Dopaminergic Facilitation of Synaptic Transmission in Layer II of the Lateral Entorhinal Cortex

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ABSTRACT

Dopaminergic facilitation of synaptic transmission in layer II of the lateral entorhinal cortex

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The lateral entorhinal cortex (LEC) plays an important role in the sensory and mnemonic functions of the medial temporal lobe, most notably in regard to object-related information and olfaction processing. The modulation of synaptic inputs in the LEC may have important implications for learning and memory, in part by affecting the synaptic output of the superficial layers of the LEC to the hippocampus. The superficial layers of the lateral entorhinal cortex are strongly innervated by midbrain dopaminergic neurons. Dopamine can modulate synaptic strength in a dose-dependent manner; high concentrations of dopamine suppress excitatory synaptic transmission, whereas lower concentrations of dopamine (1-10 μM) facilitate it. Therefore, low dopamine levels are likely to promote synaptic transmission in the LEC, and thus provide a mechanism for promoting mnemonic processes. However, the underlying intracellular signalling cascade linking dopamine receptor activation to glutamatergic transmission in layer II LEC neurons has remained hitherto unknown.

The work presented here used single-cell, patch-clamp recordings to characterize the signalling pathway linking dopamine-receptor activation to increases in synaptic transmission in layer II LEC cells. The first set of experiments in this thesis used bath application of dopamine and current-clamp recordings to investigate the dopaminergic facilitation of excitatory postsynaptic potentials (EPSPs) in layer II neurons of the rat lateral entorhinal cortex in vitro. Results indicated that activation of dopamine D_1-like receptors lead to increases in AMPA receptor-mediated responses in a manner that was dependent upon the cAMP-protein kinase A (PKA) pathway and protein phosphatase 1 (PP-1). The second series of experiments assessed the contribution of phosphatidylinositol (PI)-linked D_1-like receptors to the dopaminergic facilitation of synaptic transmission. Experiments demonstrated that, in addition to activation of the cAMP-PKA pathway, dopamine can lead to the facilitation of synaptic transmission that is reliant on a signaling cascade dependent on PI-linked D_1-like receptors, phospholipase C, release of calcium from internal stores, and protein kinase C. In a third series of experiments, fluorescence calcium imaging was used to monitor changes in intracellular calcium induced by dopamine. Bath application of dopamine and the PI-linked dopamine agonist induced a reliable and reversible increase in fluorescence in fan, but not pyramidal, entorhinal cells. This increased fluorescence was correlated with a reversible increase in the amplitude of evoked synaptic currents. Together, the results demonstrate that both the cAMP-PKA and the PLC-DAG-IP_3 signaling pathways may contribute to transient increases in synaptic strength that could mediate enhanced sensory and mnemonic function in the entorhinal cortex during release of dopamine.

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Chapter 2: Dopaminergic enhancement of excitatory synaptic transmission in layer II entorhinal neurons is dependent on D_1-like receptor-mediated signaling by Glovaci, Caruana, and Chapman.

- **Iulia Glovaci:** Designed the experiments, performed electrophysiological recordings, analyzed the data, performed statistics and created the figures, and wrote the manuscript for publication.
- **Douglas A. Caruana:** Performed electrophysiological recordings.
- **Andrew Chapman:** Assisted in designing the experiments, preparing the figures, and writing the manuscript.

Chapter 3: Activation of phosphatidylinositol-linked dopamine receptors induces a facilitation of glutamate-mediated synaptic transmission in the lateral entorhinal cortex by Glovaci and Chapman.

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Chapter 4: Dopamine induces transient increases in intracellular calcium in layer II lateral entorhinal cortex fan cells by Glovaci and Chapman.

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- **Andrew Chapman**: Assisted in designing the experiments, preparing the figures, and writing the manuscript.

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$5HT_{3a}$	5-hydroxytryptamine 3a
ACSF	artificial cerebrospinal fluid
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	analysis of variance
APV	(2R)-amino-5-phosphonopentanoate
CA1, CA3	Cornu Ammonis area 1 or 3
Ca^{2+}	calcium
CB+	calbindin-positive
cAMP	cyclic adenosine monophosphate
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CNQX	7-nitro-2,3-dioxo-1,4-dihydroquinoxaline-6-carbonitrile
DARPP-32	dopamine- and cyclic-AMP-regulated phosphoprotein
DG	dentate gyrus
DMSO	dimethyl sulfoxide
EGTA	ethylene glycol tetraacetic acid
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
GABA	gamma-aminobutyric acid
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
IP_3	inositol trisphosphate
IPSP	inhibitory postsynaptic potential
LEC	lateral entorhinal cortex
LTP	long-term potentiation
MEC	medial entorhinal cortex
Mg^{+}	magnesium
Na^{+}	sodium
NMDA	N-methyl-D-aspartate
PKA	protein kinase A
PKC	protein kinase C
PIP_2	phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
PP-1	protein phosphatase 1
PP-2A	protein phosphatase 2A
PV	parvalbumin
RE+	reelin-positive
R_{in}	input resistance
RyR	ryanodine receptors
SEM	standard error of the mean
SOM	somatostatin

CHAPTER 1

GENERAL INTRODUCTION

GENERAL OVERVIEW

Learning and memory are cognitive processes that are crucial to every aspect of our lives, and, as such, humans have evolved sophisticated mechanisms for learning new information and storing it for subsequent recall. Neurobiologically, the mammalian central nervous system has a massive capacity to encode and store a diverse range of information, and this capacity greatly shapes our behavioural potential. But how are memories formed and stored? Our current understanding of the neurobiological basis of learning and memory highlights the importance of communication between different brain regions, with changes in the strength of synaptic transmission between cells being a major means through which the nervous system forms, stores, and retrieves memories (e.g. Squire, 2004; Eichenbaum & Fortin, 2005).

Episodic memory of daily experiences consists of information regarding autobiographical events which are rich in contextual detail. In animal models, episodic memory has been defined as a memory containing information regarding the encountered stimuli, the spatial location, and the contextual and temporal information of the event (Clayton & Dickinson, 1998; Eacott & Norman, 2004; Babb & Crystal, 2006). Therefore, in order to encode an episodic memory, the brain needs to encode detailed information regarding the *when*, *where*, and *what* aspects of the event. Sensory information collected from the environment converges from multiple sensory areas to associational cortical areas, where it is further integrated prior to being conveyed to, and processed by, medial temporal lobe structures. There is a general consensus that episodic memory relies on the rhinal-hippocampal-neocortical network, within which information becomes increasingly more integrated as it is being processed (Eichenbaum, 2000; Squire, 1992), and that this circuit is critical for long-term episodic memory in both humans and nonhuman animals (Scoville & Milner, 1957; Zola-Morgan & Squire, 1990; Eichenbaum & Cohen, 2014; Izquierdo & Medina, 1997; Eichenbaum, 2017). Therefore, a detailed understanding of the anatomical and functional connections of this circuit are critical to understanding the neurobiological basis of episodic memory.

At the cellular level, memory entails changes in neuronal activity that ultimately promote encoding and storage of pertinent information, and can be viewed as the result of changes in the strength of synaptic transmission between neurons (Eichenbaum & Fortin, 2005; Squire, 2004). Synaptic transmission is modulated by a plethora of factors, including inputs from a multitude of neurotransmitter systems, that can alter synaptic activity via the activation of complex intracellular signalling cascades. Short-term changes the activity of intracellular signalling cascades can result in both short-term and long-lasting changes in synaptic transmission. Consequently, neuromodulatory transmitters can exert considerable influence over neuronal activity contributing to learning and memory. As such, the goal of the present thesis is to investigate the signalling cascades through which the modulatory neurotransmitter dopamine enhances synaptic transmission in neurons of the lateral entorhinal cortex, an area known to contribute to learning and memory processes (e.g. Young et al., 1997; Ramus & Eichenbaum, 2000).

Neurobiological Basis of Learning and Memory

In determining the neurophysiological basis of learning and memory, the hippocampus and associative areas have been extensively studied. The hippocampus is heavily involved in the formation of

long-term declarative memory, and is thought to play a central role in the encoding and consolidation of memory (O'Keefe & Nadel, 1978; Morris et al., 1982; McNaughton & Corr, 2004), as it is generally thought to serve as a short- and intermediate-duration buffer involved in maintaining memories until they are stored more permanently across regions of the neocortex (Eichenbaum, Otto, & Cohen, 1994). Proper hippocampal functioning requires inputs from nearby associative areas, the majority of which is funneled to the hippocampus via the entorhinal cortex, a region of the parahippocampal area that serves as an interface between the hippocampus and neocortex. The dentate gyrus, CA1, and CA3 regions of the hippocampus are all heavily innervated by parahippocampal areas, including the lateral entorhinal cortex (Amaral & Witter, 1989).

Because of the strength of its projections to the hippocampus, the entorhinal cortex was initially believed to be a relay station for information that required hippocampal processing (Insausti et al., 1987; Van Hoesen & Pandya, 1975a, 1975b; Van Hoesen, Pandya, & Butters, 1975), but this notion has become obsolete following subsequent tracing studies which demonstrated strong reciprocal connections between the hippocampus and the entorhinal cortex, and suggested a reciprocal feedback mechanism between the two areas (Witter et al., 1989). Most noteworthily, neurons in the superficial layers (II and III) of the entorhinal cortex project to the hippocampus, and CA1 hippocampal neurons project to the deep layers (V and VI) of the entorhinal cortex, forming a loop through which information can be highly processed between the hippocampal formation and the entorhinal cortex (Amaral & Witter, 1989; Burwell, 2000; Witter et al., 1989). This suggests that the entorhinal plays a complex role in mediating the inputs and outputs of the hippocampal formation, but the functions and characteristics of the entorhinal cortex remain relatively poorly understood in comparison to the breadth of research conducted on the hippocampus.

Anatomically, in addition to its reciprocal connections with the hippocampus, the entorhinal cortex has strong and reciprocal connections with sensory areas (Shipley & Adamek, 1984; Insausti, Herrero & Witter, 1997), as well as direct projections to neocortical areas (Swanson & Kohler, 1986). This anatomical arrangement is consistent with a central role for the entorhinal cortex in the mediation of sensory and mnemonic processes that rely on interactions between the neocortex and hippocampal formation, with the entorhinal cortex being positioned at the heart of the circuit loop through which multimodal sensory information enters the hippocampus and, exiting the hippocampus, returns to the entorhinal cortex to project back to neocortical regions (Delatour & Witter, 2002; Morecraft, Geula, & Mesulam, 1992). As such, the hippocampus and entorhinal cortex are believed to function in concert to execute complex cognitive and mnemonic processes through interactions with neocortical areas. Therefore, a more precise understanding of the cellular mechanisms governing synaptic transmission in the entorhinal cortex will lead to a better understanding of how the brain ultimately forms memories.

Overview of the Entorhinal Cortex

Ramon y Cajal was the first to highlight the significance of the entorhinal cortex, which he referred to as "the angular ganglion", after being struck by the massive number of entorhinal fibers in layers II/III

that, perforating the subiculum, terminated within the dentate gyrus and CA fields of the hippocampus. His student, Lorente de Nó (1933), was the first to publish a detailed anatomical description of the entorhinal cortex, and decades of subsequent research have added to our anatomical and functional understanding of the region, having described the complexity of this structure based on its connectivity, location, cytoarchitectonic and chemoarchitectonic aspects. Brodmann (1909) subdivided the entorhinal cortex into two subregions, namely areas 28a and 28b, which are contemporarily referred to as the lateral entorhinal cortex (LEC) and medial entorhinal cortices (MEC), respectively (van Groen, Miettinen, & Kadish, 2003; Brodmann, 1909; Steward, 1976; Witter, 2007). Although variations between species have been observed, it is now generally accepted that connectional and cytoarchitectonic evidence supports this subdivision of the entorhinal cortex into two anatomically and functionally distinct regions (Witter & Amaral, 2004). One important basis for differentiation between the MEC and LEC is based on their differing afferents, wherein the MEC receives large inputs from the presubiculum, postrhinal, and retrosplenial cortices, and the LEC receives large inputs from the olfactory bulb, perirhinal cortex and amygdala (Shipley, 1975; Room & Groenewegen, 1986; Caballero-Bleda & Witter, 1993). Another key feature for differentiation between the two regions is their relatively distinct anatomical and functional inputs to the hippocampus (e.g. Kerr et al., 2007; Insausti et al., 1997), as discussed below.

The Roles of the Lateral and Medial Entorhinal Cortex in Memory

Until recently, the functions of the entorhinal cortex have been poorly understood, when compared to the adjacent hippocampal formation. Behavioural studies in which the entirety of the entorhinal cortex was lesioned indicated that dysfunction of the entorhinal cortex leads to learning and memory impairments similar to those resulting from lesioning the hippocampus, especially in regard to contextual learning (Maren & Fanselow, 1997) and spatial navigation (Schenk & Morris, 1985). More recently, a large body of evidence has accumulated in favour of functional differences in information processing between the medial and lateral entorhinal cortices, which underlies different aspects of mnemonic processes, and likely arises from their differences in cytoarchitecture and patterns of input-output connectivity (Witter & Amaral, 2004; Witter & Moser, 2006; Kerr et al. 2007). Generally, it is accepted that MEC processes spatial information (e.g. Hafting et al., 2005), whereas LEC inputs convey nonspatial and object-related information to the hippocampus (e.g. Hargreaves et al., 2005; Deshmukh & Knierim, 2011).

Anatomically, the LEC receives strong, converging sensory and associational inputs from numerous cortical regions, and shares reciprocal connections with sensory-processing areas, which is consistent with a primarily role for the LEC in memory for objects and contextual information processing (see Figure 1.1). A large portion of afferents to the LEC arise from subcortical regions, the majority of which carry inputs from olfactory regions (Chapuis et al., 2013; Shipley & Adamek, 1984; Insausti, Herrero, & Witter, 1997; Kerr et al., 2007). Olfactory inputs to the LEC arise from both the olfactory bulb and the anterior olfactory nucleus (Haberly & Price, 1978; Kosel et al., 1981), with olfactory fibers mainly distributing in layer I of the LEC, where they synapse with dendrites of neurons from layers II and III (Wouterlood & Nederlof, 1983). Further, approximately a third of the LEC inputs arise from the piriform

cortex and associative cortical areas, including inputs from the insular and frontal cortices, regions to which the LEC is reciprocally connected (Burwell & Amaral, 1998; Beckstead, 1978; Mathiasen et al., 2015; Kondo & Witter, 2014; Kerr et al., 2007). Weaker inputs to the LEC arise from the temporal, parietal, and occipital areas (Kerr et al., 2007; Olsen et al., 2017), all of which are areas involved in aspects of sensory or context-dependent information processing. Additionally, the LEC shares reciprocal connections with the perirhinal cortex (Suzuki & Amaral, 1994; Naber, Witter, & Lopes da Silva, 1999), an area known to participate in object recognition and familiarity (Brown & Aggleton, 1999; Murray et al., 2007), as well as recognition memory (Brown & Aggleton, 2001), and with the amygdala, which provides the LEC with additional nonspatial information (Kerr et al., 2007). As such, it is clear that the LEC receives a large amount of diverse sensory and processed inputs that can provide it with information required for object and context-dependent memory.

In addition to its anatomical connections, the activity of LEC neurons also suggests an important role for the LEC in sensory and nonspatial processing (Hargreaves et al. 2005; Albasser et al. 2010; Yoganarasimha et al. 2010). Several studies demonstrate that LEC lesions cause severe non-spatial deficits (Hunsaker et al., 2013; Van Cauter et al., 2013). In one such study, Van Cauter and colleagues (2013) showed that LEC lesions do not affect the navigational abilities of rodents in a water maze or path integration task. However, rats with LEC lesions were less likely to engage with novel objects, and did not show a typical novel-object preference, as opposed to control animals or animals having received MEC lesions (Van Cauter et al., 2013). These findings lead us to infer that disruption of normal functioning in the LEC disrupts processes related to object memory, as compared to spatial processing. This is in line with evidence showing that while LEC neurons generally show weaker spatial modulation compared to cells of the MEC (Hargreaves et al., 2005; Yoganarasimha et al., 2010), they are highly responsive to object-related information. LEC neurons are known to fire to pictures of objects (Wan et al., 1999), discrete objects (Deshmukh & Knierim, 2011; Zhu et al., 1995), multiple objects (Deshmukh & Knierim, 2011), as well as individual characteristics of objects, such as odors or visual information (Zhu et al., 1995; Young et al., 1997; Wan et al., 1999). In contrast, few neurons from the superficial layers of the MEC fire specifically when animals investigate objects (Deshmukh & Knierim, 2011). Taken together, these findings highlight significant differences in functional processing between the LEC and MEC.

In turn, the LEC provides strong projections to the DG, CA3, and CA1 subdivisions of the hippocampal formation (Insausti et al., 1997; van Strien et al., 2009). Specifically, principal neurons from layer II of the LEC project to the dentate gyrus, whereas layer III LEC cells also project to CA1 (Steward & Scoville, 1976; Ruth et al., 1988). The strong, direct connectivity between the LEC and dentate gyrus (Kerr et al., 2007) provides the hippocampus with its primary source of sensory and associational input. It has been previously suggested that the LEC-DG pathway is particularly important in memory related to odor processing (Kesner, Hunsaker, & Ziegler, 2011) and odor pattern separation (Weeden, Hu, Ho, & Kesner, 2014). Additionally, given its responsivity to object-related information and its connections to the hippocampus, it is likely that information carried by the LEC also contributes to changes in spiking activity observed in CA1 cells when the animal is in the presence of objects (Manns & Eichenbaum, 2009), and to

changes in probability of expression and size of place fields in place cells when objects are present or shifted around in the environment (Burke et al., 2011). As such, the LEC and hippocampus appear to be not only anatomically, but functionally, coupled.

