenous application of dopamine,

2. to determine whether the calcium response is due to an influx of external calcium or intracellular release,

3. to determine, with the use of pharmacological techniques, if AmDOP2 is the most likely candidate for this dopamine-induced calcium response, and

4. to determine if responses to acetylcholine are modulated by the co-applications of dopamine.
4.2 Materials and methods

4.2.1 Dopamine-induced calcium responses in mushroom body Kenyon cells

4.2.1.1 Cell culture for calcium imaging

Mushroom body Kenyon cells from newly-emerged adult workers were prepared as described in Chapter 2 Section 2.2.2. Dissociated cells were transferred in 50 µL aliquots to cover slips that had been coated with poly-L-lysine (0.02 %, Sigma-Aldrich, Deisenhofen, Germany) and placed individually into the wells of a 24-well cell culture plate (Costar 3526, Corning Incorporated). The plate was then placed in a humidified incubator at 26 °C for 1 hour to allow the cells time to adhere to the cover slips. After 1 hour, each well was gently filled with 2 mL of cell culture medium supplemented with 1 % penicillin-streptomycin mix (10000 U penicillin, 10 mg/ml streptomycin; Sigma-Aldrich, Deisenhofen, Germany) and 0.1 % gentamicin (50 mg/ml; Sigma-Aldrich, Deisenhofen, Germany) and incubated for 24 hours in a humidified incubator at 26 °C.

Calcium imaging was carried out at the University of Saarland, Saarbrücken, Germany and at the University of Otago, Dunedin, New Zealand. Mushroom body Kenyon cells were incubated at room temperature in honeybee ringer (130 mM NaCl, 6 mM KCl, 4 mM MgCl, 5 mM CaCl, 160 mM sucrose, 25 mM glucose, 10 mM HEPES, pH 6.7, 500 mOsm) with 2 µM Fluo-4 (Life Technologies) for 20 minutes prior to loading into the microscope.

4.2.1.2 Calcium imaging at the University of Saarland

To measure the calcium responses in mushroom body Kenyon cells a cover slip containing dissociated Kenyon cells (as described above) was mounted into a custom-made flow chamber with a volume of 700 µL. A 2-channel pump
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(Ismatec, Germany) provided a constant flow-rate of 6 mL/min. Before recordings began, the chamber was perfused with honeybee ringer for 3-4 min from a main reservoir. Acute drug treatments were applied for 3 seconds by mechanical switching to a supplementary reservoir containing ringer and the treatment. Calcium imaging was performed using an Axiovert 200M microscope (Zeiss, Göttingen, Germany) with a Zeiss 20X Plan-Neofluar objective (NA 0.5) and a cooled CCD camera (Cool Snap HQ2; Photometrics, Tucson, USA). Fluorescence images were collected at 1 frame per 1.6 s for Fluo 4 AM at room temperature using SlidebookTM software.

4.2.1.3 Calcium imaging at the University of Otago

A cover slip was mounted into a custom-made flow chamber with a volume of 400 µL. Gravity-fed 50 mL reservoirs were used for perfusion. Before recordings began, the chamber was perfused with honeybee ringer for 3-4 min from the main reservoir. Acute drug treatments were applied for 3 seconds by mechanical switching to a supplementary reservoir containing ringer and the treatment. Calcium imaging was performed using a Zeiss LSM 510 confocal, Axiovert 200M, laser-scanning inverted microscope with motorised stage (Jena, Germany). Fluorescence images were collected at 1 frame per 1.97 s for Fluo 4 AM at room temperature using a 458, 488, 477 and 514 nm argon laser, green excitation maximum 538-562 nm bandpass/longpass emission filter 590nm, with Zeiss LSM 510 software, version 3.2.

4.2.1.4 Calcium imaging data analysis

Data were processed using SlidebookTM in Germany, or Fiji (Fiji Is Just ImageJ) in New Zealand and Microsoft Excel. Individual fluorescence traces were normalised to pre-treatment levels to establish a basal measurement (Eq. 1.). Data were further normalised to a maximum fluorescence of 1 (Eq. 2.) where
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$V_f$ is the fluorescence value, $t_n$ is a given time point, $nV_f$ is the normalised fluorescence value, and $t_M$ is the time point with the maximum fluorescence.

Equation 1.

$$V_f = \frac{t_n}{\left(\sum_{n=1}^{10}(t)\right)}$$

Equation 2.

$$nV_f = \frac{V_f}{t_M}$$

To calculate the time to peak, time points from the first point above 20 % basal fluorescence to maximum fluorescence were summed and converted to seconds using 1.6 s per frame (Germany) or 1.97 s per frame (New Zealand). The 20 % threshold frequently fell between two time points. To better approximate the time to peak from 20 %, the proportion of time between two time points that were spanning the threshold was calculated geometrically, assuming a linear relationship, using Equation 3 where $T_{f20}$ is the amount of time spent above the 20 % threshold between the two time points, $t_{a20}$ is the time point after 20 %, $t_{b20}$ is the time point before the 20 % threshold, and $s$ is the time conversion co-efficient (1.6 or 1.97).

Equation 3.

$$T_{f20} = s \left(1 - \frac{t_{a20} - t_{b20}}{0.2 - t_{b20}}\right)$$

To calculate the recovery time, time points from the maximum fluorescence to the first point below 50 % peak fluorescence were summed and converted to seconds using 1.6 s per frame (Germany) or 1.97 s per frame (New Zealand). The 50 % threshold frequently fell between two time points. To better approximate the time from peak to 50 % recovery, the proportion of time between two time points that were spanning the threshold was calculated geometrically.
assuming a linear relationship using Equation 4. $T_{f50}$ is the amount of time spent above the 50 % threshold between the two time points, $t_{a50}$ is the time point after 50 % recovery, $t_{b50}$ is the time point before the 50 % threshold, and $s$ is the time conversion co-efficient (1.6 or 1.97).

Equation 4.

$$T_{f50} = s \left(1 - \left(\frac{t_{b50} - t_{a50}}{0.5 - t_{a50}}\right)\right)$$
4.3 Results

4.3.1 Acetylcholine-induced calcium response in Kenyon cells

Previous studies have shown that Kenyon cells in primary cell culture exposed to acetylcholine respond with a calcium influx (Bicker and Kreissl 1994). To confirm this result and to examine more closely the temporal kinetics of the calcium response, Kenyon cells were exposed to 10 µM acetylcholine and their responses recorded. The Kenyon cells of newly-emerged adult workers were maintained in vitro for 24 hours. Recordings were taken from individual cells in continuous perfusion. The perfusion could be alternated between honeybee ringer alone and honeybee ringer containing 10 µM acetylcholine. Cells were exposed to a 3 second perfusion of acetylcholine. The averaged responses of all cells (n = 19) are shown in Figure 4.1 (A). Representative examples are shown in (B-H). Cells displayed a consistent response to acetylcholine with similar rates of increase and recovery in fluorescence. Calcium responses were characterised by a rapid rise in fluorescence with a time to peak ($T_{P20}$) of 5.797 s ± 0.585 s (SEM) (Table 4.1). Time to peak was defined as the time from 20 % above baseline fluorescence to maximum fluorescence (Eq. 3.). Although there was some variation in recovery time the general trend remained constant with a time to 50 % recovery ($T_{R50}$) (Eq.4) of 10.55 s ± 1.94 s (SEM) (Table 4.1). The recovery phase of the calcium response was generally smooth with no oscillation in fluorescence levels. In general calcium levels returned to basal or near basal levels with an average of 8.03 % ± 2.83 % (SEM) above basal fluorescence by the end of recording at 150 seconds post treatment.
Figure 4.1: Acetylcholine-induced calcium signal in mushroom body Kenyon cells
Cells were exposed to a 3 s perfusion of 10 µM acetylcholine. The averaged responses of all cells (n = 19) are shown in (A). Representative examples are presented in (B-H). Relative fluorescence values were calculated as $\Delta F = F/F_0$ with $\Delta F$ subsequently normalised to the initial peak in fluorescence. Black bar in (A) indicates application of treatment.
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<table>
<thead>
<tr>
<th></th>
<th>Dopamine</th>
<th>Octopamine</th>
<th>Acetylcholine</th>
<th>Caffeine</th>
<th>TEA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time to peak</strong>&lt;br&gt;(s ± SEM)</td>
<td>2.917 ± 0.351</td>
<td>3.938 ± 0.924</td>
<td>5.797 ± 0.585</td>
<td>7.277 ± 0.824</td>
<td>2.073 ± 0.21</td>
</tr>
<tr>
<td><strong>Time to recovery</strong>&lt;br&gt;(s ± SEM)</td>
<td>2.851 ± 0.2764</td>
<td>3.888 ± 1.435</td>
<td>10.55 ± 1.936</td>
<td>19.07 ± 1.863</td>
<td>5.953 ± 0.722</td>
</tr>
</tbody>
</table>

Table 4.1: **Time to peak fluorescence and time to recovery**<br>Time to peak and recovery times of compounds inducing a calcium response. Average time in seconds to peak fluorescence from 20% above basal levels, and time in seconds to return to below 50% fluorescence of the peak.