We know that encoding of episodic memories requires the integration of contextual information regarding the *what, when, and where* of the event (Clayton & Dickinson, 1998; Eichenbaum & Fortin, 2005). This integration may occur primarily within the hippocampal region, which receives contextual, spatial, and temporal information from the entorhinal cortex. Based on its role in nonspatial information processing, it has been proposed that the LEC is an extension of the 'what', or object information processing, circuit of the medial temporal lobe (Eichenbaum et al., 2007). Attractive in its simplicity, this model suggests a dissociation between a ventral 'what' circuit, extending through the temporal lobe to include the LEC, and a dorsal 'where' circuit extending through the parietal lobe, and including the MEC. Experimental data generally support this hypothesis. As aforementioned, the LEC plays a large role in nonspatial information processing (Hargreaves et al. 2005; Albasser et al. 2010; Yoganarasimha et al. 2010), and recent recordings obtained from MEC neurons have identified specialized cell types that fire in a spatially dependent manner, including "grid" cells (Hafting et al. 2005), head-direction cells, grid-by-head-direction cells (Sargolini et al. 2006), and border cells (Solstad et al. 2008; Moser et al., 2008). This has led to the current consensus that the MEC has a central role in processing spatial information, and is linked with the dorsal "where" stream of information processing.

However, such a "dorsal-where", "ventral-what" model might be too simplistic. Interestingly, recent studies have suggested that LEC processing is not completely independent of spatial context. It has been found that although LEC lesions primarily cause nonspatial deficits in rodents, they also cause weak, but significant deficits in spatial processing as well. Hunsaker and colleagues (2013) recently demonstrated that rats having received excitotoxic lesions of the LEC experienced weak deficits in *contextual* recognition, in addition to severe deficits in item recognition. The deficits in contextual recognition shown by LEC-lesioned rats were milder than those observed in MEC-lesioned animals, but rats having received LEC lesions performed more poorly than controls, which suggests a graded deficit in contextual processing, with the MEC-lesioned group showing the greatest impairment. Additionally, experimental data indicate that a subset of LEC neurons fire in relation to objects-in-space information over a prolonged period of time (Tsao, Moser, & Moser, 2013), which requires combined processing of both spatial *and* non-spatial information. These findings are backed up by several other studies suggesting that the LEC plays a role in processing the location of objects (Deshmukh & Knierim, 2011; Wilson et al., 2013). As such, while the LEC appears to primarily process 'what' information, it likely contributes to a lesser degree to spatial information processing.

Additionally, data from awake animals indicate that the LEC also contributes to *working* memory processes through ongoing interactions with olfactory and parahippocampal regions. Indeed, experimental evidence suggests that LEC neuronal activity is linked to working memory in animals engaged in an olfactory non-match-to-sample task (Young et al., 1997; Ramus & Eichenbaum, 2000). Changes in neuronal firing were linked to the period when rats sampled the odor stimulus, approached the reward, or

waited for the delay period of the trial to elapse. Cells that responded to stimulus odors were shown to be odor-specific, and appeared to process information about whether or not the stimulus was a match to the odor presented during the previous trial (Young et al., 1997). Further, the finding that the firing of lateral entorhinal cells increased during the delay period of the task suggests that these cells were actively maintaining olfactory information in working memory. These findings indicate that neurons in the lateral entorhinal cortex are not only involved in coding specific olfactory information, but that they are also involved in the retention of this information during variable-duration delay periods (Young et al., 1997). Related findings indicate that rats with EC lesions are unable to use trial-specific olfactory cues to help them remember which arms they had previously entered during an olfactory-dependent radial arm maze task (Staubli et al., 1995). Other reports suggest that while lesions of the EC may not impair the acquisition of an olfactory non-match-to-sample task, they lead to severe memory deficits when the delay is extended beyond 30 sec, suggesting deficits in either short-term or recognition memory (Otto & Eichenbaum, 1992). Overall, these findings point to a role of the EC in maintaining of information during a delay, and therefore a role for the LEC in working memory and memory maintenance which may lead to long-term memory formation.

Laminar Organization of the Lateral Entorhinal Cortex

The LEC and MEC are a part of the periallocortex, a transitional zone between the 3-layered allocortex of the hippocampus and the 6-layered neocortex. The subdivisions of the entorhinal cortex do not differ significantly in their laminar organizations, although some differences have been noted in the cell types and electrophysiological properties of neurons in superficial layers II and III (Tahvildari & Alonso, 2005; Canto et al., 2008; Witter & Amaral, 2004). Generally, the layering of the entorhinal cortex is based on relatively distinct populations of cells that vary to an extent in their morphology, connectivity, and density.

Layer I of the lateral entorhinal cortex is relatively cell-free, mainly consisting of dense fibers (Witter & Amaral, 2004). Layer I also contains a sparse population of GABAergic interneurons, with few glutamatergic cells present (Miettinen et al., 1997; Wouterlood & Nederlof, 1983; Canto & Witter, 2012).

Layer II comprises a mixture of large principal cells referred to as "fan" cells, given the distinctive structure of their dendritic arbour, pyramidal cells, and medium-sized multipolar neurons (Figure 1.2; Tahvildari & Alonso, 2005; Canto & Witter, 2012; Leitner et al., 2016). Many of these cells form ‘islands’, which are small areas of high neuronal density (Witter & Amaral, 2004). "Fan" cells are the main excitatory cell type in this layer (Canto & Witter, 2012). They are similar to layer II MEC stellate cells, and are generally reelin-positive, although some do express calbindin (Tahvildari & Alonso, 2005; Canto & Witter, 2012). Their soma are generally polygonal, with multiple, sparsely-spined primary dendrites fanning out horizontally and ascendingly throughout the superficial layers of the LEC. In contrast, pyramidal cells of layer II are generally calbindin-positive, with fewer expressing reelin. Their soma are medium sized, pyramidal-shaped, with generally thin, spiny dendrites and a thick, apical dendrite branching out into layer I of the LEC. Two different subtypes of pyramidal cells have been previously described in layer II, based primarily on their morphological properties (Tamamaki & Nojyo, 1993). Multipolar cells express both

calbindin and reelin, and have a diverse morphological profile (Canto and Witter, 2012; Leitner et al., 2016).

Electrophysiologically, layer II cells differ slightly. Typically, fan cells display a sag in their voltage response to strong hyperpolarizing current injection due to activation of the inward current I_h, and fire action potentials in intermittent clusters that are paced by theta-frequency membrane potential oscillations. In contrast, pyramidal neurons show regular firing patterns, and no sag in the voltage response during hyperpolarizing current injection. Multipolar cells express mixed electrophysiological properties that do not directly correspond to either fan or pyramidal cells (Tahvildari & Alonso, 2005). It has been recently suggested that these cell types may cluster together into sublayers (layers IIa and IIb; Leitner et al., 2016; Kobro-Flatmoen & Witter, 2017), and so they likely differ in their contribution to processing of information. Excitatory inputs to principal cells of layer II are received mainly via their layer I-spanning dendrites (Wouterlood & Nederlof, 1983). Layer II also contains a large variety of bipolar, basket, and chandelier interneurons, that express several different markers including $5HT_{3a}R$, GABA, PV, CB, calretinin, CCK, SOM, substance P, and NPY (Köhler et al., 1986; Wouterlood, 2002; Leitner et al., 2016). This diversity of interneurons serves to regulate local excitatory activity at excitatory synapses in the superficial layers of the LEC.

Principal cells in layer II of the LEC project to the dentate gyrus, CA1, and CA3 (Tamamaki & Nojyo, 1993). Axons of layer II neurons also give off thin collaterals into layers I and II, with their axonal arbor spanning approximately 400 μm (Tamamaki & Nojyo, 1993; Klink & Alonso, 1997), however the degree of interconnectivity between layer II principal cells remains controversial. Direct communication between fan cells appears to be present, but relatively rare (Nilssen et al., 2015).

Layer III in both LEC and MEC predominantly consists of a population of medium-sized, loosely arranged pyramidal neurons that share morphological and electrophysiological properties and, to a lesser degree, a population of multipolar neurons (Germroth et al., 1989). In contrast to layer II neurons, these pyramidal cells project to the CA1 region and subiculum (Tamamaki & Nojyo, 1993; Tahvildari & Alonso, 2005; Canto & Witter, 2012). Some layer III pyramidal neurons also project contralaterally to the hippocampus and entorhinal cortex (Steward & Scoville, 1976), and this contralateral innervation appears to be more common than in layer II cells (Dhillon & Jones, 2000). The microcircuitry of layer III neurons is not well known, but seems to be markedly different from that of layer II in that it shows a much stronger monosynaptic connectivity between principal neurons (Dhillon & Jones, 2000; Kloosterman et al., 2003). Neurons in layer III are the main recipients of local inputs from deep layers of the LEC, which appear to originate predominantly from neurons in layer Vb (see below; Kloosterman et al., 2003; van Haeften et al., 2003). Currently, no correlations have been reported between morphology, connectional profile and electrophysiological characteristics either in vitro or in vivo in layer III cells (Canto & Witter, 2012).

Together, layers I to III are often referred to as the superficial layers of the entorhinal cortex, and they are separated from the deep layers V and VI by layer IV, which is also known as the lamina dessicans. *Layer IV* is generally considered to be acellular, as it contains few, sparse neurons, consisting

of displaced layer III or V neurons interspersed with interneurons. Their connectivity and functionality is as of yet poorly understood (Witter & Amaral, 2004).

No significant differences have been noted between the LEC and MEC *Layer V* (Canto & Witter, 2012). Layer V is primarily composed of pyramidal cells located immediately adjacent to the lamina dissecans, whose main dendritic arbour extends within the deep layers, but whose apical dendrites extend into the superficial layers I-III of the EC, suggesting that they may receive sensory information from inputs reaching the superficial layers (Köhler, 1988). Layer V is also composed of a population of smaller multipolar and pyramidal cells that reside deeper within the layer, and whose dendritic arbour is generally restricted to layers V and VI. Neurons of layer V appear to have diverse electrophysiological profiles, but cannot be categorized based on their electrophysiological profiles alone (Hamam et al., 2002). Layer V cells receive inputs from the CA1 of the hippocampus and the subiculum (van Haeften et al., 1995; Kloosrerman et al., 2004), prefrontal cortex, and anterior cingulate cortex (Jones & Witter, 2007). In both the MEC and LEC, these neurons are considered the major output source of the EC, with widespread projections reaching diverse cortical and subcortical areas (Witter & Amaral, 2004; Rosene & Van Hoesen, 1977; Kosel et al., 1982; Cappaert et al., 2014). It has also been documented that these cells innervate, to a lesser extent, other layers of the EC (e.g. Köhler, 1988; Hamam et al., 2002).

Layer VI is the deepest cell layer of the LEC, and it is composed of multipolar cells with spherical somas and spiny dendrites that extend within the deep layers. The inputs to layer VI neurons are thought to be mainly from layer V cells, and they are thought to project mainly to the thalamus (Witter & Amaral, 2004).

The microcircuitry of the LEC is clearly complex, and despite a relatively rich neuroanatomical description of its laminar and input-output organization, the functional relevance of much of its circuitry remains poorly understood.

Dopamine in the Entorhinal Cortex

Our current understanding of the basis of learning and memory highlights the importance of changes in synaptic strength as a means through which the nervous system forms, stores, and retrieves memories. It is well established that changes in synaptic strength are modulated by an array of neurotransmitters. Although the major sensory and associational inputs to the superficial layers of the LEC are glutamatergic and GABAergic, the LEC also receives multiple inputs from neuromodulatory projections, including cholinergic (Mitchell et al., 1982), serotonergic (Köhler et al., 1980), noradrenergic (Palkovits et al., 1979), and dopaminergic inputs (Mingote et al., 2015). These inputs are therefore likely to modulate how LEC neurons process and transmit information carried by excitatory glutamatergic synaptic transmission. To understand how the entorhinal cortex contributes to sensory and mnemonic processing, we must therefore consider how LEC cellular activity and synaptic transmission is modulated by these neurotransmitters.

One of the main neuromodulatory inputs to the superficial layers of the LEC originates from midbrain dopamine neurons (Descarries et al., 1987). Considerable evidence points to the contribution of the mesolimbic dopamine system in various aspects of cognition, particularly in a diverse range of

appetitive and motivation-driven behaviors, as well as reward, addiction, stress, and learning and memory (Berridge, 2007; Hyman et al., 2006; Iversen & Iversen, 2007; Schultz, 2007; Seamans & Yang, 2004). Age-related cognitive deficits have been correlated with dysfunction in dopamine signalling in rats (Lee, Ross, et al., 1994) and non-human primates (Murphy et al., 1996), and disorders characterized by dysfunction in learning and memory such as Alzheimer's disease have also been linked to dopaminergic dysfunction (Gómez-Isla et al., 1996; De Keyser, Ebinger, & Vauquelin, 1990; Hyman, Van Hoesen, & Damasio, 1991). Interestingly, dopaminergic projections originating in the ventral tegmental area (VTA) and substantia nigra, both of which project to the LEC, appear to be the most heavily involved in cognitive and mnemonic processes (Oades & Halliday, 1987). These findings suggest an important role for dopamine in learning and memory functions, and suggest that dopaminergic modulation of glutamatergic synaptic transmission may contribute significantly to learning and memory function within the LEC.

The entorhinal cortex is one of four major cortical targets of midbrain dopamine neurons (Bjorklund & Lindvall, 1984; Fallon & Loughlin, 1987; Oades & Halliday, 1987), with early studies showing that the majority of dopaminergic inputs to the EC originate in the ventral tegmental area (VTA, A10; Collier & Routtenberg, 1977). Studies demonstrated that not only does lesioning of the VTA significantly reduce dopamine levels in the EC (Fallon, Koziell, & Moore, 1978), but that injections of the retrograde tracer horseradish peroxidase into the LEC results in labeling of VTA cells (Beckstead, 1978; Beckstead, Domesick, & Nauta, 1979). Subsequent studies indicated that the superficial layers of the LEC are also innervated by dopaminergic inputs from the substantia nigra, although to a lesser degree (Haglund, Köhler, Ross, & Kelder, 1979; Fallon & Loughlin, 1987; Oades & Halliday, 1987; Akil & Lewis, 1993). This pattern of dopaminergic innervation of the entorhinal cortex appears to be consistent across mammalian species, as it is similar in humans (Akil & Lewis, 1994), monkeys (Akil & Lewis, 1993), and rodents (Haglund, Köhler, Ross, & Kelder, 1979; Fallon & Loughlin, 1987; Oades & Halliday, 1987). Early anatomical studies in the rat demonstrated that the most robust dopaminergic projections to the cortex terminate in the deep layers of the frontal cortices, and in the superficial layers of the entorhinal cortex (Lindvall, Björklund, Moore, & Stenevi, 1974). Although present in the MEC, dopamine fibers appear denser in the LEC, where they target principal neurons more specifically (Fallon & Loughlin, 1987; Fallon, Koziell, & Moore, 1978) and make synaptic connections with dendrites of layer II cells (Collier & Routtenberg, 1977).

Dopaminergic receptors are commonly classified into two subfamilies of pharmacologically distinct receptors, namely D_1-like (D_1, D_5) receptors, traditionally linked to activation of adenylyl cyclase and cyclic AMP production, and D_2-like (D_2, D_3, D_4) family linked to inhibition of adenylyl cyclase (Missale et al., 1998). Although dopamine receptors are differentially expressed in several brain regions, ligand-binding studies (Reader et al., 1988; Savasta, Dubois, & Scatton, 1986), as well as mRNA probing (Weiner et al., 1991; Weiner & Brann, 1989) demonstrate the presence of moderate concentrations of both receptor subtypes in the superficial layers of the entorhinal cortex (Richfield et al., 1989), with D_1-like receptors appearing in higher density than D_2-like receptors (Diop et al., 1988; Reader et al., 1988). Interestingly, D_1-like and D_2-like receptors in the entorhinal cortex appear to be differentially distributed in the superficial

layers of the entorhinal cortex, with D_1-like receptors being predominantly restricted to layers II, V and VI (Huang et al., 1992; Weiner et al., 1991; Köhler et al., 1991b; Richfield, Young, & Penney, 1989), whereas D_2-like receptors are concentrated in layers I and III (Goldsmith & Joyce, 1996; Köhler et al., 1991; Köhler, Ericson, & Radesäter, 1991).

The Role of Dopamine in Learning and Memory Processes

Working memory can be defined as the ability to temporarily maintain a representation of information for further processing in order to perform a task (Baddeley, 1992), and is widely assumed to be essential for encoding and storage of longer-term memory traces (Goldman-Rakic, 1996). Dopamine modulation of prefrontal cortex activity is especially important in working memory. Several studies have noted elevations in dopamine levels in the PFC, an area involved in learning and memory processes, when rodents and non-human primates are engaged in working memory tasks (Phillips, Ahn, & Floresco, 2004; Watanabe, Kodama, & Hikosake, 1997). In contrast, depletion of dopamine in this area produces profound deficits in working memory (Brozoski et al., 1979; Simon, Scatton, & Le Moal, 1980; Bubser & Schmidt, 1990), which can be ameliorated by dopamine receptor agonists (Brozoski et al., 1979). Interestingly, the observation that dopamine contributes to memory processes is consistent across species, including rodents (Sakurai & Sugimoto, 1985), non-human primates (Passingham, 1975), and humans (Müller, von Cramon, & Pollmann, 1998), indicating that dopamine is likely crucial to normal memory function.

Dopamine typically sustains working memory by facilitating synaptic transmission (Sawaguchi & Goldman-Rakic, 1991), spontaneous activity, and firing rates of neurons (Sawaguchi, Matsumure, & Kubota, 1990; Collins et al., 1998; Seamans, Floresco, & Phillips, 1998). In the prefrontal cortex, performance on working memory tasks is linked to dopamine D_1-like receptor activation in a dose-dependent manner (Vijayraghavan et al., 2007; Seamans, Gorelova, Durstewitz, & Yang, 2001). In both rats and monkeys, D_1-like receptor activation produces an inverted U-shaped response, wherein too little (Kozlov et al., 2001; Sawaguchi & Goldman-Rakic, 1991, Seamans et al., 1998) or too much (Arnsten & Goldman-Rakic, 1998; Zahrt et al., 1997), suggesting that working memory processes require a critical range of D_1-like-receptor activation, above or below which memory impairment occurs. Optimal D_1-like receptor activation likely contributes to memory processes in part by enhancing excitatory synaptic transmission meditated by glutamate (Sawaguchi & Goldman-Rakic, 1991; Funahashi, Bruce & Goldman-Rakic, 1993; Puig & Miller, 2012). It has been previously shown that dopamine, binding to D_1-like receptors, can enhance isolated AMPA-receptor currents in layers II and III of the prefrontal cortex (Gonzalez-Islas & Hablitz, 2003; Bandyopadhyay, Gonzalez-Islas, & Hablitz, 2005) and isolated NMDA-receptor currents in layer V of the prefrontal cortex (Wang & O'Donnell, 2001), likely by increasing phosphorylation of the receptors (Snyder et al., 2000).

As previously noted, similarly to the prefrontal cortex, the entorhinal cortex appears to be an area involved in working memory (Ramus & Eichenbaum, 2000; Young et al., 1997), which receives strong dopaminergic innervation. This raises the important probability that dopamine may act in both the entorhinal cortex and PFC via similar cellular signalling mechanisms to enhance different aspects of

working memory. The following section provides a short summary of dopamine functions and underlying intracellular signalling cascades.

Dopamine Signalling

The neurophysiological effects of dopamine are mediated via the interaction of dopamine receptors with their associated G-proteins. Research by Greengard and colleagues greatly advanced our understanding of the intracellular signalling cascades triggered by dopamine-receptor binding, and how dopamine modulates excitatory synaptic transmission (e.g. Greengard, 2001 for a review). We now know that D_1-like and D_2-like receptors are coupled to different G-proteins that have distinct intracellular and electrophysiological effects (Cooper, Bloom, & Roth, 1991), and that the two receptor subtypes can activate or inhibit synaptic transmission, therefore serving different modulatory functions. Dopamine D_1-like receptors, which have been shown to be critically involved in working memory in both young and old animals (Floresco & Phillips, 2001; Sawaguchi & Goldman-Rakic, 1991), are expressed in numerous brain regions, including the cortex, hippocampus, amygdala, striatum, olfactory bulb, substantia nigra, and entorhinal cortex (Levey et al., 1993). D_1-like receptors activate $G_{s/olf}$ proteins which activate the enzyme adenylyl cyclase (AC), whose activity results in the production of cyclic adenosine 3',5'-monophosphate activity (cAMP), which, in turn, activates protein kinase A (PKA). This signaling pathway has been previously shown to be necessary for learning and memory processes, including working memory, spatial memory, instrumental learning, and conditioned fear responses, in a variety of regions (Aujla & Beninger, 2001; Baldwin et al., 2002; Berke & Hyman, 2000; Gurden, Takita, & Jay, 2000). In the amygdala, enhancing PKA activity enhances reward-dependent learning (Jentsch et al., 2002), whereas inhibiting PKA activity impairs learning (Baldwin et al., 2002). In the hippocampus, activation of the D_1-cAMP-PKA pathway has been linked to memory consolidation (Bernabeu et al., 1997). Several mechanisms of action have been suggested to underlie this, including phosphorylation of glutamate receptors by various protein kinases, such as protein kinase A and protein kinase C.

In the PFC, dopamine appears to regulate NMDA glutamate receptor-mediated currents via D_1-like-receptor activation. In vitro recordings performed in layer V prefrontal cortex neurons suggest that D_1-like receptor activation enhances NMDA-receptor currents via the classical cAMP-PKA pathway and calcium-dependent mechanisms (Wang & O'Donnell, 2001). Additionally, increases in intracellular Ca^{2+} have been linked to D_1-like receptor and dopaminergic facilitation of synaptic transmission in numerous brain regions, including the striatum (Flores-Hernandez et al., 2002), the neocortex (Cepeda et al., 1992), the prefrontal cortex (Tseng & O'Donnell, 2004) and the hippocampus (Yang, 2000). D_1-like receptor activation can also enhance glutamate-receptor currents via a mechanism linked to the activity of protein kinase C (Young & Yang, 2004; Mammen et al., 1997; Flores-Hernandez et al., 2002). The differences in intracellular pathways involved may be cell-type and location dependent. For example, it was previously observed that D_1-like receptors facilitate glutamatergic responses in dorsal striatal neurons via a mechanism dependent on PKA (Umemiya & Raymond, 1997), whereas in ventral striatal neurons, the facilitation is mediated by PKC (Chergui & Lacey, 1999), suggesting that D_1-like receptor activation can lead to facilitation of synaptic responses via different pathways in the two regions.

Stimulation of D_1-like receptors and subsequent PKA activation leads to phosphorylation of DARPP-32 and inhibitor 1, which are potent inhibitors of protein phosphatase 1 (PP-1; Snyder et al., 1998; Hemmings, Williams, Konigsberg, & Greengard, 1984; Yan et al., 1999). At basal levels, PP-1 dephosphorylates AMPA and NMDA-receptors in numerous brain regions (Snyder, Fienberg, Huganir, & Greengard, 1998; Kemp & Bashir, 2001; Malenka & Bear, 2004). Therefore, inhibition of PP-1 can promote phosphorylation of glutamatergic receptors, resulting in an increase in synaptic transmission, and there is also evidence that activation of this pathway can contribute to expression of long-term synaptic potentiation which is a well-studied cellular model of long-term memory (Centonze et al., 2001; Bourtchouladze et al., 1998).

Recently, it has been demonstrated that activation of a type of D_1-like receptor, the phosphatydilionositol-linked D_1-like (i.e, PI-linked) receptor (Panchalingam & Undie, 2001; Lezcano & Bergson, 2002), or activation of heterooligomeric D_1-like and D_2-like receptors (Hasbi, O'Dowd, & George, 2010), can lead to activation of phospholipase C (PLC) and resulting hydrolysis of the membrane lipid phosphoinositide 4,5 bisphosphate (PIP_2). This leads to generation of diacylglycerol (DAG) and release of inositol trisphosphate (IP_3) into the cytosol. IP_3 binds to IP_3 receptors (IP_3R) on the endoplasmic reticulum, which serves as an internal store of calcium, resulting in calcium release from internal stores, thereby increasing intracellular calcium concentrations (Lee, So, et al., 2004). This signalling cascade, arising from activation of PLC, could lead to phosphorylation of glutamatergic receptors via calcium-activated kinases such as PKC and CaMKII.

The role of dopaminergic modulation of glutamatergic transmission in the entorhinal cortex has been previously investigated only in a few reports, and its cellular mechanisms remained poorly understood. Previous experimental studies found that high doses of dopamine suppress the amplitude of glutamate-mediated synaptic responses in superficial layers II and III, and in deep layer V of the medial entorhinal cortex (Pralong & Jones, 1993; Stenkamp, Heinemann, & Schmitz, 1998). In the lateral entorhinal cortex, similar to findings in the prefrontal cortex (e.g. Seamans et al., 2001), dopamine exerted bidirectional effects, wherein dopamine can either suppress or facilitate synaptic transmission in a concentration-dependent manner (Caruana, Sorge, Stewart, & Chapman, 2006). Application of a high concentration of dopamine (50 or 100 μM) resulted in a strong suppression of glutamatergic synaptic transmission via activation of D_2-like receptors (Caruana et al., 2006), whereas application of a lower dose of dopamine (10 μM) resulted in a D_1-like-receptor-mediated facilitation of synaptic responses (Caruana et al., 2006). These findings indicate that dopamine, by affecting the strength of sensory and associational synaptic inputs to the superficial layers of the entorhinal cortex, may have powerful modulatory effects on cognitive functions mediated by the entorhinal cortex during learning and memory processes.

However, the exact cellular mechanisms of action through which dopamine regulates the functioning of the lateral entorhinal cortex were not fully examined in the above studies.

Summary of Experimental Chapters

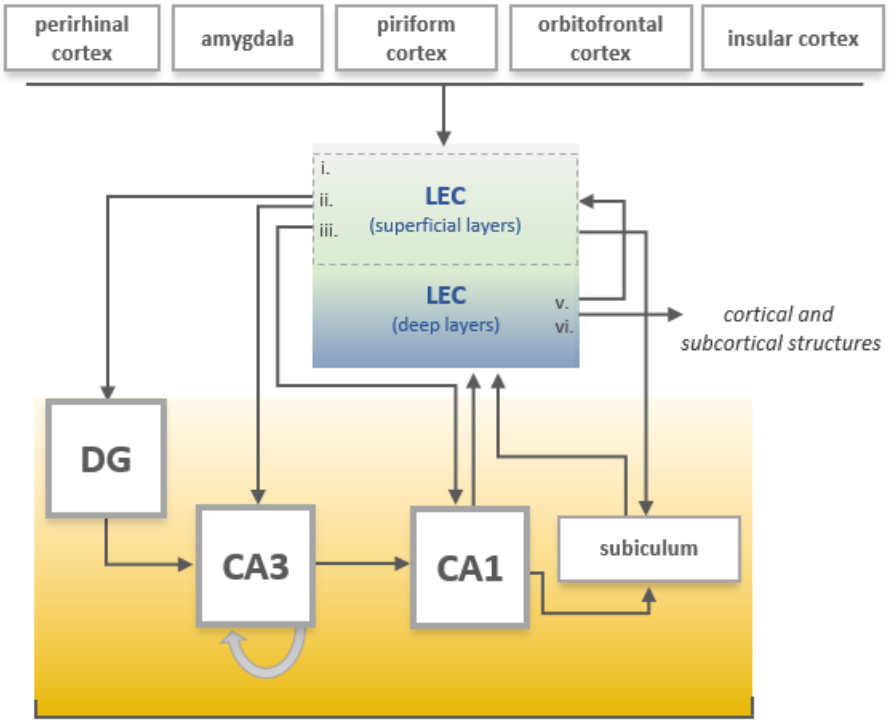
The goal of this thesis was to investigate the cellular mechanisms underlying the previously-observed facilitation of glutamatergic synaptic transmission by dopamine in layer II neurons of the lateral entorhinal cortex. Initial experiments, described in the second chapter (Glovaci, Caruana, & Chapman, 2014), used whole-cell patch clamp recordings to assess how dopamine, via binding to D_1-like versus D_2-like receptors leads to facilitation of synaptic transmission in different cell types of layer II LEC neurons. It was found that dopamine binding to D_1-like receptors induces a facilitation of AMPA-receptor-mediated, but not NMDA-receptor-mediated, synaptic currents via a signalling cascade that relies upon activation of the classical cAMP-PKA-PP-1 pathway, as observed in several other cortical regions such as the hippocampus and prefrontal cortex (Bernabeu et al., 1997; Gonzalez-Islas & Hablitz, 2003). Furthermore, this facilitation of responses was found to be dependent on the type of layer II neuron recorded from. Recordings obtained from fan and pyramidal cells, which comprise the majority of principal neurons in layer II of the LEC, demonstrated that dopamine primarily facilitates responses in fan cells in a reliable and consistent manner. In contrast, dopamine induced variable responses in pyramidal cells, wherein some cells showed a facilitation of synaptic responses in the presence of dopamine, and others did not. In contrast to the facilitation of excitatory synaptic transmission observed, inhibitory synaptic transmission was not significantly modulated by dopamine. The differing responses of LEC layer II cell types to application of dopamine suggests that dopamine may also differently modulate the manner in which they integrate sensory inputs during sensory and mnemonic tasks associated with activation of dopamine neurons, and points to the likelihood of different functional roles in sensory and mnemonic processing.

Chapter three of this thesis further assessed the intracellular signalling pathways associated with the dopaminergic facilitation of synaptic transmission in layer II LEC neurons (Glovaci & Chapman, 2015). Findings in other cortical areas suggested that, in addition to the classical cAMP-PKA pathway, binding to D_1-like receptors leads to synaptic facilitation via a mechanism that is dependent on intracellular calcium; this dependence on calcium was observed in several brain regions including the striatum (Flores-Hernandez et al., 2002), neocortex (Cepeda et al., 1992), prefrontal cortex (Tseng & O'Donnell, 2004) and hippocampus (Yang, 2000). This suggested that calcium may also be involved in the dopaminergic facilitation of glutamatergic synaptic transmission in LEC neurons. Further, since previous studies demonstrated that dopamine may facilitate synaptic transmission via a protein kinase C (PKC) dependent mechanism in the nucleus accumbens (Chergui & Lacey, 1999) and the hippocampus (Wang, Dudek, Browning, & MacDonald, 1994), we wanted to investigate whether PKC activity may play a role in the dopaminergic facilitation of responses in the LEC. The findings contained in Chapter 3 indicate that, in layer II LEC neurons, binding of dopamine to D_1-like receptors activates a secondary intracellular cascade, in addition to the classical cAMP-PKA pathway. This cascade is initiated by activation of PI-linked D_1-like which couple to G_q proteins, that upon activation, promote PLC-induced hydrolysis of PIP_2, yielding an increased production of the hydrophobic DAG and hyprophilic IP_3 (Rashid et al., 2007). In turn, increases in IP_3 can lead to increased intracellular calcium concentrations via binding of IP_3 receptors on the surface of organelles that store intracellular calcium (Cools et al., 2002, Jin et al., 2003; Zhen et al.,

2005). The IP_3-dependent release of calcium can also lead to subsequent calcium-dependent calcium-release that can elevate intracellular calcium concentrations further, via activation of ryanodine receptors. Experiments presented here demonstrate that blocking either PLC, IP_3 receptors, ryanodine receptors, intracellular calcium, or the calcium-activated protein kinase C, blocks the dopaminergic facilitation of synaptic response. This was the first demonstration of the existence of such a parallel signalling cascade involving PLC in layer II entorhinal neurons. The existence of this secondary signalling cascade suggests that dopamine-dependent signalling within layer II LEC neurons is complex, and that the resulting modulation of synaptic transmission can depend on cooperative interactions between the cAMP-PKA and PI-linked pathways.

The fourth chapter of this thesis directly investigated the nature of transient increases in intracellular calcium concentrations triggered by activation of D_1-like receptors in layer II fan and pyramidal cells. Although we previously demonstrated that calcium was essential for the dopaminergic facilitation of glutamatergic synaptic transmission in LEC neurons, the scale and time course of these transients remained unknown. Pairing single-cell, voltage-clamp recordings with fluorescence imaging of intracellular calcium concentration using the indicator fluo-4, we were able to demonstrate that binding to PI-linked D_1-like receptors, but not classical D_1-like receptors, leads to significant and reversible transient increases in calcium via release of calcium from internal stores in layer II fan cells. The transient increases in intracellular calcium induced by dopamine were more prominent in fan cells versus pyramidal cells, and this matches the finding of more reliable dopamine-induced facilitation of synaptic responses in fan cells observed in the previous Chapter, and further highlights a difference in processing in different cell types of the LEC.

Together, the studies within this thesis provide novel insights into the complex intracellular mechanisms that mediate the facilitation of glutamatergic synaptic transmission by dopamine in layer II neurons of the lateral entorhnial cortex. These results provide a solid foundation for additional studies needed to further clarify these intracellular mechanisms, and the results also point to the potential importance of differences in the neuromodulatory effects of dopamine across the different cell types of the LEC. The precise functional role of dopamine in modulating sensory and mnemonic processes within the lateral entorhinal cortex remains unknown, but the results of this thesis strongly suggest that dopamine has an important role in modulating the strength or salience of sensory and associational inputs to the entorhinal cortex under physiologically significant conditions associated with activation of midbrain dopamine neurons.



Hippocampal formation

Figure 1.1. Simplified schematic showing the major connections of the superficial layers of the LEC in the rat. The entorhinal-hippocampal areas form a loop through which multimodal sensory information enters the hippocampal formation via the superficial layers of the LEC, and returns via hippocampal projection to the deep layers of the LEC. DG: dentate gyrus; CA: Cornu Ammonis.