### 4.3.2 Dopamine-induced calcium responses in Kenyon cells

With the intense interest in the role of dopamine in Kenyon cells and previous data showing that AmDOP2 responds to dopamine with a calcium response (Beggs, Tyndall et al. 2011), Kenyon cells were exposed to dopamine. Recordings were taken from individual cells exposed to a 3 second perfusion of dopamine. Cells did not show any response to a 3 second perfusion of 10 µM dopamine (data not shown); however, Kenyon cells gave robust responses to 100 µM dopamine. The averaged responses of all cells (n = 33) are shown in Figure 4.2 (A). Representative examples are shown in (B-H). Dopamine-induced calcium responses were characterised by a rapid initial peak, with a time to peak from 20 % above basal fluorescence ($T_{P20}$) of 2.917 s ± 0.351 s (SEM) and an equally rapid time to 50 % recovery ($T_{R50}$) of 2.851 s ± 0.276 s (SEM). The average level of calcium fluorescence at the end of the recording at 120 s post treatment was 33.71 % ± 1.4 % (SEM) above the initial baseline level. This indicates that, on average, the cells maintain elevated intracellular calcium levels long after the initial calcium spike induced by dopamine. The summation in (A) obscures a range of calcium response Types (B-H). Type A responses were characterised by a rapid return to basal or near basal levels, with little or no further activity (see B). The proportion of traces that showed this trace type was 5/33. Type B responses (e.g. C, D) were characterised by
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a rapid initial peak followed by a series of rhythmical spikes. The proportion of traces that showed this trace type was 5/33. Type C responses (e.g. E, F) were characterised by the initial peak and recovery followed by a series of small fast peaks with slower, smaller rises and recoveries. The traces were more common than either type A or type B with 9/33 being recorded. Type D responses (e.g. G, H) were characterised by a rapid return to near basal levels after the initial peak followed by a slower increase to a secondary maximum which were occasionally higher than the initial peaks (see G) and concluded with a second slower recovery phase. This trace type was the most common with 14/33 being recorded.

4.3.3 Kenyon cells that respond to dopamine also respond to acetylcholine

The integration of separate inputs is central to learning, therefore whether cells that respond to dopamine are also responsive to acetylcholine was investigated. Individual dopamine-responsive cells were washed with honeybee ringer for a minimum of 180 s then exposed to 10 µM acetylcholine. The averaged responses of all cells (n = 19) are shown in Figure 4.3 (A). Representative traces are presented in (B-H). Dopamine responses are generally smaller in amplitude than responses to acetylcholine, with a relative value of 0.427 ± 0.075 (SEM) compared to that of acetylcholine. As expected, the dopamine responses were similar to those seen in Figure 4.2. The acetylcholine responses on the other hand were not identical to those obtained from cells that were exposed to acetylcholine alone (Figure 4.1). The calcium response recovery tended to be delayed and did not always return to the baseline. There were often additional spikes observed in the recovery phase of the acetylcholine response (Figure 4.3 B,C,F), and also extended plateaus (e.g. D, E) of elevated fluorescence. Nine of the 19 cells that also responded to acetylcholine had an
Figure 4.2: Dopamine-induced calcium signal in Kenyon cells
Cells were exposed to a 3 s perfusion of 100 µM dopamine. The averaged responses of all cells (n = 33) are shown in (A). Representative examples are presented in (B-H). Relative fluorescence values were calculated as $\Delta F = F/F_0$ with $\Delta F$ subsequently normalised to the initial peak in fluorescence. Black bar in (A) indicates application of treatment.
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initial fluorescence above 20 % of baseline due to on-going effects of dopamine exposure. The remaining 10 cells responses had a time to peak fluorescence from 20 % of baseline (T_{P20}) of 4.586 s ± 0.5452 s (SEM). The time to 50 % recovery (T_{R50}) of all calcium responses was 15.76 s ± 2.95 s (SEM). After a minimum 200 s wash with honeybee ringer after the final treatment the average fluorescence had only recovered to 34 % ± 5.1 % (SEM) above baseline. This was not significantly different to the end point fluorescence seen in dopamine alone.

4.3.4 Co-application of acetylcholine and dopamine to Kenyon cells in primary cell culture

As both acetylcholine and dopamine induced a calcium response in Kenyon cells in vitro, the effects of co-application were also assessed. Co-application produced large and robust calcium oscillations (Figure 4.4). No Type A responses were observed, with the majority of the responses being similar in form to Type B responses seen in the application of dopamine alone. Interestingly, amplitude of the calcium response with the co-application of dopamine and acetylcholine were similar to that of acetylcholine alone (data not shown).

4.3.5 Responses to dopamine are abolished when extracellular calcium is replaced with cobalt

To determine the effect of blocking calcium channels, Kenyon cells were perfused with a calcium-free honeybee ringer containing cobalt chloride. Kenyon cells were perfused with dopamine (100 µM) for 3 seconds. Kenyon cells were then washed with honeybee ringer for at least 30 seconds before a second 3 second perfusion of dopamine. The averaged responses of all cells (n = 12) are shown in Figure 4.5 (A). Representative traces are presented in (B-H). No
Figure 4.3: Mushroom body Kenyon cells that respond to dopamine also respond to acetylcholine

Kenyon cells that responded to a 3 s perfusion of 100 µM dopamine also responded to 3 s perfusion of 10 µM acetylcholine. The averaged responses of all cells (n = 19) are shown in (A). Representative examples are presented in (B-H). Relative fluorescence values were calculated as \( \Delta F = F/F_0 \) with \( \Delta F \) subsequently normalised to the maximal peak of the acetylcholine response. Black bar in (A) indicates application of treatment.
Figure 4.4: Co-application of acetylcholine and dopamine to Kenyon cells
Kenyon cells were exposed to a 3 s perfusion of 10 μM acetylcholine and 100 μM dopamine simultaneously. The averaged calcium responses are summed in (A) (n = 9). Representative examples are presented in (B-H). Relative fluorescence values were calculated as \( \Delta F = F/F_0 \) with \( \Delta F \) subsequently normalised to the initial peak in fluorescence. Black bar in (A) indicates application of treatment.
responses to dopamine were observed in the absence of extra-cellular calcium and the presence of cobalt chloride. Responses to dopamine returned when cells were resupplied with regular honeybee ringer. Type A responses were observed in 1/12 responses, Type B in 2/12, Type C in 3/12 and Type D was the most common with 6/12 traces being recorded. The dopamine responses had a $T_{P20}$ of 5.455 s ± 0.158 s (SEM). In 3/12 cells the fluorescence remained > 50 % baseline, the remaining 9/12 cells had a $T_{R50}$ of 4.369 s ± 0.975 s (SEM).