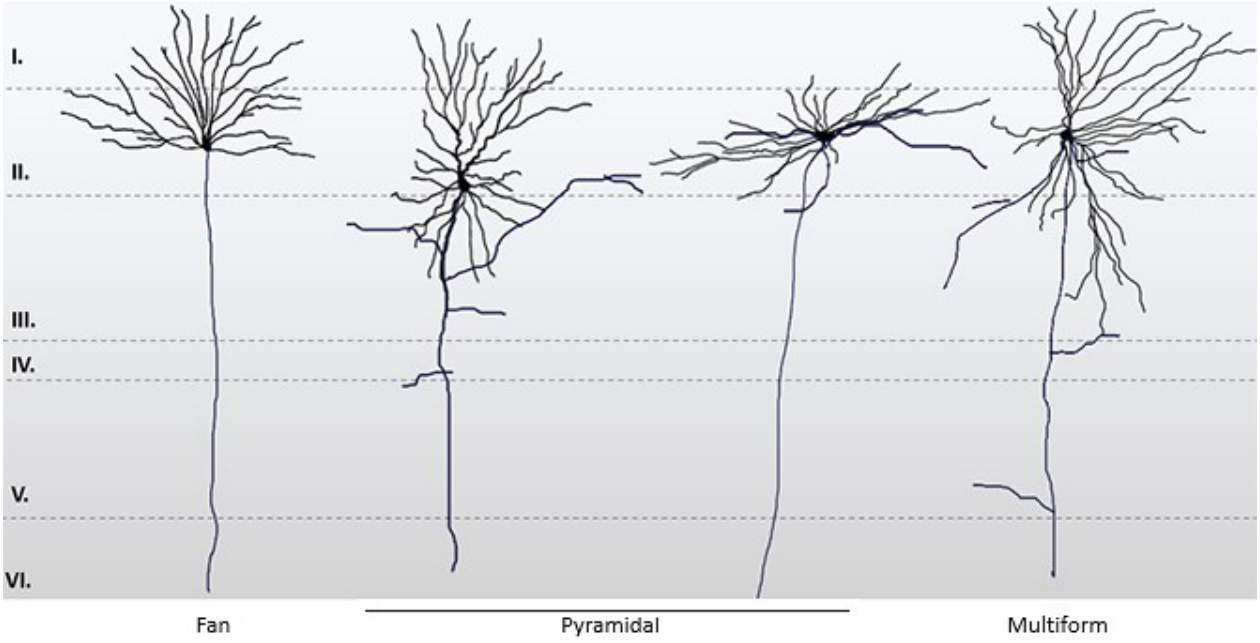

I.
II.
III.
IV.
V.
VI.
Fan
Pyramidal
Multiform

Figure 1.2. Approximate drawing of general dendritic and axonal morphology of LEC layer II principal cells; drawing based on tracing studies by Tahvildari and Alonso (2005), and Canto and Witter (2011).

CHAPTER 2

DOPAMINERGIC ENHANCEMENT OF EXCITATORY SYNAPTIC TRANSMISSION IN LAYER II ENTORHINAL NEURONS IS DEPENDENT ON D1-LIKE RECEPTOR-MEDIATED SIGNALING

ABSTRACT

The modulatory neurotransmitter dopamine induces concentration-dependent changes in synaptic transmission in the entorhinal cortex, in which high concentrations of dopamine suppress evoked excitatory postsynaptic potentials (EPSPs) and lower concentrations induce an acute synaptic facilitation. Whole-cell current-clamp recordings were used to investigate the dopaminergic facilitation of synaptic responses in layer II neurons of the rat lateral entorhinal cortex. A constant bath application of 1 μM dopamine resulted in a consistent facilitation of EPSPs evoked in layer II fan cells by layer I stimulation; the size of the facilitation was more variable in pyramidal neurons, and synaptic responses in a small group of multiform neurons were not modulated by dopamine. Isolated inhibitory synaptic responses were not affected by dopamine, and the facilitation of EPSPs was not associated with a change in paired-pulse facilitation ratio. Voltage-clamp recordings of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) glutamate receptor-mediated excitatory postsynaptic currents (EPSCs) were facilitated by dopamine, but *N*-methyl-d-aspartate receptor-mediated currents were not. Bath application of the dopamine D_1-like receptor blocker SCH23390 (50 μM), but not the D_2-like receptor blocker sulpiride (50 μM), prevented the facilitation, indicating that it is dependent upon D_1-like receptor activation. Dopamine D_1-like receptors lead to activation of protein kinase A (PKA), and including the PKA inhibitor H-89 or KT 5720 in the recording pipette solution prevented the facilitation of EPSCs. PKA-dependent phosphorylation of inhibitor 1 or the dopamine- and cAMP-regulated protein phosphatase (DARPP-32) can lead to a facilitation of AMPA receptor responses by inhibiting the activity of protein phosphatase 1 (PP-1) that reduces dephosphorylation of AMPA receptors, and we found here that inhibition of PP-1 occluded the facilitatory effect of dopamine. The dopamine-induced facilitation of AMPA receptor-mediated synaptic responses in layer II neurons of the lateral entorhinal cortex is therefore likely mediated via a D_1-like receptor-dependent increase in PKA activity and a resulting inhibition in PP-1-dependent dephosphorylation of AMPA receptors.

The superficial layers of the entorhinal cortex receive widespread, converging inputs from sensory and associational cortical areas, and neurons in layers II and III provide the hippocampal region with most of its cortical sensory input (Burwell, 2000). The medial entorhinal area receives large inputs from visual and posterior parietal cortices, and the lateral entorhinal area receives strong inputs from piriform and insular cortices (Kerr et al., 2007); these patterns of connectivity are consistent with a greater involvement of the medial entorhinal cortex in spatial processing mediated in part by "grid" cells (Derdikman & Moser, 2010), and a greater involvement of the lateral entorhinal cortex with non-spatial processing and olfaction (Lavenex & Amaral, 2000; Petrulis et al., 2005). In addition, the entorhinal cortex, with the prefrontal, cingulate and suprarhinal cortices, is one of four major cortical regions that are targeted by midbrain dopamine neurons (Björklund & Dunnett, 2007a) with the strongest inputs to the cell islands in layers II and III of the lateral entorhinal cortex (Bjorklund & Lindvall, 1984; Loughlin & Fallon, 1984; Oads & Halliday, 1987; Akil & Lewis, 1994; Erickson et al., 1998; Björklund & Dunnett, 2007). Dopamine is known to play important roles in learning and motivated behaviors (Berridge, 2007), and the strong dopaminergic input to the entorhinal cortex suggests that dopamine modulates the sensory and mnemonic functions of the medial temporal lobe by affecting neuronal firing and synaptic processing of neurons in the superficial layers of the entorhinal cortex (Lavenex & Amaral, 2000; Liu et al., 2000; Clark et al., 2012).

In the prefrontal cortex, the effects of dopamine on neuronal function are dependent on the extent of D_1-like and D_2-like receptor activation, and working memory function is enhanced by moderate D_1-like receptor activation (Goldman-Rakic et al., 2000; Seamans & Yang, 2004; Vijayraghavan et al., 2007; Puig & Miller, 2012). Effects of dopamine on excitatory synaptic transmission in the entorhinal cortex are also dependent upon the concentration of dopamine applied; high concentrations of dopamine (50–100 μM) result in a suppression of glutamate-mediated synaptic responses in layers II, III and V of the entorhinal cortex (Pralong &Jones, 1993; Stenkamp et al., 1998; Caruana & Chapman, 2008) and the suppression in layer II neurons is mediated largely by a D_2-like receptor-dependent suppression of transmitter release (Stenkamp et al., 1998; Caruana & Chapman, 2008). Lower concentrations of 1–10 μM dopamine induce a D_1-like receptor-mediated *facilitation* of synaptic responses (Caruana et al., 2006). This facilitation may enhance the processing of reward-relevant stimuli in the lateral entorhinal cortex, but the intracellular signaling mechanisms that mediate it are not clear.

Dopamine also has bidirectional effects on excitatory synaptic transmission in the prefrontal cortex and striatum and, while dopamine can lead to reduced excitatory responses via an inhibition of transmitter release (Law-Tho et al., 1994; Gao et al., 2001; Seamans & Yang, 2004; Tritsch & Sabatini, 2012), D_1-like receptor activation can also enhance α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and *N*-methyl-d-aspartate (NMDA)-mediated synaptic responses through postsynaptic mechanisms (Zheng et al., 1999; Seamans et al., 2001; Gonzalez-Islas & Hablitz, 2003; André et al., 2010; Chen et al., 2012; see also Yang, 2000). Enhancement of AMPA and NMDA currents in layer II and III neurons in the prefrontal cortex is induced by D_1-like receptor-mediated increases in cAMP and protein kinase A (PKA) (Gonzalez-Islas & Hablitz, 2003; Bandyopadhyay et al., 2005). PKA can facilitate synaptic transmission by catalyzing phosphorylation of glutamate receptors (Snyder et al., 2000; Sun et al., 2005). In addition, PKA

can also result in the inhibition of protein phosphatase 1 (PP-1) through activation of either inhibitor 1 (Huang et al., 2010) or the dopamine- and cAMP-regulated phosphoprotein (DARPP-32; Nishi et al., 2000); because PP-1 catalyzes the dephosphorylation of AMPA receptor subunits leading to a reduction in synaptic responses, the PKA-dependent inhibition of PP-1 can enhance synaptic responses (Yan et al., 1999; Neve et al., 2004).

Here, we have used whole-cell recordings in acute brain slices to characterize signaling mechanisms that mediate the dopaminergic facilitation of excitatory synaptic responses in neurons in layer II of the lateral entorhinal cortex. Recordings were obtained from electrophysiologically identified fan, pyramidal, and multiform cells (Tahvildari & Alonso, 2005; Canto & Witter, 2012) and effects of dopamine on isolated AMPA and NMDA receptor-mediated synaptic currents were assessed. The contributions to the dopamine-dependent facilitation of synaptic responses of D_1-like and D_2-like receptor subtypes, PKA signaling, and PP-1 were also assessed pharmacologically.

METHOD

In Vitro Slice Preparation

Preparation of brain slices for whole-cell recordings was conducted in accordance with guidelines of the Canadian Council on Animal Care. Acute brain slices were collected from 4- to 7-week-old male Long–Evans rats (Charles River). Animals were deeply anesthetized with halothane prior to decapitation, and brains were quickly extracted and submerged in ice-cold, high-sucrose artificial cerebrospinal fluid solution (ACSF; saturated with 95% $O_2$and 5% CO_2, pH 7.4) containing (in mM) 250 sucrose, 2 KCl, 1.25 NaH_2PO_4, 7 $MgCl_2$, 26 $NaHCO_3$, 0.5 $CaCl_2$ and 10 dextrose. All drugs were obtained from Sigma–Aldrich, MO, USA, unless indicated otherwise. Horizontal slices containing the entorhinal region were obtained using a vibratome (300 μM thick; WPI, Vibroslice, Sarasota, USA) and allowed to recover for at least one hour in normal ACSF containing 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 dextrose (pH ≈7.3; 300–310 mOsm; ~22 °C). Individual slices were submerged in ASCF (2 ml/min) using a nylon net and visualized using an upright microscope (Leica, DM-LFS) equipped with a 40x objective and differential interference contrast optics. Layer II of the lateral entorhinal cortex was identified based on the presence of cell ‘islands’ (Burwell, 2000).

Stimulation and Recording

Recording pipettes used for whole-cell recordings were pulled from borosilicate glass (1.0 mm OD, 3–6 MΩ) and filled with a solution containing (in mM) 140 K-gluconate, 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.5 EGTA, 2 ATP-Tris, and 0.4 GTP-Tris (pH adjusted to 7.2–7.3 with KOH). After forming a tight seal between the pipette and soma (1–2 GΩ) using gentle negative pressure, stronger pressure was used to obtain whole-cell configuration, and cells were allowed to stabilize for 3–5 min prior to current-clamp experiments, and 10–15 min prior to voltage-clamp experiments. Synaptic responses were evoked using a bipolar stimulating electrode made from two tungsten electrodes (~1 MΩ, FHC Inc., ME, USA) placed in layer I of the entorhinal cortex about .2–.4 mm rostral to the recording electrode. Synaptic responses were

evoked by 0.1-ms-duration constant current pulses delivered using a stimulus timer and isolation unit (WPI, Models A300 and A360). Current- and voltage-clamp recordings were obtained using an Axopatch 200B amplifier, filtered at 10 kHz, and digitized at 20 kHz (Axon Instruments, CA, USA, Digidata 1322A).

Electrophysiological characteristics of entorhinal neurons were analyzed using the Clampfit 8.2 software package (Axon Instr.). Series resistance was estimated in current-clamp recordings by compensating for the discontinuity in the voltage response to −50-pA current pulses, and recordings were accepted if series resistance was <20 MΩ (14.3 ± 1.3 MΩ). Input resistance was monitored regularly, and was determined from the peak voltage response to a 500-ms, −100-pA current pulse from a holding level of −60 mV. Membrane potential responses to 500-ms-duration current pulses (range −200 to +60 pA) were used to characterize input resistances and firing properties of neurons. Inward rectification was quantified as the ratio between peak and steady-state input resistances in response to a −200-pA hyperpolarizing current pulse (rectification ratio). In voltage-clamp recordings, series resistance was monitored from the transient response at the onset of 100-ms 3-mV voltage steps (21.1 ± 1.7 MΩ), and input resistance was measured based on the current response at the end of the voltage steps. Recordings were discontinued if either value changed by >15%. Spike properties were measured from the first action potential evoked in response to a 500-ms-duration positive current injection. Action potential amplitude was calculated from resting membrane potential, and action potential duration and afterhyperpolarization were measured from action potential threshold. Neurons in the lateral entorhinal cortex were classified based on their electrophysiological profiles as fan, pyramidal, or multiform cells (Tahvildari & Alonso, 2005); fan cells display a sag in the voltage response to strong hyperpolarizing current injection, and fire action potentials in intermittent clusters that are paced by theta-frequency membrane potential oscillations, while pyramidal neurons show a regular firing pattern and no sag response. Multiform neurons show a mix of properties of fan and pyramidal neurons.

Synaptic Potentials and Currents

The effect of dopamine on excitatory postsynaptic potentials (EPSPs) was assessed by comparing EPSPs recorded in normal ACSF to those recorded following addition of 1 μM dopamine for 5 min, and following 20-min washout of dopamine. Dopamine HCl was dissolved just prior to bath application, and sodium metabisulfite was added to slow the degradation of dopamine (50 μM; Yang & Seamans, 1996; Stenkamp et al., 1998). Pairs of stimulation pulses (30-ms interpulse interval), were used to evoke EPSPs once every 15 s, and 5 to 10 responses were averaged under each recording condition. The effect of dopamine on mixed IPSPs was also assessed during a constant bath application of either kynurenic acid (1 mM) or a combined application of 7-nitro-2,3-dioxo-1,4-dihydroquinoxaline-6-carbonitrile (CNQX, 50 μM) and (2R)-amino-5-phosphonopentanoate (APV, 50 μM). Drugs were stored as concentrated stock solutions at −20 °C.

Effects of dopamine on isolated AMPA and NMDA glutamate receptor-mediated excitatory postsynaptic currents (EPSCs) were also assessed. AMPA-receptor-mediated responses were isolated by either recording EPSCs at a holding potential of −70 mV to reduce the contribution of NMDA receptor-mediated currents, or by recording EPSCs in the presence of the NMDA receptor blocker APV (50 μM).

Ten EPSCs were evoked during baseline recordings in normal ACSF, after a 5-min application of dopamine, and following a 20-min wash in normal ACSF. NMDA receptor-mediated EPSCs were not reliably obtained at depolarized potentials in the presence of CNQX with the regular intracellular recording solution, and so NMDA receptor-mediated currents were assessed using a Cs^+-based intracellular solution at a holding potential of +40 mV in the presence of CNQX (50 μM) and picrotoxin (20 μM) (135 CsCl, 5 Na, 0.5 EGTA, 10 HEPES, 2 $MgCl_2$, 10 HEPES, 2 ATP-Tris, and 0.4 GTP-Tris; pH adjusted to 7.2–7.3 with CsOH).

The dependence of the dopaminergic facilitation of EPSCs on D_1- vs. D_2-like receptors was assessed using a constant bath application of selective dopamine receptor antagonists. Following a baseline period in normal ACSF, responses were recorded in the presence of either the $D_{1/5}$ receptor antagonist SCH23390 (10 or 50 μM) or the D_2-like receptor antagonist sulpiride (50 μM; Caruana et al., 2006), and following application of 1 μM dopamine for 5 min. Sulpiride was dissolved daily in 6% DMSO in ACSF titrated with 0.1 N HCl and diluted to a final concentration of 50 μM in ACSF with 0.1% DMSO. We have previously found no effect on basal synaptic transmission of sulpiride in 0.1% DMSO in both field and intracellular recordings from lateral entorhinal cortex neurons (Caruana et al., 2006; Caruana & Chapman, 2008). The role of PKA was assessed by including the PKA inhibitors H-89 (10 μM; Ascent Scientific, Bristol, UK) or KT 5720 (1 μM; Tocris Bioscience, Bristol, UK) in the intracellular recording pipette solution. The role of PP-1 was assessed using the mixed PP-1/PP-2A inhibitor okadaic acid (10 μM) and the specific protein phosphatase 2A (PP-2A) inhibitor fostriecin (250 nM; Tocris Bioscience).

Changes in synaptic responses were analyzed using Clampfit 8.2 software (Axon Instr.). Ten consecutive synaptic responses were averaged for each phase of recordings and the amplitudes of averaged responses were measured relative to the pre-stimulus baseline. Paired-pulse facilitation ratio was determined by expressing the amplitude of the response to the second pulse as a percentage of the response to the first pulse. Changes in cellular properties and synaptic responses were analyzed using planned dependent samples *t*-tests to compare responses obtained during the baseline period with those obtained after dopamine application, and with those obtained after drug-washout. Data are presented as group means ± standard error of the mean.

RESULTS

Dopaminergic Effects on Cellular Properties and EPSPs

Effects of dopamine on cellular properties and evoked EPSPs were assessed in current-clamp recordings from 29 layer II lateral entorhinal cortex neurons. Neurons were classified as either fan cells, pyramidal cells, or multiform cells based on their electrophysiological properties (Tahvildari & Alonso, 2005; Figure 2.1A). Fan cells (n = 16) were characterized by a marked sag in the voltage response to strong depolarizing current injection and voltage-dependent theta-frequency membrane potential oscillations that paced the firing of action potentials. Pyramidal neurons fired more regularly, and showed no substantial sag response (n = 10). Three neurons that had intermediate properties, including a clustering discharge without a sag response, were classified as putative multiform neurons (n = 3).

Dopamine had similar effects on the electrophysiological properties of fan and pyramidal neurons except for a small, consistent reduction in medium afterhyperpolarization in fan cells (3.0 ± 0.5 vs. 3.6 ± 0.4 mV; $t = 8.20$, $p < 0.01$), that was not observed in pyramidal neurons ($t = 0.13$, $p = 0.89$). The reduced afterhyperpolarization in fan cells was not, however, associated with a significant change in the number of spikes evoked by a 500-ms-duration, 20-pA current pulse delivered at −60 mV (2.68 vs. 2.50, $t = 1.86$, $p = 0.083$). Other cellular properties, including action potential waveform, rectification ratio, and input resistance (105 ± 7 vs. 106 ± 8 MΩ, $t = 0.46$, $p = 0.65$) were not affected by dopamine (Caruana & Chapman, 2008).

Dopamine increased the amplitude of EPSPs in layer II entorhinal neurons evoked by stimulation of layer I. Among the 29 neurons recorded, dopamine increased EPSP amplitude to 124.4 ± 8.4% of baseline values (from 2.7 ± 0.4 mV to 3.4 ± 0.5 mV; $t = 4.18$, $p < 0.001$) and responses returned to baseline values after a 20-min wash in normal ACSF (99.6 ± 8.4%; $t = 0.351$, $p = 0.73$). The facilitation, however, was partly dependent on cell type (see responses to the initial pulses in Figure 2.1B, and mean amplitudes in Figure 2.1C). Fan cells showed a moderate and consistent facilitation of EPSP amplitude (117.9 ± 5.4% of baseline, $n = 16$; $t = 3.62$, $p = 0.002$) and pyramidal cells had more variable changes in EPSP amplitude, with a mean increase to 142.2 ± 22.3% of baseline levels, ($n = 10$; $t = 2.30$, $p = 0.037$). EPSPs recorded in the small group of multiform cells were not markedly modulated by dopamine ($n = 3$; 103.1 ± 4.3% of baseline), and multiform cells were not included in later experiments on the mechanisms of the facilitation.

The enhancement of EPSPs induced by dopamine in fan and pyramidal neurons was not associated with a change in paired-pulse facilitation ratio (pyramidal, 216 ± 36 vs. 235 ± 37%; fan, 172 ± 12 vs. 182 ± 12%) suggesting that the synaptic enhancement is expressed via postsynaptic mechanisms (Figure 2.1B). We also tested the possibility that reduced synaptic inhibition contributed to the enhancement of EPSPs (Seamans et al., 2001) by testing effects of dopamine on pharmacologically isolated inhibitory postsynaptic potentials (IPSPs) recorded by blocking AMPA and NMDA receptors with either CNQX and AP5 (20 and 50 μM; $n = 6$) or with kynurenic acid (1 mM; $n = 5$). Dopamine had no significant effect on either putative early $GABA_A$ ($n = 11$; 103.9 ± 15.1% of baseline; $t = -0.48$, $p = 0.64$) or late $GABA_B$ IPSPs (90.7 ± 8.1% of baseline; $t = 1.09$, $p = 0.30$; Figure 2.1D).

Effects on AMPA vs. NMDA-mediated EPSCs

Voltage-clamp recordings of EPSCs were used to investigate the transmitter receptors and postsynaptic signals involved in the dopamine-induced facilitation of synaptic responses. AMPA receptor-mediated EPSCs were first isolated by holding cells at −70 mV to reduce the contribution of NMDA receptors. Dopamine significantly increased the amplitude of EPSCs in these cells, including two cells recorded in the presence of picrotoxin to block $GABA_A$-mediated inputs (Figure 2.2A; $n = 7$, 6 fan, 1 pyramidal; 133.2 ± 5.9% of baseline; $t = 2.79$, $p = 0.031$). The facilitation of EPSCs in the presence of picrotoxin, and the lack of an effect of dopamine on isolated IPSPs (see Figure 2.1D) suggest that changes in inhibition did not contribute to the facilitation of AMPA responses. We also evaluated the effect of dopamine on AMPA EPSCs in the presence of the NMDA receptor blocker APV (50 μM). Application of

APV alone had no effect on the amplitude of EPSCs (Figure 2.2B; *n* = 5, 3 fan, 2 pyramidal; 107.5 ± 4.1% of baseline; *t* = 1.95, *p* = 0.124) suggesting only a minimal contribution of NMDA receptors to EPSCs. Addition of dopamine resulted in an increase in the peak amplitude of AMPA-mediated EPSCs to 135.5 ± 12.5% of baseline values (*t* = 3.40, *p* = 0.027) and responses returned to baseline during a 20-min washout period in APV (97.9 ± 6.7%; *t* = 0.05, *p* = 0.966). Some cells showed a broadening of the EPSC, but there was no consistent effect of dopamine on the amplitude of responses during the falling phase of EPSCs (92.8 ± 4.5% of baseline; *t* = 1.63, *p* = 0.18).

No clear NMDA receptor-mediated EPSC could be observed at holding potentials near rest (−53 to −65 mV) in 50 μM CNQX and 20 μM bicuculline (*n* = 5, data not shown), and we therefore used a Cs^+-based intracellular solution to record pharmacologically isolated NMDA receptor-mediated EPSCs at a holding potential of +40 mV in CNQX and bicuculline. Dopamine failed to modulate isolated NMDA-mediated EPSCs (Figure 2.2C; *n* = 4, 2 fan, 2 pyramidal; 97.1 ± 2.4% of baseline; *t* = 0.37, *p* = 0.74), indicating that effects on mixed EPSCs are mediated by a selective effect of dopamine on AMPA receptor-mediated currents.

Dopamine Receptors

The dopamine receptors that mediate the facilitation of EPSCs were investigated by applying dopamine in the presence of D_1-like or D_2-like receptor blockers. Application of either the D_2-like receptor antagonist sulpiride (50 μM) or the D_1-like receptor antagonist SCH23390 (10 or 50 μM; Kuzhikandathil & Oxford, 2002) alone had no significant effect on EPSC amplitudes (Figure 2.3; sulpiride: *t* = 1.41, *p* = .207; SCH23390, 50 μM: *t* = 0.06, p = 0.96; 10 μM: *t* = 1.76, *p* = 0.18) or other cellular properties measured. The dopamine-induced facilitation of EPSCs was not blocked by the presence of sulpiride (*n* = 7, 3 fan, 4 pyramidal; 153.0 ± 29.3% of baseline; *t* = 2.56, *p* < 0.05), but was completely blocked by either a low (10 μM) or high dose (50 μM) of SCH23390 (10 μM: 100.6 ± 2.3%; *n* = 4, 3 pyramidal, 1 fan; *t* = 2.07, *p* = 0.13; 50 μM: 97.2 ± 12.2%; *n* = 5, 4 fan, 1 pyramidal; *t* = 0.06, *p* = 0.955) indicating that activation of D_1-like receptors is required for the dopamine-mediated facilitation of EPSCs (Figure 2.3).

Intracellular Signaling

Activation of D_1-like receptors increases cAMP and PKA, and we therefore assessed the effect of including the PKA inhibitor H-89 (10 μM) in the recording pipette. The amplitude of EPSCs remained stable during dopamine application in H-89-filled neurons (102.5 ± 6.3% of baseline values; *n* = 6, 4 fan, 2 pyramidal; *t* = 0.27, *p* = 0.80; Fig. 4A1). A similar pattern of results was also obtained using the more specific PKA inhibitor KT 5720 (1 μM; 96.7 ± 2.9% of baseline values; *n* = 6, 2 fan, 4 pyramidal; *t* = 1.05, *p* = 0.34; Figure 2.4A_2) indicating that D_1-like receptor activation likely facilitates AMPA-mediated EPSCs through a signaling pathway involving PKA. PKA can modulate AMPA receptors through phosphorylation of inhibitor 1 (Huang et al., 2010) or the DARPP-32 (Nishi et al., 2000) which are inhibitors of PP-1. Here, we tested whether PP-1 is required for the facilitation of EPSCs by including the PP-1/PP-2A-inhibitor okadaic acid (10 μM) in the recording pipette to occlude effects of dopamine (Figure 2.4B_1). Baseline synaptic responses were not larger in cells filled with okadaic acid (77 ± 4 vs. 87 ± 19 pA in cells filled with either H-89, KT 5720 or fostriecin), but a lower stimulus intensity was required to evoke baseline

responses in cells filled with okadaic acid (81 ± 10 vs. 143 ± 13 μA for cells fills with H-89, KT 5720 or fostriecin; t = 2.59, p = 0. 016) suggesting that suppression of PP-1 activity enhanced synaptic responsivity in these cells. In addition, okadaic acid blocked the dopamine-induced enhancement of EPSCs (98.8 ± 0.9% of baseline, t = 1.143, p = 0.336; n = 4, 2 fan, 2 pyramidal cells; Figure 2.4B$_1$), consistent with the idea that D$_1$-like receptor activation leads to a facilitation of EPSCs via a PKA-mediated inhibition of PP-1 activity. Okadaic acid is a mixed inhibitor of PP-1 and PP-2A, and we therefore used the specific PP-2A inhibitor fostriecin to test the possible involvement of PP-2A. Including fostriecin in the recording solution did not block the dopamine-induced facilitation of EPSCs (126.3 ± 8.6% of baseline, t = 3.502, p = 0.025; n = 5, 2 fan, 3 pyramidal cells; Figure 2.4B$_2$), suggesting that okadaic acid acts via PP-1 to block the dopamine-induced facilitation.

DISCUSSION

Excitatory synaptic transmission in the entorhinal cortex is facilitated by lower concentrations of dopamine and suppressed by higher concentrations of dopamine (Pralong & Jones, 1993; Caruana et al., 2006; Caruana & Chapman, 2008), and this suggests that the effects of dopamine on sensory and mnemonic function within the entorhinal cortex are also strongly dependent on the intensity of dopamine release (Schultz, 2007; Clark et al., 2012). The facilitation of synaptic transmission in layer II entorhinal neurons induced by 1 μM dopamine may reflect a modulatory effect of low, tonic release of dopamine that could facilitate the salience of sensory input to the lateral entorhinal area or modulate the induction of learning-related synaptic plasticity (Caruana et al., 2007; Stramiello & Wagner, 2008). The facilitation is expressed through enhanced AMPA receptor-mediated responses, without a substantial contribution of NMDA receptors or inhibitory synaptic transmission. Changes in paired-pulse facilitation ratio do not definitively indicate pre- vs. post-synaptic effects, but the lack of an effect of dopamine on paired-pulse ratio is consistent with a synaptic facilitation expressed via a postsynaptic enhancement of AMPA receptor function, rather than by increased transmitter release (Caruana &Chapman, 2008). Similar to mechanisms described for prefrontal cortex neurons (Gonzalez-Islas & Hablitz, 2003; Bandyopadhyay et al., 2005), the facilitation was dependent on D$_1$-like receptor activation, and intracellular signaling via PKA and PP-1.

Dopaminergic Modulation of EPSPs is Dependent on Cell Type

Dopamine had a slightly different pattern of modulatory effects within electrophysiologically differentiated fan, pyramidal, and multiform neurons (Tahvildari & Alonso, 2005; Canto & Witter, 2012). Fan cells are the most numerous cell type in layer II, and showed a consistent moderate facilitation of EPSPs in response to dopamine. Fan cells display theta-frequency membrane potential oscillations and a sag in their voltage response to strong hyperpolarizing current steps (Tahvildari & Alonso, 2005), and dopamine may help maintain the strength of synaptic inputs to these neurons which may be suppressed by cholinergic inputs during theta activity (Hamam et al., 2007; Sparks & Chapman, 2013). The size of the synaptic facilitation effect was more variable in pyramidal cells which have neither a sag response nor membrane potential oscillations. Variability in the size of the facilitation may be related to the presence of two morphologically distinct pyramidal neuron types that are not electrophysiologically distinct: one with

vertically oriented soma and dendritic branching in layers II and III, and a second type with horizontal soma and dendrites mainly within layers I and II (Canto & Witter, 2012). Responses recorded from a small group of three multiform neurons, which are the least common type of cell in layer II (Tahvildari & Alonso, 2005), were not markedly affected by dopamine. Additional recordings from morphologically identified pyramidal and multiform neurons will be required to verify the differential effects of dopamine observed here.

D_1-like Receptor-Dependent Modulation of AMPA Receptor-Mediated EPSCs

The synaptic facilitation induced by dopamine was blocked by the $D_{1/5}$ receptor antagonist SCH23390, indicating that it is dependent on D_1-like receptor activation, and it was expressed by increases in AMPA, but not NMDA, receptor-mediated EPSCs. SCH23390 can block G-protein-coupled inwardly rectifying potassium channels (Kuzhikandathil & Oxford, 2002), but effects of SCH23390 on input resistance are unlikely to have occluded effects of dopamine because SCH23390 alone did not enhance EPSCs (Figure 2.3B). Dopamine facilitated AMPA-mediated responses that were isolated either by holding cells at −70 mV to enhance the Mg^{2+} block on NMDA receptors, or by recording at resting potential in the presence of APV. In contrast, NMDA receptors, which made a minimal contribution to baseline EPSCs (see also Kourrich et al., 2008), were not significantly affected by dopamine. The NMDA antagonist APV caused a small, non-significant reduction in the falling phase of EPSCs recorded at subthreshold potentials, and EPSCs recorded at resting potential were completely suppressed by CNQX (not shown). We therefore used a Cs^+-based intracellular recording solution and a holding potential of +40 mV in the presence of CNQX to isolate more robust NMDA-mediated currents, but found that dopamine had no effect on the isolated NMDA-mediated responses. D_1-like receptor activation can selectively enhance NMDA receptor-mediated responses in the prefrontal cortex and striatum (Cepeda et al., 1998; Zheng et al., 1999; Wang & O'Donnell, 2001; Seamans et al., 2001b; Kruse et al., 2009) but AMPA receptor-mediated responses are also enhanced by D_1-like receptor activation in layer II–III prefrontal neurons (Gonzalez-Islas & Hablitz, 2003; Bandyopadhyay et al., 2005; Sun et al., 2005) and in striatal neurons (Yan et al., 1999). This selective and transient modulation of AMPA-mediated EPSCs suggests a role for dopamine in the modulation of reward-relevant sensory processing, and a resistance of the lateral entorhinal area to enhancements in NMDA receptor function that might contribute to lasting synaptic plasticity (Racine et al., 1983; Caruana et al., 2007).