4.3.6 Calcium responses to dopamine in Kenyon cells are also inhibited by cadmium

To confirm that calcium channels are likely to be involved in the dopamine-induced calcium responses, Kenyon cells were perfused with 50 µM of cadmium. Cells were then exposed to a 3 second perfusion of 100 µM dopamine and responses recorded for at least 60 seconds. Cells were washed with regular honeybee ringer for a further 30 s, followed by a 3 second perfusion of dopamine. The averaged responses of all cells (n = 12) are shown in Figure 4.6 (A). Representative examples are presented in (B-H). The amplitude of the dopamine-induced calcium responses were markedly reduced in the presence of cadmium, with no fast peaks characteristic of the regular dopamine response with the exception of one trace (G). The dopamine-induced calcium responses recovered after cells were perfused with cadmium-free honeybee ringer. The dopamine responses had a $T_{P20}$ of 3.084 s ± 0.54 s (SEM). Only 5/12 cells showed 50 % recovery after a minimum of 100 s, and these showed great variability with a $T_{R50}$ of 7.203 s ± 4.20 s (SEM). The responses were split between Type B with 8/12 and Type D with 4/12.
Figure 4.5: Dopamine-induced calcium responses are abolished by replacement of extra cellular calcium with cobalt. Kenyon cells were exposed to a 3 s perfusion of 100 µM dopamine in honeybee ringer with calcium replaced by cobalt. Cells were washed in regular honeybee ringer and exposed to a second 3 s perfusion of dopamine. The averaged responses of all cells (n = 12) are shown in (A). Representative examples are presented in (B-H). Relative fluorescence values were calculated as ΔF = F/F₀ with ΔF subsequently normalised to the maximal peak of the second dopamine response. Black bar in (A) indicates application of treatment.
Figure 4.6: Dopamine-induced calcium responses in mushroom body Kenyon cells are disrupted by cadmium
Kenyon cells were continuously perfused with honeybee ringer containing 50 µM cadmium. During perfusion, cells were treated with a 3 s exposure of 100 µM dopamine. Cells were washed in regular honeybee ringer, and exposed to a second 3 s perfusion of dopamine. The averaged calcium responses of all cells examined (n= 12) are shown in (A). Representative examples are presented in (B-H). Relative fluorescence values were calculated as $\Delta F = F/F_0$ with $\Delta F$ subsequently normalised to the maximal peak of the second dopamine response. Black bar in (A) indicates application of treatment.
4.3.7 Kenyon cells exposed to TEA exhibit spontaneous calcium spikes

To induce spontaneous calcium spikes to compare with spikes induced by dopamine, the $K^+$ channel blocker tetraethylammonium (TEA) was applied to cells. Cells were continuously perfused with 22 mM TEA in honeybee ringer, starting 30 s before recordings began. In the presence of TEA multiple spontaneous peaks in fluorescence were recorded. Representative traces are presented in Figure 4.7 (A-H). TEA induced spontaneous, random, and often continuous spiking. TEA-induced spikes had a $T_{P20}$ of 2.07 s $\pm$ 0.21 s (SEM) and a $T_{R50}$ of 5.95 s $\pm$ 0.72 s (SEM). In the presence of TEA the average fluorescence of the cells overall increased to 18.7 % $\pm$ 3.2 % (SEM) to the baseline.

4.3.8 Dopamine-responsive Kenyon cells do not respond to octopamine

To investigate if the dopamine-induced calcium responses were indeed dopamine-specific, cells were also exposed to octopamine. During continuous perfusion with honeybee ringer, 100 $\mu$M octopamine was perfused for 3 s then perfused with honeybee ringer for at least 180 s followed by a 3 s perfusion of 100 $\mu$M dopamine. The averaged calcium responses of all cells ($n = 6$) are shown in Figure 4.8 (A). Representative traces are presented in (B-F). The cells did not respond to octopamine. The cells did respond to dopamine with a $T_{P20}$ of 3.94 s $\pm$ 0.924 s (SEM) and a $T_{R50}$ of 3.888 s $\pm$ 1.42 s (SEM). There were 3/6 Type A responses and 3/6 Type D responses.
Figure 4.7: Kenyon cells exposed to TEA exhibit spontaneous calcium spikes
Kenyon cells were continuously perfused with honeybee ringer containing 22 mM tetraethylammonium (TEA). Representative examples are presented in (A-H). Relative fluorescence values were calculated as $\Delta F = F/F_0$ with $\Delta F$ subsequently normalised to the maximal peak of the first response.
Figure 4.8: Octopamine does not induce a calcium response in Kenyon cells that respond to dopamine
During continuous perfusion with honeybee ringer cells were exposed to a 3 s perfusion of 100 µM octopamine. Cells were then perfused for at least 120 s, then exposed to a 3 s perfusion of 100 µM dopamine. The averaged calcium responses of all cells (n = 6) are shown in (A). Representative examples are presented in (B-F). Relative fluorescence values were calculated as ΔF = F/F₀ with ΔF subsequently normalised to the maximal peak of the dopamine response. Black bar in (A) indicates application of treatment.
4.3.9 Effect of cis-(Z)-flupentixol on dopamine–induced calcium responses

To determine whether dopamine-induced calcium responses are affected by the potent AmDOP2 antagonist cis-(Z)-flupentixol (Mustard, Blenau et al. 2003, Beggs, Tyndall et al. 2011), Kenyon cells were exposed to cis-(Z)-flupentixol. Cells were continuously perfused with 10 µM cis-(Z)-flupentixol then exposed to a 3 second perfusion of 100 µM dopamine. After a minimum of 60 s cells were washed with honeybee ringer for a minimum of 30 s before a second 3 second perfusion of dopamine. The averaged calcium responses of all cells (n = 6) are summed in Figure 4.9 (A). Five individual traces are presented in (B-F). In the presence of 10 µM cis-(Z)-flupentixol the calcium response to dopamine was abolished. The cells recovered their ability to respond to dopamine after the cells had been washed with honeybee ringer. The dopamine response had a $T_{P20}$ of $2.94 \pm 0.746$ s (SEM) and a $T_{R50}$ of $2.581 \pm 0.429$ s (SEM). The dopamine-induced calcium responses could all be categorised as Type A.

4.3.10 Spiperone alters the form of the dopamine-induced calcium response in Kenyon cells

To determine whether the dopamine receptor antagonist spiperone (Mustard, Blenau et al. 2003, Beggs, Tyndall et al. 2011) affects the dopamine-induced calcium responses, Kenyon cells were exposed to a continuous perfusion of 10 µM spiperone then exposed to a 3 second perfusion of 100 µM dopamine. After a minimum of 60 s, cells were washed with honeybee ringer for a further 30 s before a second 3 second perfusion of 100 µM dopamine. The averaged calcium responses of all cells (n = 11) are shown in Figure 4.10 (A). Representative traces are presented in (B-H). In the presence of spiperone, cells responded to dopamine with a rapid increase in calcium, with a $T_{P20}$ of $5.709 \pm 0.882$ s.
Figure 4.9: Effect of cis-(Z)-flupentixol on dopamine-induced calcium responses in mushroom body Kenyon cells

Kenyon cells were perfused with 10 µM cis-(Z)-flupentixol for 60 s, before being washed for at least a further 30 s in honeybee ringer, and exposed to a second 3 s perfusion of dopamine. The averaged calcium responses of all cells (n = 6) are shown in (A). Representative examples are presented in (B-F). Relative fluorescence values were calculated as ΔF = F/F₀ with ΔF subsequently normalised to the maximal peak of the second dopamine response. Black bar in (A) indicates application of treatment.
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s (SEM) and a $T_{R50}$ of $20.04 \pm 4.89$ s (SEM). The majority of traces had a Type B response (10/11) and one had a Type D response. The time to peak of the second response to dopamine was $5.33 \pm 0.674$ s (SEM). No discernable recovery phase was observed as none of the traces dropped below 50 % of the baseline fluorescence for the remaining 60 s of recording after the second perfusion of dopamine. Spiperone was the only antagonist that did not abolish the dopamine-induced calcium response. The dopamine-induced calcium response in the presence of spiperone appeared to greatly slow the TP20 with it being significantly slower than with dopamine alone ($p = 0.0011$) (Figure 4.11).

4.3.11 Calcium responses to dopamine in Kenyon cells are inhibited by epinastine

As activation of the honeybee dopamine receptor AmDOP2 expressed in vitro has been found to induce a calcium signal that can be blocked by epinastine (Beggs, Tyndall et al. 2011), the effects of this ligand on the dopamine-induced calcium responses in Kenyon cells were investigated. Cells were continuously perfused with 10 µM epinastine, then exposed to a 3 second perfusion of 100 µM dopamine. After a minimum of 60 second, cells were washed with honeybee ringer for a further of 30 second before a second 3 second perfusion of dopamine. The averaged calcium responses of all cells ($n = 11$) are shown in Figure 4.12 (A). Representative traces are presented in (B-H). In the presence of epinastine, the calcium responses to dopamine were abolished. The cells were still able to respond to dopamine after the 30 s wash with honeybee ringer. The dopamine responses had a $T_{P20}$ of $2.935 \pm 0.771$ s (SEM) and a $T_{R50}$ of $7.05 \pm 1.69$ s (SEM). There were 3/11 Type A responses, 6/11 Type B responses, and 2 intermediate traces (A,G).
Figure 4.10: Effect of spiperone on dopamine-induced calcium responses in Kenyon cells
Kenyon cells were continuously perfused with honeybee ringer containing 10 µM spiperone. During perfusion, cells were treated with a 3 s exposure of 100 µM dopamine. Cells were washed for 30 s in honeybee ringer, and exposed to a second 3 s perfusion of dopamine. The averaged calcium responses recorded in all cells (n = 11) is shown in (A). Representative examples are presented in (B-H). Relative fluorescence values were calculated as $\Delta F = F/F_0$ with $\Delta F$ subsequently normalised to the maximal peak of the first dopamine response.
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4.3.12 Caffeine induces a calcium signal in Kenyon cells

In order to compare the time course of calcium responses involving intracellular release with responses elicited by dopamine, cells were exposed to a 6 second perfusion of 20 mM caffeine. Shorter lengths of exposure were insufficient to elicit a consistently detectable response (data not shown). The averaged calcium responses of all cells (n = 19) are shown in Figure 4.13 (A). Representative traces are presented in (B-H). Cells displayed consistent responses to caffeine. These responses were generally smaller in amplitude than the acetylcholine response, with a relative value of 0.632 s ± 0.066 of the acetylcholine response and a $T_{p_{20}}$ of 4.67 s ± 0.662 s (SEM). The recovery phase was smooth, with no peaks or oscillations in fluorescence with a $T_{r_{50}}$ of 19.07 s ± 1.863 s (SEM).