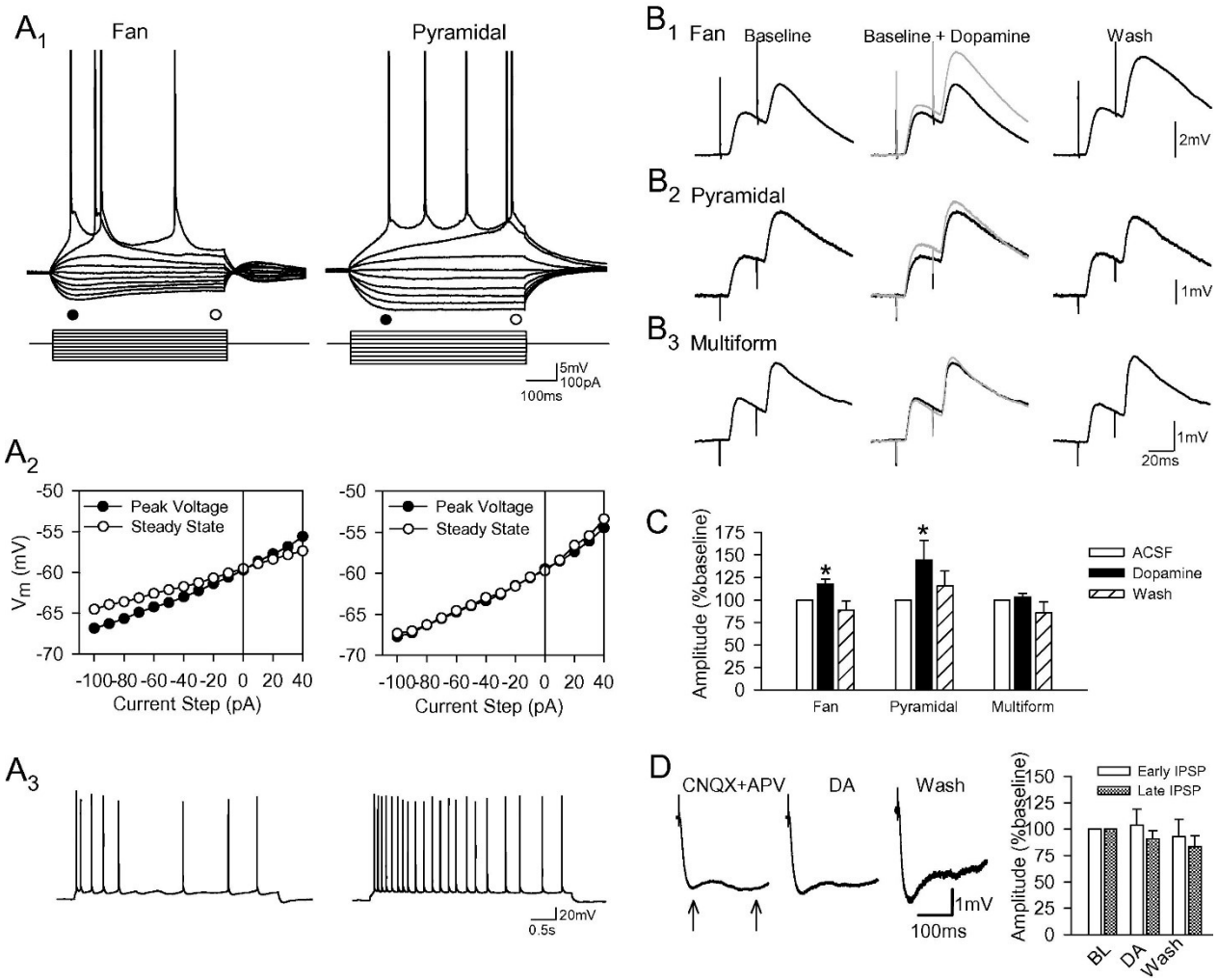
The cAMP–PKA Pathway

D_1-like receptors are positively coupled to adenylate cyclase to increase production of cAMP and activation of PKA (Neve et al., 2004), and we found that loading cells with the PKA inhibitor H-89 or the more specific PKA inhibitor KT 5720 prevented the dopamine-induced facilitation of EPSCs. PKA can modulate AMPA receptors through PKA-catalyzed phosphorylation of the AMPA GluR1 subunit to increase the channel open time probability (Roche et al., 1996; Snyder et al., 2000). However, the block of the dopamine-induced facilitation by the mixed PP-1/PP-2A inhibitor okadaic acid, but not the specific PP-2A blocker fostriecin, suggests that PKA may modulate AMPA channels through effects on PP-1 which contributes to dephosphorylation of AMPA receptors; PKA can lead to activation of the DARPP-32, and

the resulting inhibition of PP-1 by DARPP-32 can lead to facilitation of AMPA-mediated synaptic responses by reducing AMPA receptor dephosphorylation (Yan et al., 1999; Neve et al., 2004). There is substantial expression of DARPP-32 in the entorhinal cortex (Rosa et al., 2008) and the involvement of DARPP-32 in the transient synaptic facilitation observed here would be consistent with other findings showing rapid effects of D_1-like receptor activation on phosphorylation of DARPP-32 and facilitation of AMPA receptor-mediated currents (Yan et al., 1999; Nishi et al., 2000). In addition, PKA can also activate inhibitor 1 which inhibits PP-1 activity (Huang et al., 2010), and this provides an alternative route for the facilitation of AMPA receptor responses observed here.

Functional Significance

The entorhinal cortex is a major cortical target of midbrain dopamine neurons (Loughlin & Fallon, 1984; Björklund & Dunnett, 2007) but there is a limited understanding of the functional role of dopamine in the entorhinal cortex (Liu et al., 2000; Clark et al., 2012). High concentrations of dopamine suppress synaptic transmission in the entorhinal cortex (Pralong & Jones, 1993; Stenkamp et al., 1998; Caruana & Chapman, 2008), and the studies reported here indicate major intracellular signals through which a lower concentration of dopamine can facilitate AMPA-mediated excitatory synaptic responses in layer II entorhinal neurons. The number of dopamine neurons engaged in slow regular firing sets the extent of tonic availability of low concentrations of dopamine in the cortex, and burst firing of dopamine cells triggered by reward-relevant stimuli can phasically elevate dopamine concentrations (Schultz, 2007). The low concentration of bath-applied dopamine used here may be more analogous to levels of tonic dopamine release rather than to higher levels induced by strong phasic release of dopamine, but applied dopamine can sum with endogenous tonic or evoked dopamine release from the slice, and the relationship of applied dopamine to levels of dopamine release *in vivo* is not clear. Trains of rewarding lateral hypothalamic stimulation induce a transient D_2-like receptor-dependent *suppression* of synaptic responses in piriform cortex inputs to the lateral entorhinal cortex (Hutter et al., 2013), and we have recently found that exposure to cues that predict food reward also induces a dopamine-dependent suppression of entorhinal synaptic responses (Hutter & Chapman, 2013). It is therefore possible that strong phasic release of dopamine may suppress synaptic input to the entorhinal cortex and that the facilitation effects observed here may reflect the effects of lower tonic release of dopamine that may occur during task performance and facilitate the salience of cortical sensory inputs to the lateral entorhinal cortex.


A1
Fan
Pyramidal
5mV
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A2
Peak Voltage
Steady State
Vm (mV)
-50
-55
-60
-65
-70
-100 -80 -60 -40 -20 0 20 40
Current Step (pA)
A3
20mV
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B1 Fan
Baseline
Baseline + Dopamine
Wash
2mV
B2 Pyramidal
1mV
B3 Multiform
1mV
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Amplitude (%baseline)
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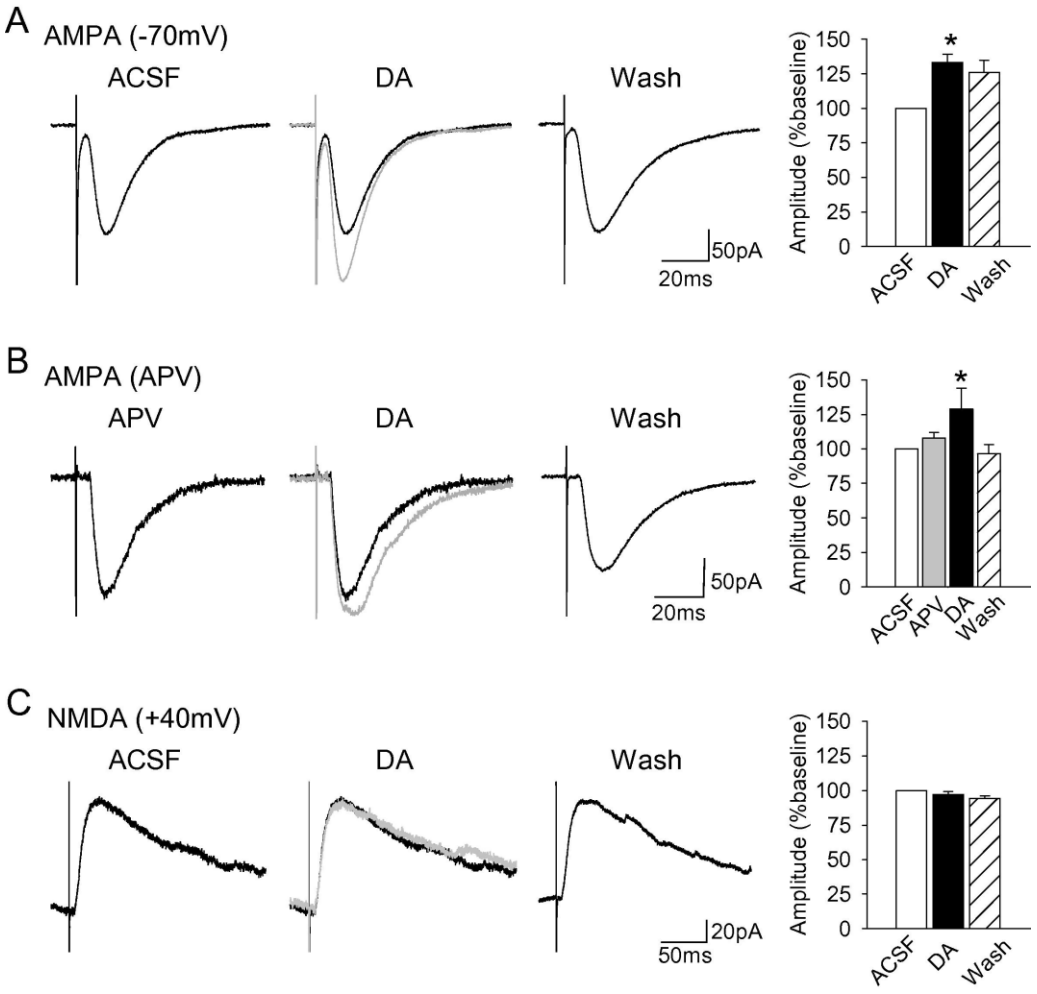
Figure 2.1. Dopamine facilitates excitatory postsynaptic potentials in electrophysiologically identified fan and pyramidal cells in layer II of the lateral entorhinal cortex**. (A)** Membrane potential responses to positive and negative current steps have been superimposed for representative fan and pyramidal cells **(A_1)**. Note the hyperpolarization-dependent inward rectification in the fan cell, that is absent in the pyramidal cell. Current–voltage plots in **A_2** show peak and steady-state voltage responses to current steps measured at the time-points indicated by open and closed circles in A_1. Fan cells tended to fire periodically compared to the more regular discharge in pyramidal cells in response to prolonged positive current injection **(A_3)**. **(B)** Dopamine (1 μM) facilitates EPSPs in fan **(B_1)** and pyramidal **(B_2)** cells but not in multiform **(B_3)** neurons. Intracellular EPSPs evoked by paired-pulse stimulation (30-ms interval) are shown for representative fan, pyramidal, and multiform neurons before, during, and 20 min after application of 1 μM dopamine. Traces recorded in the presence of dopamine (gray lines) have been superimposed with baseline traces for comparison. **(C)** The mean amplitude of EPSPs was significantly and reversibly increased by dopamine in fan cells (n = 16, * indicates $p < 0.05$ relative to baseline) and pyramidal cells (n = 10) but not in a small group of multiform neurons (n = 3). **(D)** Dopamine had no significant effect on the amplitude of early or late IPSPs (arrows, n = 11) recorded in the presence of CNQX and APV.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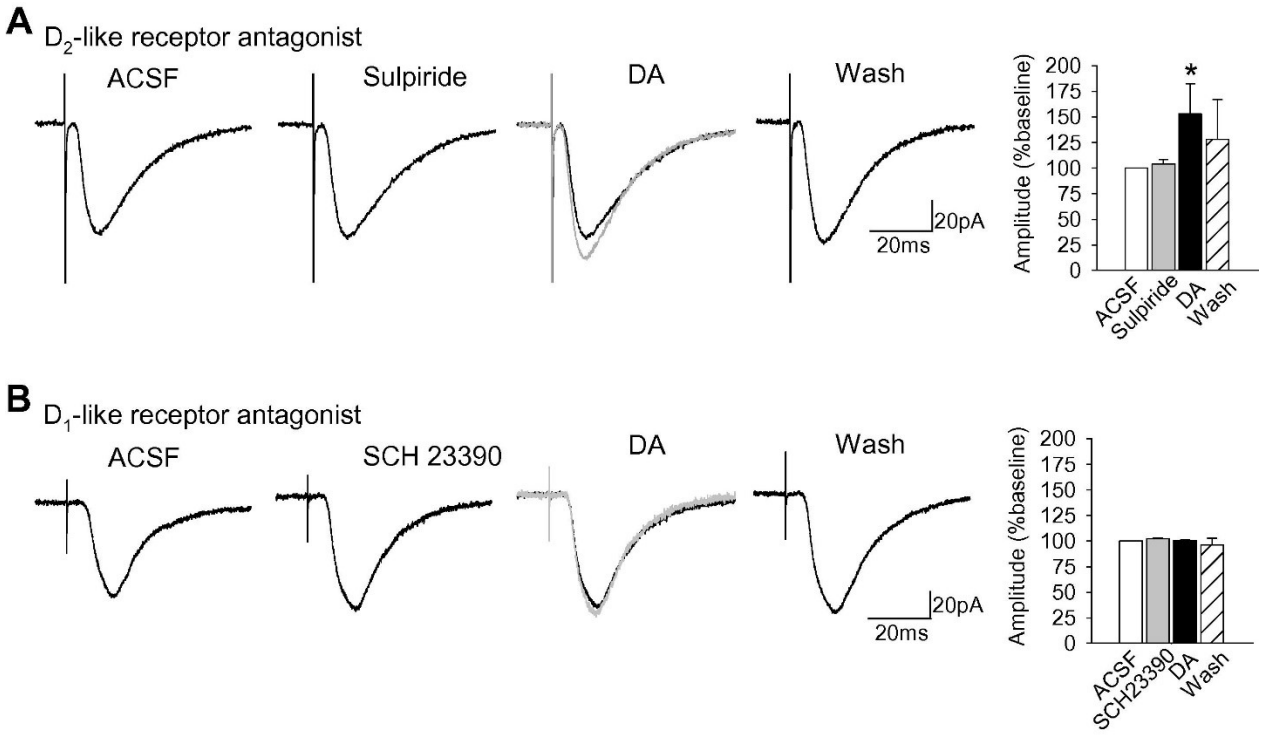
Wash

Figure 2.2. Application of dopamine increases the amplitude of AMPA receptor-mediated excitatory postsynaptic currents (EPSCs) in neurons in layer II of the lateral entorhinal cortex. **(A)** Representative-averaged EPSCs recorded at a holding potential of −70 mV to reduce the contribution of NMDA receptors are shown before, during, and 20 min after application of 1 μM dopamine. The histogram at right shows a facilitation of mean EPSC amplitudes in response to dopamine. **(B)** Isolated AMPA-mediated EPSCs recorded at −60 mV in the presence of APV showed a reliable and reversible facilitation in response to dopamine. **(C)** In contrast, isolated NMDA-mediated EPSCs recorded at +40 mV in the presence of CNQX and bicuculline were not significantly affected by dopamine.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D2-like receptor antagonist
ACSF
Sulpiride
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B
D1-like receptor antagonist
ACSF
SCH 23390
DA
Wash
20pA
20ms
Amplitude (%baseline)
200
175
150
125
100
75
50
25
0
ACSF
SCH23390
DA
Wash

Figure 2.3. The dopaminergic facilitation of EPSCs in layer II entorhinal neurons depends on activation of D_1-like, but not D_2-like receptors. **(A)** Traces show representative-averaged EPSCs obtained at baseline, during application of the D_2-like receptor antagonist sulpiride (50 μM), following addition of dopamine (1 μM), and after washout of dopamine. Dopamine induced a reversible facilitation of EPSC amplitudes in the presence of sulpiride ($p < 0.05^*$). **(B)** The facilitation of EPSC amplitudes induced by dopamine was blocked in the presence of SCH23390 (10 μM) indicating that D_1-like receptors are required for the effect.

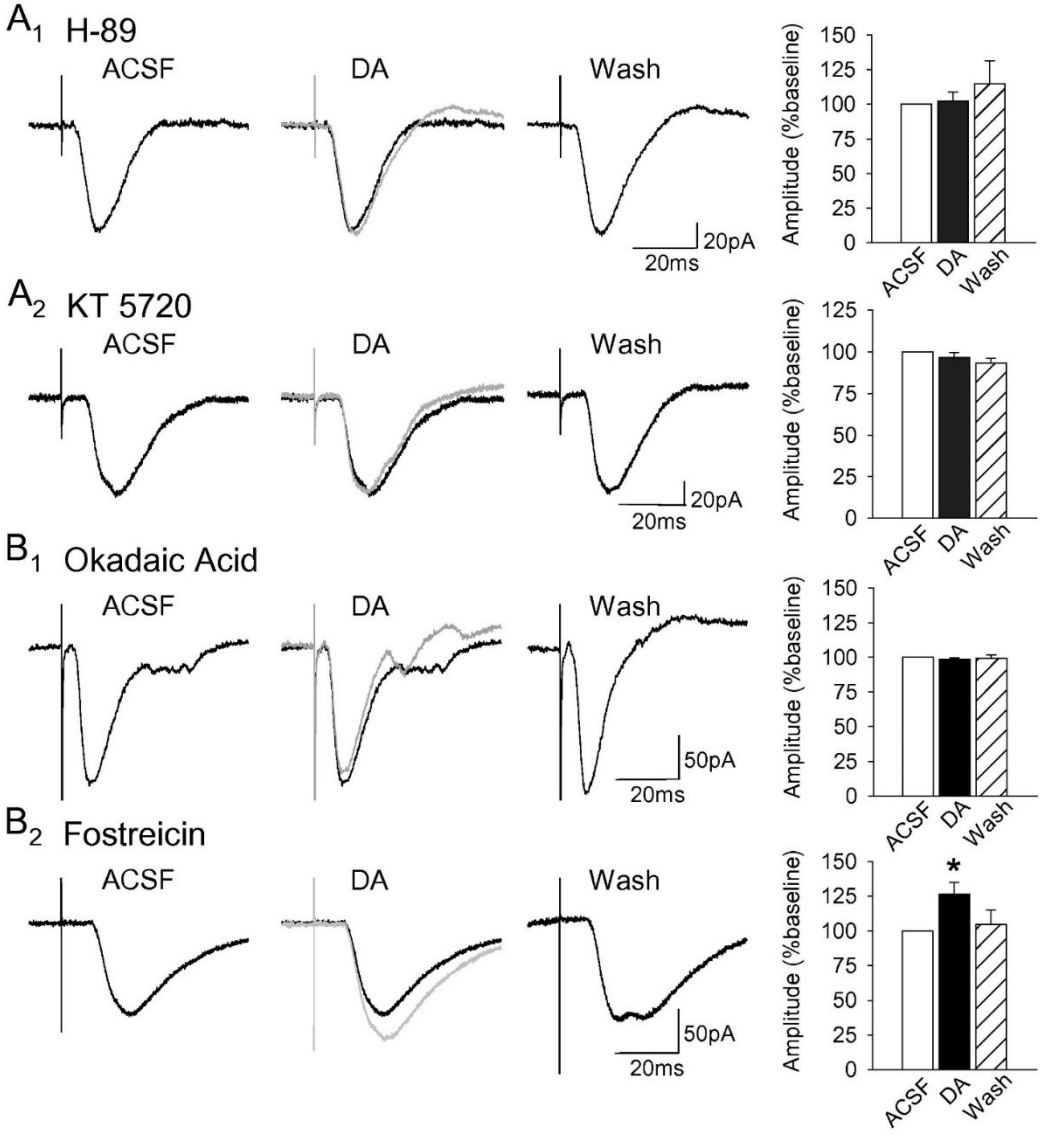

A1 H-89
ACSF
DA
Wash
20pA
20ms
Amplitude (%baseline)
150
125
100
75
50
25
0
ACSF
DA
Wash
A2 KT 5720
ACSF
DA
Wash
20pA
20ms
Amplitude (%baseline)
125
100
75
50
25
0
ACSF
DA
Wash
B1 Okadaic Acid
ACSF
DA
Wash
50pA
20ms
Amplitude (%baseline)
150
125
100
75
50
25
0
ACSF
DA
Wash
B2 Fostreicin
ACSF
DA
Wash
50pA
20ms
Amplitude (%baseline)
150
125
100
75
50
25
0
*
ACSF
DA
Wash

Figure 2.4. Inhibition of protein kinase A (PKA) or protein phosphatase 1 (PP-1), but not protein phosphatase 2A (PP-2A), blocks the dopaminergic facilitation of EPSCs in layer II entorhinal neurons. **(A_1)** Representative-averaged EPSCs are shown for cells filled with the PKA inhibitor H-89 (10 μM) during baseline recordings in normal ACSF, after a 5 min bath application of dopamine (10 μM), and following a 20-min washout period in normal ACSF. Group averages of EPSC amplitudes remained stable**. (A_2)** Similar results were obtained in cells filled with the more selective PKA inhibitor KT5720 (1 μM). **(B_1)** The facilitation of EPSCs induced by dopamine was also blocked in recordings obtained from cells filled with the PP-1/PP-2A inhibitor okadaic acid (10 μM). **(B_2)** The selective PP-2A inhibitor fostriecin fails to block the dopaminergic facilitation, suggesting that dopamine mediates the facilitation of EPSPs via activation of PP-1.