Figure 4.11: Peak fluorescence of the dopamine-induced calcium response in the presence of spiperone

In the presence of 10 µM spiperone the dopamine-induced calcium response was significantly slower than when treated with dopamine alone. Student’s unpaired t-test, $p = 0.0011$. 
Figure 4.12: Effect of epinastine on dopamine-induced calcium responses

Kenyon cells were perfused with honeybee ringer containing 10 µM epinastine. During perfusion cells were treated with a 3 s exposure of 100 µM dopamine. Cells were washed in honeybee ringer, and exposed to a second 3 s perfusion of dopamine. The averaged calcium responses of all cells (n = 11) are shown in (A). Representative examples are presented in (B-H). Relative fluorescence values were calculated as ΔF = F/F₀ with ΔF subsequently normalised to the maximal peak of the second dopamine response. Black bar in (A) indicates application of treatment.
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Figure 4.13: Caffeine-induced calcium signals in mushroom body Kenyon cells
During continuous perfusion with honeybee ringer cells were exposed to a 6 s perfusion of 20 mM caffeine. The averaged calcium responses of all cells (n=19) are shown in (A). Representative examples are presented in (B-H). Relative fluorescence values were calculated as $\Delta F = F/F_0$ with $\Delta F$ subsequently normalised to the maximal peak of the caffeine response. Black bar in (A) indicates application of treatment.
4.3.13 Mushroom body Kenyon cells can respond to both caffeine and acetylcholine

As Kenyon cells can respond to both acetylcholine and caffeine individually, Kenyon cells ability to respond to both treatments was also assessed. To investigate the effect of combined treatment, Kenyon cells were exposed to a 6 second burst of 20 mM caffeine as in Section 4.3.12, cells were then perfused for at least 180 s with honeybee ringer before they were subsequently exposed to a 3 second perfusion of 10 μM acetylcholine. Recordings were maintained for at least 120 s after the acetylcholine exposure. The average calcium responses are summed in Figure 4.14 (A). Representative examples of individual traces are presented in (B-H). Cells displayed a consistent response to caffeine with very similar rates of increase in fluorescence; however, the recovery rate was more variable between cells. Responses to acetylcholine were similar to previous acetylcholine responses; however, recovery was occasionally impaired (B, E, H).
Figure 4.14: Mushroom body Kenyon cells that respond to caffeine also respond to acetylcholine
Examples of calcium-induced fluorescence from individual mushroom body Kenyon cells, which were exposed to a 6 s burst of 20 mM caffeine were subsequently exposed to a 3 s burst of 10 µM acetylcholine. The average calcium responses are summed in (A) (n = 16). An assortment of individual traces is presented in (B-H). Relative fluorescence values were calculated as $\Delta F = F/F_0$ with $\Delta F$ subsequently normalised to the maximal peak of the second dopamine response. Black bar in (A) indicates application of treatment.
4.4 Discussion

Techniques advanced by Raccuglia and Müller (2014) have provided an effective tool to analyse dopamine-induced calcium responses in honeybee mushroom body neurons. Results presented in this Chapter show that dopamine can induce a calcium response in these cells. This is in line with results from the previous Chapter, which showed that Kenyon cells \textit{in vitro} express the dopamine receptor genes, \textit{Amdop1}, \textit{Amdop2} and \textit{Amdop3}. It is also consistent with previous work showing that these same genes are expressed in Kenyon cells \textit{in vivo} (Kurshan et al., 2003, McQuillan et al., 2012).

The calcium responses elicited by dopamine all shared similar times-to-peak, and they were significantly faster than responses to either acetylcholine or caffeine. It is clear from the acetylcholine results that the vast majority of the cells in culture were capable of a detectable calcium response. However, the number of cells responding to dopamine was less than 1% of cells responding to acetylcholine, and a relatively high concentration of dopamine (100 µM) was necessary to elicit calcium signals in these cells. A likely mediator of the dopamine-induced calcium responses observed in this study is the honeybee dopamine receptor AmDOP2. \textit{In vitro} studies have shown that activation of the AmDOP2 receptor, like its orthologue in \textit{Drosophila}, DAMB (Han et al., 1996), leads to a rapid increase in intracellular calcium (Beggs et al., 2011). Beggs et al., (2011) found that calcium signals mediated by AmDOP2 could be reduced by inhibiting PLC\textsubscript{β} activity, whereas cAMP signaling via the same receptor could not. This suggests that AmDOP2 couples to calcium via a PLC\textsubscript{β} dependent pathway. PLC hydrolyses the membrane phospholipid PIP\textsubscript{2} to form IP\textsubscript{3} and diacylglycerol (DAG). DAG, together with calcium, activates the enzyme protein kinase C (PKC), whereas IP\textsubscript{3} binds to receptors on the endoplasmic reticulum, releasing calcium (Berridge, 1993, Kamikouchi et al., 1998, Kamikouchi et al., 2000). Whether dopamine-mediated calcium re-
sponses observed in this study involve calcium release from intracellular stores remains unclear. What is clear from the results of this study is that calcium transients triggered by dopamine require a calcium influx from the extracellular medium. Replacement of extracellular calcium with cobalt abolished dopamine-induced calcium responses and responses returned when cobalt was removed and extracellular calcium resupplied. Furthermore, in the presence of cadmium, and with extracellular calcium, dopamine responses were either abolished or greatly reduced. Potentially the concentrations of cadmium used were not sufficient to completely block the calcium channel, allowing some influx of calcium. However, the effectiveness of cadmium as a calcium channel blocker has also been shown to be sensitive to the level of cell polarization, potentially this could also account for the reduced calcium responses as cadmium might be less effective at the voltage potentials expected in the KCs (Brown et al., 1983, Lansman et al., 1986, Armstrong and Chow 1987, Swandulla and Armstrong 1989). In the presence of spiperone, the time-to-peak of dopamine-induced calcium responses was slowed significantly, and the fast, transient component of the response tended to become lost as the calcium signal became prolonged. These observations are consistent with evidence suggesting that spiperone can interfere with both signaling via AmDOP2 and AmDOP1; however, it is a relatively weak antagonist of these receptors (Mustard et al., 2003, Beggs et al., 2011).

AmDOP2 shares a close evolutionary relationship with the octopamine receptor, AmOA1, a receptor that also signals through calcium (Grohmann et al., 2003). AmOA1 and AmDOP2 receptors have similar pharmacological profiles, and epinastine has been shown to be a potent antagonist of both AmOA1– and AmDOP2– mediated calcium signals (Beggs et al., 2011). In this present study, dopamine-induced calcium responses were completely abolished in the presence of epinastine, which provides evidence that AmDOP2 mediates
the dopamine-induced calcium signals observed in this study.