CHAPTER 3

ACTIVATION OF PHOSPHATIDYLINOSITOL-LINKED DOPAMINE RECEPTORS INDUCES A FACILITATION OF GLUTAMATE-MEDIATED SYNAPTIC TRANSMISSION IN THE LATERAL ENTORHINAL CORTEX

ABSTRACT

The lateral entorhinal cortex receives strong inputs from midbrain dopamine neurons that can modulate its sensory and mnemonic function. We have previously demonstrated that 1 μM dopamine facilitates synaptic transmission in layer II entorhinal cortex cells via activation of D_1-like receptors, increased cAMP-PKA activity, and a resulting enhancement of AMPA-receptor mediated currents. The present study assessed the contribution of phosphatidylinositol (PI)-linked D_1-like receptors to the dopaminergic facilitation of transmission in layer II of the rat entorhinal cortex, and the involvement of phospholipase C activity and release of calcium from internal stores. Whole-cell patch-clamp recordings of glutamate-mediated evoked excitatory postsynaptic currents were obtained from pyramidal and fan cells. Activation of D_1-like receptors using SKF38393, SKF83959, or 1 μM dopamine induced a reversible facilitation of EPSCs which was abolished by loading cells with either the phospholipase C inhibitor U-73122 or the Ca^{2+} chelator BAPTA. Neither the L-type voltage-gated Ca^{2+} channel blocker nifedipine, nor the L/N-type channel blocker cilnidipine, blocked the facilitation of synaptic currents. However, the facilitation was blocked by blocking Ca^{2+} release from internal stores via inositol 1,4,5-trisphosphate (IP_3) receptors or ryanodine receptors. Follow-up studies demonstrated that inhibiting CaMKII activity with KN-93 failed to block the facilitation, but that application of the protein kinase C inhibitor PKC(19-36) completely blocked the dopamine-induced facilitation. Overall, in addition to our previous report indicating a role for the cAMP-PKA pathway in dopamine-induced facilitation of synaptic transmission, we demonstrate here that the dopaminergic facilitation of synaptic responses in layer II entorhinal neurons also relies on a signaling cascade dependent on PI-linked D_1-like receptors, PLC, release of Ca^{2+} from internal stores, and PKC activation which is likely dependent upon both DAG and enhanced intracellular Ca^{2+}. These signaling pathways may collaborate to enhance sensory and mnemonic function in the entorhinal cortex during tonic release of dopamine.

The entorhinal cortex is an essential parahippocampal region through which multimodal sensory information from the neocortex reaches the hippocampal formation (Burwell, 2000), and it is thought to contribute significantly to the sensory and mnemonic functions of the medial temporal lobe (Lavenex & Amaral, 2000). Functionally, the medial entorhinal cortex is linked to areas that are involved in spatial processing such as the subicular complex, postrhinal, and retrosplenial cortex (Hargreaves, Yoganarasimha, & Knierim, 2007; Sharp, 1997), whereas the lateral division is linked to object and odor recognition and familiarity, and receives strong inputs from the perirhinal cortex (Murray, Bussey, & Saksida, 2007; Wan, Aggleton, Brown, 1999; Petrulis, Alvalez, & Eichenbaum, 2005; Kerr, 2007; Deshmukh & Knierim, 2011). In addition, the cell islands of layer II of the lateral entorhinal cortex receive one of the largest cortical projections from midbrain dopamine neurons that contribute to appetitive motivation and learning (Berridge, 2007; Björklund & Lindvall, 1984; Loughlin & Fallon, 1984; Akil & Lewis, 1994; Björklund & Dunnett, 2007; Clark et al., 2012).

Although the exact functions played by dopamine in the superficial layers of the lateral entorhinal cortex are presently poorly understood, effects of dopamine on synaptic transmission suggest that low concentrations of dopamine may act to enhance the salience of synaptic inputs received from sensory regions. The effects of dopamine on synaptic transmission in the entorhinal cortex are concentration-dependent, wherein high concentrations of dopamine (50–100 μM) result in a *suppression* of glutamate-mediated synaptic responses (Pralong & Jones, 1993; Stenkamp, Heinemann, & Schmitz, 1998) via a D_2-like receptor-dependent mechanism (Caruana & Chapman, 2008) and lower concentrations of dopamine (1–10 μM) induce a D_1-like-receptor-mediated *facilitation* of synaptic responses (Caruana, Sorge, Stewart, & Chapman, 2006; Glovaci, Caruana, & Chapman, 2014). Functionally, low to moderate levels of D_1-like receptor activation enhance synaptic transmission and working memory function in the prefrontal cortex (Goldman-Rakic, Muly, & Williams, 2000; Vijayraghavan et al., 2007; Puig & Miller, 2012), and dopamine may act similarly in the superficial layers of the lateral entorhinal cortex.

Our laboratory has recently investigated the intracellular signaling pathways mediating the dopaminergic facilitation of glutamatergic transmission in fan and stellate cells of layer II of the entorhinal cortex. Similar to findings in the prefrontal cortex and hippocampus (Gonzalez-Islas & Hablitz, 2003; Young & Yang, 2004), we established that binding of dopamine to D_1-like, but not D_2-like, receptors leads to a rapid and reversible increase in the amplitude of glutamatergic excitatory postsynaptic currents (EPSCs) via a signaling cascade that is dependent on increased activation of the cyclic AMP-protein kinase A (PKA) pathway (Glovaci, Caruana, & Chapman, 2014). Increased PKA activity can increase the phosphorylation of the dopamine- and cAMP-regulated phosphoprotein, 32 kDa, (DARPP-32) and of inhibitor 1 (I-1), which are potent inhibitors of protein phosphatase 1 (PP-1), a phosphatase that reduces synaptic responses by dephosphorylating AMPA receptors during basal conditions (Yan et al., 1999; Yger & Girault, 2011; Song & Huganir, 2002). We also found that the dopaminergic facilitation of AMPA-mediated EPSCs was dependent upon PP-1 activity (Glovaci, Caruana, & Chapman, 2014). These results provided the first evidence that PKA-mediated inhibition of PP-1 contributes to the dopaminergic facilitation of AMPA-mediated synaptic responses.

In addition to the cAMP-PKA pathway, D_1-like receptor activation leading to a facilitation of synaptic transmission has also been reported to depend on increases in postsynaptic calcium in the striatum (Flores-Hernández et al., 2002; Cepeda et al., 1998), prefrontal cortex (Gonzalez-Islas & Hablitz, 2003; Tseng & O'Donnell, 2004), and hippocampus (Yang, 2000). Our findings are similar, wherein intracellular application of the calcium chelator BAPTA completely blocked the dopamine-induced facilitation of EPSCs in the entorhinal cortex. However, the intracellular cascade linking dopamine to increased intracellular calcium, and the mechanism through which calcium may contribute to the synaptic enhancement remained unclear.

The present experiments aimed to determine the origin of increased intracellular calcium and the signaling cascade required for dopamine-induced enhancement of AMPA-mediated synaptic transmission in layer II principal cells of the entorhinal cortex. Recordings in other areas have shown that D_1-like receptors linked to G_s proteins that stimulate cAMP-PKA can enhance calcium currents via PKA-mediated phosphorylation of both L- and N-type voltage-gated calcium channels (VGCCs) (Schultz et al., 1990; Gross et al., 1990; Hartzell et al., 1991). Because we found that PKA signaling was required for the dopamine-mediated facilitation of EPSCs (Glovaci, Caruana, & Chapman, 2014), and PP-1 also regulates phosphorylation of L- and N-type VGCCs (Hescheler et al., 1987; Shenolikar & Nairn, 1991; Lai, Peterson, & Catterall, 1993), our first experiments tested the involvement of L- and N-type VGCCs in the dopaminergic facilitation of synaptic transmission. In addition to G_s-linked D_1-like receptors, phosphatidylinositol (PI)-linked D_1-like receptors coupled to G_q proteins that stimulate PLC to increase production of diacylglycerol (DAG) and inositol triphosphate (IP_3) can lead to increased cytosolic calcium levels by binding to IP_3 receptors that mediate calcium release from internal stores (Ming et al.,2006; Zhen, Goswami, & Friedman, 2005; Jin et al., 2004; Zhen et al., 2004). We therefore also assessed the role of PI-linked D_1-like receptors, PLC, and release of Ca^{2+} from internal stores. Our results show a role for PI-linked D_1-like receptors in the facilitation of EPSCs that is dependent on IP_3-medited release from internal stores, and further indicate that the Ca^{2+}-dependent kinase PKC is required for the dopaminergic enhancement of EPSCs in the entorhinal cortex.

METHOD

In Vitro Slice Preparation

Recordings were obtained from brain slices collected from 4–9 week-old male Long-Evans rats (Charles River). Brains were quickly extracted and submerged into an ice cold, high-sucrose artificial cerebrospinal fluid cutting solution (ACSF; saturated with 95% O_2 and 5% CO_2, pH ≈7.4) containing (in mM) 250 sucrose, 2 KCl, 1.25 NaH_2PO_4, 7 $MgCl_2$, 26 $NaHCO_3$, 0.5 $CaCl_2$and 10 dextrose. All drugs were obtained from Sigma-Aldrich unless indicated otherwise. Horizontal slices containing the entorhinal region were obtained using a vibratome (300 µM thick; WPI, Vibroslice, Sarasota, USA). There was a recovery period of at least one hour in normal ACSF containing (in mM) 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 dextrose (pH ≈7.4; 300–310 mOsm; ~22 ºC). During recordings, individual slices were submerged in ASCF (2 ml/min) and fixed using a nylon net, and were visualized using an

upright microscope (Leica, DM-LFS) equipped with a 40x objective and differential interference contrast optics. Layer II of the lateral entorhinal cortex was distinguished from layers I and III based on the presence of clusters of cells (Burwell, 2000).

Stimulation and Recording

Recording pipettes used for whole-cell recordings were pulled from borosilicate glass (1.0 mm OD, 2.7 to 6 MΩ) and were filled with a solution containing (in mM) 140 K-gluconate, 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.5 EGTA, 2 ATP-Tris, and 0.4 GTP-Tris (pH adjusted to 7.2–7.3 with KOH). The formation of a tight seal between the pipette and soma (1–4 GΩ) was obtained using gentle negative pressure, and a stronger pressure was applied to obtain whole-cell configuration. Neurons were allowed to stabilize for 10–15 minutes prior to recordings to allow for diffusion of intracellular drugs where applicable. Synaptic responses were evoked using a bipolar stimulating electrode made from two tungsten electrodes (~1 MΩ, FHC Inc.) placed in layer I of the lateral entorhinal cortex, approximately .1 to .2 mm rostral to the recording electrode. Synaptic responses were evoked by 0.1 ms-duration constant current pulses delivered using a stimulus timer and isolation unit (WPI, Models A300 and A360). Stimulus intensity was adjusted to evoke responses of roughly 70% of maximal without evoking action potentials (131 ± 15 μA). Current and voltage clamp recordings were obtained using an Axopatch 200B amplifier, and were filtered at 5–10 kHz, then digitized at 20 kHz (Axon Instruments, Digidata 1322A).

Electrophysiological characteristics and firing properties of entorhinal neurons were characterized prior to synaptic recordings by recording membrane potential responses to 500 ms duration current pulses (range -200 to +60 pA). Responses were analyzed using the Clampfit 8.2 software package (Axon Instruments). Inward rectification was quantified as the ratio between peak and steady-state input resistances in response to -200 pA hyperpolarizing current pulses (rectification ratio). Spike properties were measured from the first action potential evoked in response to positive current injection. Action potential amplitude was calculated from resting membrane potential and action potential duration and afterhyperpolarization were measured from action potential threshold.

Synaptic Currents

The effects of dopamine receptor activation on evoked synaptic response in the entorhinal cortex were assessed by recording excitatory postsynaptic currents (EPSCs) at a holding potential of -60 mV before, during and after 5-min bath application of dopamine or dopamine receptor agonists. We previously found that early and late inhibitory synaptic potentials are not affected by dopamine application in layer II entorhinal neurons (Glovaci, Caruana, & Chapman, 2014) and GABA receptor blockers were therefore not included in experiments. Cells occasionally showed outward currents at latencies following the peak of EPSCs during drug application or wash (e.g., Figures 3.1B and $3.2A_1$), but these were not associated with altered patterns of drug effects on EPSC amplitude. Ten to twenty EPSCs were evoked by single pulses delivered once every 15 sec during each recording condition. Input resistance, access resistance and capacitance were monitored via responses to -10 mV 50 ms voltage steps delivered before each evoked synaptic response, and recordings were discontinued if the values changed by >15%. The dopamine-induced facilitation of AMPA receptor-mediated transmission (Glovaci, Caruana, & Chapman, 2014) was

replicated by recording EPSCs at a holding potential of -60 mV during a baseline period, following 5-min application of the D_1-like receptor agonists SKF38393 (10 μM) or SKF83959 (5 or 10 μM), and following a 20 min washout period in normal ACSF. Possible increases in glutamate release induced by the PI-linked dopamine receptor agonist SKF83959 (10 μM) were assessed in some neurons by monitoring responses to pairs of stimulation pulses separated by 30 ms (Glovaci, Caruana, & Chapman, 2014).

The role of intracellular calcium in the dopamine-induced facilitation of EPSCs was assessed by including the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N ′-tetracetic acid (BAPTA, 10 μM) in the intracellular solution, and EPSCs were recoded prior to and during 5-min application of dopamine (1 μM), and after 20 min of washout. The dependence of the dopaminergic facilitation on voltage-gated calcium channels was assessed by applying 1 μM dopamine during constant bath application of either the selective L-type channel blocker cilnidipine (10 μM), or the mixed L/N-type blocker nifedipende (10 μM). There was a 10 min wash-out period in the presence of the blocker, and during an additional 10 min in normal ACSF. The contribution of IP_3-receptors and ryanodine receptors was assessed by including either heparin (1 mM) or dantrolene (20 μM) in the recording solution, and by comparing EPSCs recorded before, during, and after dopamine. Other signaling molecules were tested using the bath application of the PLC inhibitor U-73122 (10 μM), and intracellular application of the PKC inhibitor PKC(19–36) (1 μM; Tocris Bioscience), or the calcium/calmodulin-dependent protein kinase II (CaMKII) blocker KN-93 (5 μM, dissolved in dimethylsulfoxide (DMSO) with a final concentration of 0.1%; Tocris Bioscience). All recording solutions were stored at –20°C.

Changes in synaptic responses were analyzed using Clampfit 8.2 software (Axon Instruments). For each cell, at least 10 consecutive synaptic responses free from artifacts or action potentials were averaged for each phase of recordings, and the amplitudes of averaged responses were measured relative to the pre-stimulus baseline. Raw data were analyzed using modified Bonferroni planned comparisons and an alpha level of .05 (Keppel & Wickens, 2004) to assess changes in cellular properties and synaptic responses prior to and after drug application, as well as between baseline and washout to assess the reversibility of effects and stability of recordings. The data met the requirements for normality (Lilliefors' corrected Kolmogorov-Smirnov test), and are presented here as group means ± one standard error of the mean. Cohen's *d* was calculated as a measure of effect size.