The form of the dopamine-induced calcium signals following exposure of cells to cis-flupentixol was particularly interesting. Like epinastine, cis-flupentixol completely abolished dopamine-induced calcium responses, which is consistent with previous studies showing that this dopamine receptor antagonist blocks AmDOP2 receptors (Beggs et al., 2011). Interestingly, cis-flupentixol is also a potent antagonist of AmDOP1. After flushing briefly with normal saline, dopamine-induced calcium signals returned, but unusually, all responses were fast, transient (A-type) responses. This suggests that other dopamine receptors, in addition to AmDOP2, may contribute to the responses to dopamine seen in this study. For example, it is possible that other dopamine receptors play a role in prolonging the duration of calcium signals mediated by AmDOP2. Calcium influx into Kenyon cells activates calcium-activated potassium currents (IKCa) (Schäfer et al., 1994). Once activated, these currents would be expected to hyperpolarise the cell membrane and inactivate voltage-dependent calcium channels in the cells. If IKCa currents were blocked, then dopamine-evoked calcium signals could potentially be prolonged. In honeybee antennal lobe neurons, dopamine reduces the amplitude of calcium-activated potassium currents in the cells (Perk and Mercer, 2006). If dopamine has a similar action on Kenyon cells, this might be expected to prolong the duration of calcium responses mediated via AmDOP2. Interestingly, most of the dopamine-induced calcium signals observed in this study consisted of a fast, transient component at the outset, followed by a slow rise in intracellular calcium that sometimes gave rise to calcium oscillations in the cells. Responses of this kind were still apparent under spiperone. They recovered soon after the washout of epinastine, but late components of the dopamine response remained blocked after treatments with cis-flupentixol.

One possible explanation for the increased prevalence of A-type responses
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following treatment with cis-flupentixol could be that in the presence of this antagonist, a receptor which normally inhibits calcium-activated potassium currents in the cells, potentially AmDOP1, remains blocked. Therefore dopamine-induced calcium influx would be terminated rapidly by IKCa currents. As cis-flupentixol is a potent antagonist of AmDOP1 (Mustard et al., 2003, Beggs et al., 2011), it is possible that after brief washing of the cells the effects of this dopamine receptor antagonist on AmDOP2 are at least partially reversed, but the AmDOP1 receptor remains blocked. If AmDOP1 activation reduces calcium activated potassium currents thereby prolonging calcium signaling in the cells, then blocking the receptor with cis-flupentixol would be expected to enhance the rate of termination of AmDOP2-induced calcium responses and increase the prevalence of A-type responses in the cells. Consistent with this model, epinastine, which is a potent blocker of AmDOP2 but not of AmDOP1, did not promote the early curtailment of dopamine-induced calcium signals.

4.4.1 Proposed mode of action for the honeybee dopamine receptors

The data presented in this Chapter indicate that calcium influx is mediated by AmDOP2, potentially via a voltage-gated calcium channel which may be prolonged by release from intracellular stores and/or the activation of AmDOP1 receptors (Figure 4.15). Activation of AmDOP3 receptors, which down-regulate intracellular levels of cAMP, are predicted to have an action opposite to that of AmDOP1. The AmDOP3 receptor may inhibit effects mediated via AmDOP1 and enhance the termination of dopamine-induced calcium responses. Cells with A-type responses may represent cells that have a low AmDOP1 complement, or express higher levels of AmDOP3. In support of the hypothesis that voltage-gated calcium channels are central to this dopamine mediated calcium response, calcium oscillations similar in form to Type B
Proposed model of signalling for the three honeybee dopamine receptors. Binding of dopamine to AmDOP1 leads to an increase in cAMP levels, inhibiting K⁺ channels. Binding of dopamine to AmDOP2 also increases cAMP levels in the cell, but also a calcium influx via voltage-gated calcium channels. Increase in intracellular calcium levels leads to activation of K⁺ channels. AmDOP3 activation reduces cAMP levels, inhibiting the suppression of K⁺ channel activity via AmDOP1. DA dopamine, AC adenylyl cyclase, PLC phospholipase C, Ca²⁺ calcium, K⁺ potassium.

responses could be induced by treating cells with the K⁺ channel blocker tetraethylammonium (TEA). TEA would be predicted to block K⁺ channels and depolarise the cell membrane, leading to an increase in spontaneous opening of voltage-gated calcium channels.

It is possible that AmDOP1 and AmDOP2 receptors work together to promote the likelihood of prolonged responses in the form of calcium oscillations resulting from depolarising stimuli such as cholinergic inputs from the antennal lobe. Calcium activation of cellular components such as PKC play an important role in long-term memory (Fiala et al., 1999, Müller, 2000, Kemenes et al., 2006a, Kemenes et al., 2006b Michel et al., 2008), in particular transcription and de novo protein synthesis, which is critical for long-term memory formation (Tully et al., 1994, Lefer et al., 2013, Eisenhardt, 2014).

The prolonged activation of calcium oscillations could lead to greater levels of transcription and protein synthesis within the specific areas of the mush-
room body relevant to the conditioning event (Greenberg et al., 1986, Morgan and Curran, 1986, Dolmetsch et al., 1998, Barbado et al., 2009). This could be expected to produce long-term changes to levels of excitability and synaptic strengthening in the Kenyon cells. Interestingly, there are critical times during and after conditioning where transcription is required for the formation of effective long-term memory (Lefer et al., 2013). For example, blocking transcription during subsequent spaced conditioning events, blocks long-term memory. In this study, co-application of acetylcholine and dopamine often produced clear calcium oscillations in the cells. It is predicted that acetylcholine is inducing a calcium influx through nicotinic acetylcholine receptors (nAChR) (Lee and O’Dowd, 1999, Oleskevich, 1999, Gauglitz and Pfluger, 2001, Gu and O’Dowd, 2006, Campusano et al., 2007, Dupuis et al., 2012), and that the influx of calcium leads to depolarisation of the cell membrane. Under these conditions, applying dopamine to the cells enhances calcium influx and may help establish the strong calcium oscillations observed in this study. Elevation of intracellular calcium levels using either caffeine or dopamine prior to acetylcholine treatment generally failed to induce calcium oscillations in the acetylcholine treatments. Interestingly, when non-A type responses were observed in the dopamine response prior to acetylcholine application, oscillations in the acetylcholine responses were subsequently observed. These results elucidated two points; firstly, the co-application of acetylcholine and dopamine enhances the likelihood of calcium oscillations, and secondly, cells that responded to dopamine with short A-type calcium transients, failed to modulate responses to acetylcholine.

Variations in the complement and levels of expression of dopamine receptors in different populations of mushroom body Kenyon cells would be predicted to have a significant influence on the modulatory role of dopamine on these cells. Following the model, AmDOP1 and AmDOP2 receptors are predicted
to work together to enhance the likelihood of prolonged responses to olfactory cholinergic inputs leading to changes in gene expression, whereas this may be less likely in cells expressing low levels of AmDOP2 or high levels of AmDOP3. *Amdop*1 is expressed at similar levels across all cell populations while there appears to be a much more cell population-specific expression of *Amdop*2 and *Amdop*3 (Kurshan et al., 2003, McQuillan et al., 2012). The data suggest that Kenyon cells may require some depolarisation for dopamine to be maximally effective, as robust calcium oscillations were consistently recorded with co-application of dopamine and acetylcholine, and under these conditions no fast calcium transients typical of A-type responses were seen. The co-application of acetylcholine and dopamine in this system was undertaken to represent an associative learning event *in vitro*, with acetylcholine representing the input to Kenyon cells of a conditioned odour stimulus, and dopamine representing the input from an unconditioned stimulus such as an electric shock which, *in vivo*, induces a predictable response, sting extension (Vergoz et al., 2007a, Vergoz et al., 2007b). Honeybee Kenyon cells are innervated extensively by dopaminergic neurons (Schäfer and Rehder, 1989, Schurmann et al., 1989, Blenau et al., 1995, Blenau et al., 1999, Kokay et al., 1999). Dopamine can be released in this region of the brain by aversive stimuli, such as stimulation with electric shock (Jarriault et al., unpublished observations).

*In vivo*, the signal from antennal lobe projection neurons shows sparsening in an odour specific manner at the level of the Kenyon cells (Perez-Orive et al., 2002, Szyszka et al., 2005). The data gathered in this study suggest that while dopamine may be released widely in the mushroom body neuropil, it is likely to have a significant effect only on those cells that are active at the time that dopamine is released. The results of this Chapter provide important new information on the possible mode of action of dopamine in the mushroom bodies of the honeybee brain. They suggest a complex interaction between
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cholinergic inputs to mushroom body Kenyon cells and dopamine modulation of cell excitability. Future work will be required to confirm the identity of the calcium channels modulated by dopamine and the possible interaction between the different dopamine receptor populations in these cells.
Chapter 5

Concluding remarks

This thesis used honeybee Kenyon cells in primary cell culture to investigate dopamine receptor function in these intrinsic mushroom body neurons. The development of this technique represents an important step forward in expanding the possible approaches available for future research. However, using dissociated Kenyon cells in primary cell culture is not without its limitations. The dissociation of the Kenyon cells necessarily results in the loss of connectivity between the Kenyon cells within the mushroom bodies as well as to other regions of the honeybee brain such as the antennal lobes, and dopaminergic neuron clusters, such as C1, C2, and C3. The specific sub-population origin of any given Kenyon cell in vitro responding to dopamine could not be identified, and therefore the composition of dopamine receptors in responsive cells is also unknown. However, a clear advantage of this technique is that the dopamine receptors themselves are likely to be in a cellular milieu that is much more similar to that found in vivo than if transfected cell lines had been used. Supporting this is that the dopamine induced calcium responses observed in this thesis differ in important ways from those observed previously in transfected cell. Thus while losing the connectivity and hormonal environment of the whole brain, primary cell culture of Kenyon cells does give a greater confidence that
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the cellular responses to dopamine and hormone treatment observed in this thesis are similar to those present in vivo.

Previous reports identified dopamine-induced calcium signals in heterologous cells expressing the honeybee dopamine receptor AmDOP2 (Beggs et al., 2011), a receptor shown to be blocked effectively by the vertebrate antihistamine epinastine (Beggs et al., 2011). The results presented in this thesis provide evidence that dopamine-induced calcium signals detected in mushroom body Kenyon cells are mediated by this invertebrate-specific dopamine receptor. Dopamine-induced calcium responses and dopamine modulation of responses elicited by acetylcholine have been reported also in vitro in mushroom body cells taken from Drosophila (Leyton et al., 2014). The dopamine receptor responsible was not identified in the fly, but previous studies have shown that the Drosophila orthologue of AmDOP2, known as DAMB, is expressed by Kenyon cells and like AmDOP2, the fly receptor DAMB couples both to cAMP and to calcium. Interestingly, the DAMB receptor has been shown to be essential for the formation of appetitive long-term memories in the fly (Musso et al., 2015) and it appears to play a role also in forgetting as low levels of dopaminergic neuron activity in the fly activate the receptor, leading to memory degradation (Berry et al., 2012, Berry and Davis, 2014).

In contrast to DAMB, relatively little is known about the role of the honeybee orthologue AmDOP2. However, a study using RNAi to reduce levels of Amdop2 expression in the honeybee suggests that AmDOP2 plays a role in locomotion (Mustard et al., 2010). The study by Mustard et al., showed that application of cis-flupentixol reduced locomotion, as does the injection of dsRNA of Amdop2. Yet, the reduction on locomotion was not equivalent between the two treatments, as Amdop2 RNAi also showed an increase in grooming behaviour suggesting that Amdop2 may be regulating other behaviours in the honeybee. While RNAi has been used successfully to study AmDOP2’s
role in locomotion in the honeybee, the effect of Amdop2 RNAi on honeybee memory and learning has not yet been assessed (Mustard et al., 2010). Knockdown of Amdop2 could be used to assess the function of the AmDOP2 receptor in learning and memory, as well as potentially active forgetting. A clearer understanding of the functional similarities between AmDOP2 and the Drosophila orthologue DAMB, through the use of RNAi would be a very useful tool for guiding future investigations of the AmDOP2 receptor.

To understand the role of AmDOP2 in the honeybee, a more thorough understanding of the impact of AmDOP2 activation on cell excitability is needed. The use of RNAi has revolutionized functional studies in Drosophila from dissecting signal transduction pathways and ion channel signaling (e.g. Clemens et al., 2000, Elbashir et al., 2001, Hammond et al., 2001, Roos et al., 2005), to investigating learning and memory mechanisms (e.g. Presente et al., 2004, Qi and Lee, 2014, Chambers et al., 2015, Matsuno et al., 2015, Walkinshaw et al., 2015). Knockdown of Amdop2 transcript levels in Kenyon cells in primary cell culture may prove useful to confirm the contribution AmDOP2 makes to dopamine-induced calcium responses in mushroom body neurons. A pilot study using dsRNA to target Amdop2 via RNAi conducted during this thesis was unsuccessful (data not shown) and more stable siRNAs may be necessary for in vitro RNAi in primary cell culture.

The model proposed in Chapter 4, based on pharmacological analyses undertaken in this study, proposes receptors other than AmDOP2 may contribute to shaping calcium responses induced by dopamine. For example, the presence of Type A responses only during the washout of cis-flupentixol, and the large continuous calcium responses in presence of spiperone both suggest that dopamine receptors such as AmDOP1 and AmDOP3, which are reported to be more sensitive to these particular antagonists than AmDOP2 (Mustard et al., 2003, Beggs et al., 2011), may contribute to the form of dopamine-
induced calcium signals. Consistent with this possibility, all three dopamine receptor genes appear to be expressed in all mushroom body Kenyon cells (Blenau et al., 1998, Humphries et al., 2003, Mustard et al., 2003, McQuillan et al., 2012, Kaneko et al., 2013, Kaneko et al., 2016). Interestingly, the levels of expression of these genes (as suggested by levels of gene transcript) are not uniform suggesting that levels of dopamine receptor expression within Kenyon cells may be subpopulation specific (Kurshan et al., 2003, Mustard et al., 2003, McQuillan et al., 2012). Dopamine receptor gene transcript levels within these subpopulations change over time (Kurshan et al., 2003, Mustard et al., 2003, McQuillan et al., 2012), suggesting that the responses of Kenyon cells to dopamine may also alter with age. The increased transcript levels of Amdop2 in the ICC is particularly interesting when compared with the expression pattern of CaMKII, which is preferentially phosphorylated in the NCC, in direct contrast to Amdop2 (Mustard et al., 2003, Pasch et al., 2011, Kaneko et al., 2013). Future work targeting the potential relationship between AmDOP2 and CaMKII may provide interesting insight to honeybee learning and memory as RNAi of CaMKII has been shown to disrupt both early and late long-term memory formation in the honeybee (Scholl et al., 2015).

In this study, dopamine receptor gene transcript levels were used to indicate changes in dopamine receptor gene expression in Kenyon cells in vitro. One weakness of using this approach is that transcript levels do not always correlate well with levels of receptor protein (Abreu et al., 2009, Maier et al., 2009, Vogel and Marcotte, 2012). However, at present, the ability to investigate the distribution of dopamine receptor protein in honeybees is limited by the absence of specific dopamine receptor antibodies. It will be important in future studies to determine where in the mushroom bodies AmDOP1, AmDOP2 and AmDOP3 receptors are expressed. Mushroom bodies are extensively innervated by dopaminergic neurons (Schafer and Rehder, 1989, Schurmann et al.,
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1989) and appear to contain high densities of dopamine binding sites (Schafer and Rehder, 1989, Schurmann et al., 1989, Kokay and Mercer, 1996, Kokay et al., 1999), but the distribution of dopamine receptor types in the brain remains unclear. Dopamine immunoreactive fibres make synaptic contact not only with cell projections in the lobes of the mushroom body, but also with Kenyon cell somata (Blenau et al., 1999). However, which dopamine receptors are expressed, and whether their distribution changes over the lifetime of the honeybee are questions that remain to be answered.

This thesis also revealed that levels of dopamine receptor gene transcript in mushroom body Kenyon cells in vitro can be altered by exposing cells to hormone treatment and specifically to JH and 20HE. The question still remains however, whether JH or 20HE-induced changes in dopamine receptor gene transcript levels have an impact on the amplitude or kinetics of responses induced by dopamine. For example, work presented in Chapter 2 of this thesis suggests that Amdop3 transcript levels are increased in the presence of JH in cell culture, this trend is also seen in pollen foragers in vivo where both JH titres and Amdop3 transcript levels are higher than in newly-emerged adult workers (Beggs et al., 2005, McQuillan et al., 2012). It would be interesting to determine the effects of elevated Amdop3 expression levels on the amplitude of dopamine-induced changes in cAMP, and on the form of dopamine-induced calcium signals in mushroom Kenyon cells. Results presented in this thesis and in earlier studies suggest that JH may be at least partly responsible for age-related changes in dopamine signaling in the honeybee brain (Sullivan et al., 2000, Jassim et al., 2000, Sasaki et al., 2012, McQuillan et al., 2014).

Recent work suggests that 20HE may also be active in the brain of the adult worker honeybee (Paul et al., 2005, Paul et al., 2006, Yamazaki et al., 2006, Geddes et al., 2013, Ishimoto et al., 2013.). Interestingly, in the present investigation, mushroom body calyces of newly-emerged adult workers responded
to dopamine with a significant increase in cAMP levels, but only in the presence of 20HE. An important question that remains is how 20HE enhances the cAMP response to dopamine in these young honeybees. One possibility is that 20HE acts via the putative dopamine/ecdysone receptor AmGPCR19. While there is currently no published data on AmGPCR19 receptor activity, work on the *Drosophila* orthologue DmDopEcR has revealed that DmDopEcR can signal both via cAMP and MAPK/ERK (Srivastava et al., 2005, Petruccelli et al., 2016). Until more information is known about the characteristics of AmGPCR19 it is difficult to evaluate its role in honeybee behaviour. It is tempting to speculate however, that the early peak in 20HE titres observed in honeybees shortly after eclosion may be important developmentally for dopamine signaling in the brain of the honeybee.

This thesis used honeybee Kenyon cells in primary cell culture to investigate dopamine modulation of the intrinsic mushroom body neurons. The development of this approach and the findings presented suggest several new lines of inquiry. Including if dopamine receptors other than AmDOP2 contribute to the shaping of dopamine-induced calcium responses, and whether dopamine-modulation of Kenyon cells changes with age. Further, if dopamine-induced calcium responses are influenced by changing hormone titres, this begs the question; what role, if any, do these hormones play in the development of responses to dopamine in the honeybee brain? Addressing such issues should help reveal what role dopamine, and dopamine-induced calcium signals, play in the behavioural modulation of the honeybee.
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Appendices

5.1 Modulation of dopamine receptor gene transcript levels in Kenyon cells in cell culture normalised qPCR results

5.1.1 Effect of HVA on dopamine receptor transcript levels in Kenyon cells in cell culture
Figure 5.1: **Effect of homovanillyl alcohol on dopamine receptor transcript levels in newly-emerged adult worker Kenyon cells with normalisation to rpn2**

Kenyon cells from newly-emerged adult workers were treated with 1 μM HVA and maintained in culture for 4 days. Normalisation to rpn2 did not change the trend of the dopamine receptor gene transcript levels, however due to the increased variance in Amdop1, statistical significance of the increase transcript levels seen in HVA treated cell culture was lost. Unpaired Student’s t-tests, mean ± SEM of 5 independent samples per group.
Figure 5.2: Effect of homovanillyl alcohol on dopamine receptor transcript levels in newly-emerged adult worker Kenyon cells with normalisation to \( rpn2 \)

Kenyon cells from pollen foragers were treated with 1 \( \mu \text{M} \) HVA and maintained in culture for 4 days. Normalisation to \( rpn2 \) did not change the trend of the dopamine receptor gene transcript levels, however due to the increased variance in \( Amdop2 \), statistical significance of the increase transcript levels seen in HVA treated cell culture was lost. Unpaired Student’s t-tests, mean ± SEM of 5 independent samples per group.
5.1.2 Effect of 20HE on dopamine receptor transcript levels in Kenyon cells in cell culture
Figure 5.3: **Effect of 20-hydroxyecdysone on gene transcript levels in Kenyon cells harvested from newly-emerged adult workers in 24 hour cell culture**

Kenyon cells from newly-emerged adult workers were treated with 30 nM 20HE and maintained in culture for 24 hours. Normalisation to *rps8* or *rpn2* did not change the trend of the dopamine receptor gene transcript levels. The statistical significant changes in *Amgpcr19* (*p = 0.0411*) when normalised to *rps8* or *rpn2* was lost. Unpaired Student’s t-tests, mean ± SEM of 5 independent samples per group.
Figure 5.4: Effect of 20-hydroxyecdysone on gene transcript levels in Kenyon cells harvested from newly-emerged adult workers in 4 day cell culture

Kenyon cells from newly-emerged adult workers were treated with 30 nM 20HE and maintained in culture for 4 days. Normalisation to rps8 or rpn2 did not change the trend of the dopamine receptor gene transcript levels. The statistical significant changes in Amdop1 and Amgpcr19 when normalised to rps8 (p = 0.0304, p = 0.0092) and rpn2 (p = 0.0172, p = 0.044) remained. Unpaired Student’s t-tests, mean ± SEM of 5 independent samples per group.
Figure 5.5: Effect of 20-hydroxyecdysone on gene transcript levels in Kenyon cells harvested from pollen foragers in 4 day cell culture
Kenyon cells from pollen forager were treated with 30 nM 20HE and maintained in culture for 4 days. Normalisation to rps8 or rpn2 did not change the trend of the dopamine receptor gene transcript levels. However, due to the increased variance in rpn2 and rps8 some of the statistically significant changes seen in Amdop2, Amdop3 and Amgpcr19 was lost. The statistical significant changes in Amdop1 when normalised to rps8 (p = 0.0016) and rpn2 (p = 0.024) remained. Amgpcr19 when normalised to rps8 (p = 0.0031) remained significant, but was lost when normalised to rpn2. Unpaired Student’s t-tests, mean ± SEM of 5 independent samples per group.
Figure 5.6: Effect of 20-hydroxyecdysone and juvenile hormone on gene transcript levels of newly-emerged adult worker Kenyon cells

Mean gene transcript levels detected in newly-emerged adult worker Kenyon cells that were treated with JH, 20HE or JH and 20HE in combination, and maintained in vitro for 4 days. Groups that share letters are not significantly different. Transcript levels are expressed relative to the average transcript levels of the control. ANOVA with Tukey’s post hoc, SEM, mean of 5 independent samples per group.
Appendix 2

Modulatory actions of dopamine and serotonin on insect antennal lobe neurons: insights from studies *in vitro*

Modulatory actions of dopamine and serotonin on insect antennal lobe neurons: insights from studies in vitro

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Abstract Biogenic amines play diverse roles in the development and modulation of invertebrate neurons and ultimately also, in the regulation of animal behaviour. Here we examine the contribution that analyses of antennal lobe neurons in vitro have made towards our understanding of the mechanisms through which dopamine and serotonin operate in primary olfactory centres of the brain of the moth, Manduca sexta and the honey bee, Apis mellifera. This chapter reviews evidence suggesting that these biogenic amines function as regulators of neuronal development and as mediators of cellular and behavioural plasticity, in part at least, through the modulation of K⁺ conductances in the cells. Insect neurons in vitro provide an excellent model for exploring basic principles of amine function and their impact on neuronal excitability.

Keywords Biogenic amines · Modulation · Olfactory interneurons · Insect

Introduction

Insect responses to olfactory cues are seldom, if ever, “hard wired”. On the contrary, in insects, as in other animals, behavioural responses to sensory signals can vary dramatically depending on factors such as age, reproductive maturity and physiological state. One group of compounds that contribute to this behavioural plasticity are the biogenic amines. Analyses of insect neurons in culture have provided important insights into the mechanisms through which these modulatory compounds operate, mechanisms that appear to be highly conserved throughout the animal kingdom.

The detection and discrimination of floral odours by honey bees, and the exquisite sensitivity of male moths to female sex pheromone illustrate well the fundamentally important role of olfaction in the life and survival of some insect species. In this brief review, developmental studies and studies of olfactory information processing in primary olfactory centres (antennal lobes) of the brain of the honey bee, Apis mellifera, and the moth, Manduca sexta, are used to highlight questions related to amine-modulation of neural circuits in insects that have been addressed using in vitro techniques. We focus in particular on techniques that we have found invaluable, not only for analysing the functional properties of biogenic amine receptors, but also for identifying targets of amine modulation and for investigating effects of amines on cell excitability. We discuss in addition, how in vitro studies complement studies in vivo and enhance understanding of the developmental functions of biogenic amines, as well as their role as mediators of insect behavioural plasticity.

Analysis of antennal lobe neurons in culture

Antennal-lobe (AL) neurons can be maintained for prolonged periods in culture and their activity recorded in voltage-clamp (or in current-clamp) mode using whole-cell patch-clamp recording techniques described by Hamill et al. (1981). Membrane currents in AL neurons have been isolated and identified using a combination of pharmacological blockers, voltage inactivation and digital current subtraction protocols (see Hayashi and Hildebrand 1990; Kloppenburg et al. 1999a; Mercer and Hildebrand 2002a, b) and the ionic currents identified in these cells are similar.
to equivalent currents described in many other species, including other types of insect neurons (e.g. Byerly and Leung 1988; Baker and Salkoff 1990; Kloppenburg et al. 1999b; Schäfer et al. 1994). AL neurons in vitro can be monitored also in cell-attached mode, which is particularly useful for long-term recording of spike activity in the cells. In *Manduca*, for example, cell-attached recordings have been used to investigate effects of serotonin on odour-induced responses prior to examining modulation of ionic currents by serotonin in the same cells using whole-cell recording techniques (Kloppenburg et al. 1999a).

**A developmental role for dopamine and serotonin**

Dopamine- and serotonin-immunoreactive neurons invade the antennal lobes early in development, before the lobes undergo rapid growth and compartmentalization into functional subunits of synaptic neuropil called glomeruli (Kent et al. 1987; Oland et al. 1995; Kirchhof et al. 1999). Processes of amine-containing neurons are ideally placed to influence developmental events occurring within the AL neuropil, and analyses of AL neurons in vitro suggest that amines released at this level may affect the structure, as well as the functional development of these cells.

Immature AL neurons are spontaneously active (Oland et al. 1996; Mercer and Hildebrand 2002a). This is of significant interest, firstly, because early forms of electrical activity are known to affect the growth and differentiation of developing neurons, and contribute also to activity-dependent tuning of neuronal connections (reviewed by Spitzer 1994; Katz and Shatz 1996) and secondly, because spontaneous activity of AL neurons in vivo coincides with critical periods of glomerular development and synaptogenesis (Oland et al. 1996). Early in development, action potentials recorded in *Manduca* AL neurons are small in amplitude, long in duration, and Ca$^{2+}$-dependent (Mercer and Hildebrand 2002a), and many immature AL neurons at this time exhibit spontaneous sustained membrane depolarisations that resemble plateau potentials (Mercer et al. 2005). Interestingly, exposing immature AL neurons in vitro to serotonin increases the likelihood that a small depolarizing current pulse will trigger a cell’s entry into a plateau state (Mercer et al. 2005), an effect that could serve in vivo to promote the translation of brief synaptic inputs into long-lasting activity, perhaps enhancing the growth of developing cells. Consistent with this possibility, both dopamine and serotonin have been found to increase the growth of AL neurons in vitro (Mercer et al. 1996a; Kirchhof et al. 1999).

As development proceeds, action potential waveforms in *Manduca* AL neurons become larger in amplitude, shorter in duration, and increasingly Na$^{+}$-dependent (Mercer and Hildebrand 2002a). These shifts in cell excitability are accompanied by changes in the density of Na$^{+}$, Ca$^{2+}$ and K$^{+}$ currents, and as development proceeds, cell-type-specific response characteristics emerge (Mercer and Hildebrand 2002b). Results showing that a significantly higher percentage of immature neurons in vitro display Na$^{+}$-based action potentials following the addition of serotonin to the culture medium (Mercer and Hildebrand 2002a) suggest that not only the structural development of AL neurons may be influenced by serotonin, but also their functional development.

**Identifying cellular targets of amine modulation**

AL neurons in vitro have proven to be invaluable also for identifying cellular targets of amine modulation in these cells. Both dopamine and serotonin appear to target K$^{+}$ currents in AL neurons, but the currents modulated by these amines are not identical. Serotonin has been found to reduce at least two voltage-activated K$^{+}$ conductances in *Manduca* AL neurons in vitro; a fast-activating transient A-type K$^{+}$ current ($I_{A}$) and a more slowly-activating, sustained current K$^{+}$ current [$I_{K(V)}$] that resembles the delayed rectifier (Mercer et al. 1995, 2005; Kloppenburg et al. 1999a). While K$^{+}$ currents in AL neurons of the honey bee are also targets of dopamine modulation, $I_{A}$ and delayed rectifier-like $I_{K(V)}$ currents in these neurons do not appear to be modulated by this amine. Instead, dopamine targets Ca$^{2+}$-dependent K$^{+}$ currents in these cells (Perk and Mercer 2006).

**Effects of amines on cell excitability**

As extensive evidence shows that resting membrane potentials, action potential waveforms and the frequency of neuronal firing are sculpted by K$^{+}$ channel activity (reviewed by Salkoff et al. 1992), amine modulation of K$^{+}$ conductances would be expected to impact on cell excitability. While this has yet to be clearly demonstrated in *Apis* AL neurons, analyses of *Manduca* AL neurons in vitro have shown that serotonin increases cell excitability and broadens action potential waveforms in these cells (Mercer et al. 1996b; Mercer and Hildebrand 2002a). Importantly, these changes are consistent with serotonin’s effects on the responses of AL neurons in situ both, to electrical stimulation of the olfactory receptor cells (Kloppenburg and Hildebrand 1995), and to stimulation with sex pheromone (Kloppenburg et al. 1999a; Kloppenburg and Heinbockel 2000). Together, evidence from studies of AL neurons in vitro and in situ strongly support the hypothesis that modulation of K$^{+}$ conductances underpins serotonin-induced
changes in cell excitability and spike waveform in these cells.

**In vitro analysis of amine receptor function**

While it is important to determine the identity and functional properties of amine receptors, the identity of receptors expressed by target cells often remain unresolved, even after effects of amines on behaviour and cell excitability have been clearly established. This in part, is because analysing biogenic amine receptors in situ can be extremely difficult. Not only are biogenic amine receptors expressed in many different regions of the brain, but also in invertebrates, as with vertebrates, the actions of any one biogenic amine are generally mediated by more than one receptor type, and the complement of receptors expressed by central neurons can vary markedly in different regions of the brain.

While the identities of the receptors that mediate the effects of dopamine and serotonin on AL neurons have yet to be fully revealed, first steps made in this direction highlight further the value of in vitro techniques. By utilising expression vectors to express a receptor gene of interest in cells normally devoid of that receptor type, the functional properties of the receptor in question can be investigated in isolation. For example, 3 honey bee dopamine receptor genes have been cloned and the receptors characterized using heterologous expression and pharmacological techniques (Blenau et al. 1998; Humphries et al. 2003; Beggs et al. 2005; Mustard et al. 2005). These in vitro studies have shown that 2 of the 3 honey bee dopamine receptors, AmDOP1 and AmDOP2, are positively coupled to adenylyl cyclase (Blenau et al. 1998; Humphries et al. 2003; Mustard et al. 2003), whereas activation of the 3rd receptor type (AmDOP3) generally downregulates intracellular levels of cAMP (Beggs et al. 2005; Beggs and Mercer 2009). Interestingly, AmDOP2 receptors signal not only through cAMP but also through calcium (Beggs et al. 2011), a property that it shares with the related honey bee octopamine receptor, AmOAc1 (Grohmann et al. 2003; Beggs et al. 2011).

Significant progress has been made also towards identifying the receptors that mediate the actions of serotonin in insect AL neurons. Two putative serotonin receptors have been cloned in the moth, *M. sexta* (Dacks et al. 2006), and in the honey bee, four serotonin receptor types have been identified (reviewed in Hauser et al. 2006; Blenau and Baumann 2001; Blenau and Thamm 2011). Heterologous expression techniques and pharmacological analyses have been used to characterize 2 of the honey bee serotonin receptors, Am5HT7 (Schlenstedt et al. 2006) and Am5HT1A (Thamm et al. 2010). These studies have revealed that while activation of Am5HT7 increases intracellular cAMP levels (Thamm et al. 2010), cAMP synthesis is inhibited by activation of Am5HT1A (Schlenstedt et al. 2006). The identification and characterization of receptors mediating the effects of dopamine and serotonin is important as it promises exciting advances in our understanding of the mechanisms through which these amines operate, not only in the antennal lobes, but also elsewhere in the brain.

**Integrating studies in vivo and in vitro**

Analyses of AL neurons in culture have provided important evidence that dopamine and serotonin target K+ conductances in the cells and that amine-induced reduction of K+ currents contributes to changes in cell excitability likely to affect the development and functional plasticity of antennal lobes. These studies have provided a valuable complement to studies of AL neurons in intact brains, in semi-intact brain preparations and in brain slices, studies that describe effects of serotonin not only on the output of neural circuits in the antennal lobes, but also on behavioural responses to olfactory cues (Kloppenburg and Hildebrand 1995; Kloppenburg et al. 1999a, b; Kloppenburg and Heinbockel 2000). Taken together, these studies indicate that biogenic amines are involved in events associated not only with the development of the antennal lobes but also in regulating the sensitivity of AL neurons to incoming olfactory signals, in part at least, through the modulation of K+ channel activity in these cells. As mechanisms through which amines operate are highly conserved, insect AL neurons in vitro provide a valuable model for exploring basic principles of amine function.

**References**