RESULTS

Effects of dopamine on evoked EPSCs were assessed in recordings from 97 layer II lateral entorhinal cortex neurons. Neurons were classified by their electrophysiological profile in current clamp recordings (Tahvildari & Alonso, 2005) as either fan cells that are characterized by a marked sag in the voltage response to strong hyperpolarizing current injection (n = 45; rectification ratio: 1.23 ± 0.09) or pyramidal neurons that fire regularly with no substantial sag response (n = 52; rectification ratio: 1.01 ± 0.01). Drug effects on synaptic responses were not dependent on cell type, and the data reported here reflect mean responses in mixed groups of neurons.

The Facilitation of Responses Induced by D_1-like-Receptor Activation is Calcium-Dependent

Consistent with our previous findings that 1 μM dopamine induces a reversible D_1-like receptor-dependent facilitation of AMPA receptor-mediated EPSCs in entorhinal cells (Glovaci, Caruana, & Chapman, 2014), we found here that 5 min bath application of the selective D_1-like-receptor agonist SKF38393 (10 μM) facilitates EPSCs in lateral entorhinal cortex cells held at -60 mV (Figure 3.1A_1). Responses were increased by SKF38393 to 126.3 ± 3.5% of baseline responses (t = 2.63, p = 0.04, n = 6, d = 1.3). The facilitation was reversible, and responses recorded after 20 min washout did not differ significantly from baseline (105.9 ± 3.7%). The facilitation of synaptic responses was not associated with changes in cellular input resistance (89 ± 7 vs. 87 ± 7 MΩ; t = 0.98, p = 0.36) or capacitance (169 ± 31 vs. 184 ± 30 pF; t = 1.32, p = 0.24). The facilitation induced by SKF38393 4 was also blocked by the D_1-like receptor antagonist SCH23990 (10 μM; 102.7 ± 4.4% of baseline responses; t = 0.11, p = 0. 92, n = 6), indicating that it is dependent upon activation of D_1-like dopamine receptors (Figure 3.1A_2), consistent with our previous findings that the dopaminergic facilitation of synaptic responses is dependent upon activation of D_1-like receptors (Glovaci, Caruana, & Chapman, 2014).

Increases in intracellular calcium contribute to the D_1-like receptor-dependent enhancement of glutamatergic responses in other regions (Gonzalez-Islas & Hablitz, 2003; Galarraga et al., 1997; Hernández-López et al., 1997). We therefore assessed the role of intracellular Ca^{2+} in the facilitation of EPSCs by including the Ca^{2+} chelator BAPTA (10 μM) in the intracellular solution. Comparisons of action potential waveforms recorded immediately, and 10 to 15 min after break-in in three of the cells filled with BAPTA showed an increase in action potential duration (5.6 ms to 8.2 ms) and a reduction in medium afterhyperpolarization (2.9 mV to 1.5 mV), consistent with reduced intracellular Ca^{2+}. The dopaminergic facilitation of EPSC amplitudes was blocked by BAPTA (94.6 ± 3.1% of baseline; n = 7, t = 0.51, p = 0.63; Figure 3.1B) indicating that increases in intracellular calcium are required for the facilitation effect.

Dopamine is known to enhance calcium influx via PKA-mediated phosphorylation of L-type voltage-gated calcium channels in other neurons (Cepeda et al., 1998; Galarraga et al., 1997; Hernández-López et al., 1997), and we have shown that facilitation of AMPA-receptor currents in entorhinal neurons is dependent on increased PKA activity (Glovaci, Caruana, & Chapman, 2014). However, we found here that bath-application of the selective L-type voltage-gated calcium channel blocker nifedipine (10 μM) did not block the dopaminergic facilitation of EPSCs (n = 8; 118.7 ± 8.5% of baseline, t = 2.67, p = 0.03, d = .41), indicating that calcium influx through L-type VGCCs is not required for the facilitation of EPSCs. The facilitation induced by dopamine reversed during washout in nifedipine (see Figure 3.1C), and remained at baseline values during 10 min subsequent washout in ACSF (105.8 ± 7.4% of baseline, t = 0.33, p = 0.76).

N-type voltage-gated calcium channels are also present in the entorhinal cortex (Kelly et al., 2001), and calcium influx via N-type channels could also be enhanced by D_1-like receptor activation and PKA-mediated phosphorylation (Bergson et al., 2003). Bath-application of the combined L/N type VGCC blocker cilnidipine (10 μM) alone did not change the amplitude of EPSCs (n = 6; 103.0 ± 2.5% of baseline, t = 0.16, p = 0.9), but subsequent co-application of dopamine increased EPSCs in these cells to 139.3 ±

6.5% of baseline (t = 8.56; p = 0.0004, d = 1.07), and responses returned to baseline during 20 min washout in ACSF (110.3 ± 5.4% of baseline, t = 0.55, p = 0.6; Figure 3.1D). In addition, the dopaminergic facilitation of EPSCs during application of nifedipine and cilinidipine was not associated with a change in holding current or input resistance. Thus, neither L- nor N-type VGCCs are required for the dopaminergic facilitation of EPSCs in entorhinal neurons.

The Facilitation is Dependent upon Phosphatidylinositol-Linked Dopamine Receptors and Internal Calcium Stores

Because voltage-gated calcium channels were not required, we then investigated the role of phosphatidylinositol (PI)-linked D_1-like receptors that lead to activation of PLC and production of IP_3 that could result in release of calcium from internal stores (Jin et al., 2003; Undie et al.,1994; Jin et al., 2001). We tested the involvement of this receptor using SKF83959 which is the most selective agonist available for PI-linked D_1-like receptors (Arnt, Hyttel, & Sánchez, 1992; Panchalingam & Undie, 2001; Lezcano & Bergson, 2002; Shen, Goswami, & Friedman, 2005; Jin et al., 2003). The amplitude of EPSCs increased during application of 5–10 μM SKF83959 to 110.9 ± 1.9% of baseline values (n = 6; t = 4.06, p = 0.009, d = .44), indicating that these receptors could be involved in the synaptic effects of dopamine (Figure 3.2A$_1$). Subsequent co-application of 1 μM dopamine was associated with a further, but statistically non-significant, increase in EPSC amplitudes to 123.2 ± 7.3% of baseline values (n = 7; t = 2.18, p = 0.06, d = .35) that is consistent with the added involvement of cAMP-PKA-linked D_1-like receptors described previously (Glovaci, Caruana, & Chapman, 2014). The effects were reversible, and EPSC amplitudes returned to baseline values during a 20 min washout period in ACSF (97.3 ± 5.0% of baseline, n = 3; t = 2.68, p = 0.12; Figure 3.2A$_1$). The facilitation of synaptic responses was not associated with changes in resting membrane potential (-55.7 ± 2.6 mV vs -54.3 ± 3.1 mV; t = -2.35, p = 0.07), holding current (-4.6 ± 16.5 vs -7.3 ± 17.6 pA; t = 1.05, p = 0.33), or membrane resistance (115.8 ± 34.7 vs 110.2 ± 34.3 MΩ; t = 1.95, p = 0.12). Because activation of presynaptic PI-linked D_1-like receptors can lead to increases in glutamate (Chu et al., 2010), we also assessed paired-pulse facilitation ratios before and after application of SKF83959 in a separate group of neurons. The significant facilitation of responses (119.2 ± 8.5% of baseline values, n = 6; t = 2.74, p = 0.04, d = 0.32) was not associated with significant alteration in paired-pulse ratio, however (Figure 3.2A$_2$; 119.5 ± 6.9 vs. 119.6 ± 6.2, t = 0.095, p = 0.93) suggesting that changes in presynaptic release do not contribute to the effect. We also found that the facilitation induced by SKF83959 (5 μM) was blocked in the presence of the D_1-like receptor antagonist SCH23990 (10 μM; 101.8 ± 3.9% of baseline responses; t = 0.19, p = 0. 86, n = 6), indicating that it is dependent on D_1-like receptors (Figure 3.2A$_3$).

Because PI-linked D_1-like receptors stimulate phospholipase C (Rashid et al., 2007; Berridge, 1998), we tested whether or not PLC is required for the dopaminergic facilitation of EPSCs using bath-application of the PLC inhibitor U-73122 (10 μM). Responses remained stable following application of U-73122 (n = 8; 106.6 ± 5.4% of baseline, t = 0.11, p = 0.91), and the presence of U-73122 also blocked the dopamine-induced enhancement of EPSCs (100.5 ± 3.3%, t = 0.03, p = 0.98; 107.1 ± 11.9% of baseline during washout, t = 0.01, p = 0.70; Figure 3.2B$_1$) indicating that the dopaminergic facilitation requires

activation of PLC. Intracellular application of U-73122 (10 μM) also prevented facilitation of EPSCs induced by the putative PI-linked D_1-like receptor agonist SKF83959. The responses remained stable following application of SKF83959 (5 μM; n = 7; 99.9 ± 4.3% of baseline, t = 0.52, p = 0.62; Figure 3.2B_2), as well as during washout (102.8 ± 7.6% of baseline, t = 0.005, p = 0.99) indicating that the facilitation induced by SKF83959 is dependent on activation of PLC.

PLC-induced production of IP_3 leads to increased cytosolic Ca^{2+} via binding to IP_3 receptors and the resulting release of Ca^{2+} from internal stores (Berridge, 1998). To test whether IP_3 receptors might mediate increases in Ca^{2+} required for the dopaminergic facilitation of EPSCs, we included the IP_3R blocker heparin (5 μM) in the intracellular recording solution. Bath application of dopamine had no significant effect on EPSC amplitudes in heparin-filled neurons (97.6. ± 8.1% of baseline responses, n = 8; t = 0.86, p = 0.45; Figure 3.3A) indicating that activation of IP_3 receptors is required for the facilitation effect.

Calcium release from internal stores is also mediated by ryanodine receptors (RyR), which are themselves activated by low to moderate increases in cytosolic Ca^{2+} (Berridge, 1998). In order to assess the role of Ca^{2+} release via RyR, we included the RyR blocker dantrolene (20 μM) in the intracellular solution. Application of dopamine had no effect on EPSC amplitudes in cells filled with dantrolene (99.8 ± 3.7% of baseline, n = 7; t = 0.20, p = 0.85; Figure 3.3B) indicating that the dopamine-induced facilitation of EPSCs is dependent upon activation of RyR. The requirement for both IP_3R and RyR activation suggests that IP_3 receptor activation followed by calcium-induced calcium-release via RyR may be required for the facilitation effect.

Calcium-Dependent Kinases

Increases in cytosolic calcium can enhance protein kinase activity in neurons, and protein kinases can rapidly regulate AMPA-receptor channel kinetics via receptor subunit phosphorylation (Krebs, 1994; Mammen, Kameyama, Roche, & Huganir, 1997; Derkach, Barria, & Soderling, 1999; Lee et al., 2003). In the hippocampus, dopamine D_1-like receptor activation can lead to activation of calmodulin and subsequent activation of the Ca^{2+} and calmodulin-dependent protein kinase II (CaMKII) which may enhance AMPA single channel conductance via phosphorylation of the GluR1 subunit at Ser^{831} (Derkach, Barria, & Soderling, 1999; Lee et al., 2003; Wang et al., 1994). Further, blocking CaMKII activity prevents the transient D_1-like-receptor-mediated enhancement of EPSCs in prefrontal neurons (Gonzalez-Islas & Hablitz, 2003). However, we found that blocking CaMKII activity in layer II entorhinal cortex neurons using intracellular application of 5 μM KN-93 did not block the dopamine-induced facilitation of EPSCs (n = 8; 120.8 ± 7.2% of baseline; t = 2.84, p = 0.025, d = 0.74; washout: 103.7 ± 6.8% of baseline; t = 0.64, p = 0.54; Figure 3.4A), indicating that activation of CaMKII is not required for the transient facilitation of synaptic transmission observed here.

Several studies also indicate that protein kinase C, which is activated by PLC-DAG and by Ca^{2+}, can potentiate AMPA receptor-mediated currents in hippocampal neurons (Wang et al., 1994; Tan, Wenthold, &Soderling, 1994). In order to determine if increased Ca^{2+} might enhance EPSCs via increased PKC activity, we included the PKC inhibitor PKC(19–36) in the intracellular recording solution. The

dopaminergic facilitation of synaptic responses was blocked by PKC(19–36) (100.5 ± 3.6% of baseline during dopamine application, $n = 8$; $t = 0.55$, $p = 0.6$), and responses also remained stable during washout (100.6 ± 5.1%, $t = 1.06$, $p = 0.33$; Figure 3.4B). Therefore, increases in EPSCs amplitudes induced by dopamine are dependent upon activation of PKC that may lead to phosphorylation of AMPA receptors.

DISCUSSION

The lateral entorhinal cortex processes multimodal sensory information and has been linked to object recognition, olfaction, and mnemonic processes (Lavenex & Amaral, 2000; Murray, Bussey, & Saksida, 2007; Wan, Aggleton, & Brown, 1999; Petrulis, Alvalez, & Eichenbaum, 2005; Kerr, 2007; Deshmukh & Knierim, 2011). As the lateral entorhinal cortex is one of the four major cortical targets of midbrain dopamine neurons (Björklund & Dunnett, 2007), it is likely that dopamine availability in this area promotes the processing of reward-relevant stimuli (Hutter et al., 2013). Given that a low concentration of dopamine facilitates synaptic transmission onto principal cells of the lateral entorhinal cortex (Caruana & Chapman, 2008; Glovaci, Caruana, & Chapman, 2014), low, tonic levels of dopamine may enhance the salience4 of sensory inputs and promote memory formation during exploratory behavior in awake animals. However, the intracellular pathways through which dopamine modulates glutamatergic transmission in layer II lateral entorhinal cortex neurons have hitherto remained unclear.

It is well-acknowledged that activation of D_1-like receptors linked to $G_{s/olf}$ proteins can lead to increased glutamatergic transmission via increased activity in the cAMP-PKA pathway in other brain regions (Gonzalez-Islas & Hablitz, 2003; Wang & O'Donnel, 2001) and we previously reported a similar PKA-dependent dopaminergic potentiation of AMPA currents in lateral entorhinal cortex slices (Glovaci, Caruana, & Chapman, 2014). In the present paper, we describe an additional intracellular pathway that depends on activation of PI-linked D_1-like receptors that are coupled to G_q proteins, which lead to increases in PLC activity, IP_3-dependent release of calcium from internal stores, and a PKC-dependent facilitation of glutamate-mediated synaptic responses (Figure 3.5). Full potentiation of glutamate transmission appears to require parallel activation of both PLC-dependent and PKA-dependent pathways, because activation of PI-linked D_1-like receptors alone produces a partial facilitation effect (Figure 3.2A_1), and blocking signaling steps within either the PKA- or PLC-dependent signaling pathways blocks the full facilitation effect (Glovaci, Caruana, & Chapman, 2014). Although the role of SKF83959 as a selective PI-linked D_1-like receptor agonist has been recently questioned (Lee et al., 2014), our current findings demonstrate that the dopaminergic facilitation is dependent upon D_1-like receptors, PLC activity, Ca^{2+} signaling and PKC activity. These data point strongly to a role for PI-linked dopamine receptors and subsequent activation of the PLC-DAG-IP_3 signaling cascade. The involvement of both PKA- and PLC-dependent signaling cascades provides increased means through which other neuromodulators may gate synaptic transmission in lateral entorhinal cortex neurons, to either promote or restrict synaptic transmission.

The experiments presented here indicate the blockade of postsynaptic calcium or PKC activity blocks the facilitation effect of dopamine, indicating an involvement of postsynaptic signals, but it is also

possible that activation of PI-linked D_1-like receptors on presynaptic terminals could contribute to the facilitation effect by enhancing glutamate release (Hernández et al., 2007; Chu et al., 2010). However, similar to our previous findings for the application of dopamine (Glovaci, Caruana, & Chapman, 2014), we found here that the facilitation of synaptic responses induced by the PI-linked D_1-like receptor agonist SKF83959 was not associated with a change in paired-pulse facilitation ratio, suggesting that changes in presynaptic release do not contribute substantially.

Dopamine-Induced Increases in Intracellular Ca^{2+}

The present study used the Ca^{2+} chelator BAPTA to demonstrate that, similar to findings reported in the prefrontal cortex and the hippocampus (Puig & Miller, 2012;Yang, 2000), the dopamine-mediated facilitation of synaptic currents in the lateral entorhinal cortex is dependent on increases in cytosolic calcium (Figure 3.1B). Dopamine is unlikely to enhance cytosolic calcium via either T-type voltage-gated calcium channels (VGCCs) which are inhibited by dopamine (Bender, Ford, & Trussell, 2010; Braun, 2009) or via P/Q type channels which are located primarily presynaptically and are typically involved in suppression of synaptic transmission (Wheeler, Randall, & Tsien, 1994). However, the entorhinal cortex contains both L-type and neuronal N-type voltage-gated calcium channels (Hernández-López et al.,1997) that can be phosphorylated by PKA (Hell et al., 1995), and others have also found that D_1-like receptor activation can enhance calcium influx via L-type and N-type VGCCs (Surmeiner et al., 1995). However, we found that blocking either L-type channels with the specific blocker nifidipine, or N-type channels using the L/N type blocker cilnidipine, did not prevent the dopaminergic facilitation of EPSC, indicating that they are not necessary for the dopaminergic facilitation of synaptic transmission.

Cytosolic calcium can also be increased via calcium release from internal sources by activation of IP_3 receptors or ryanodine receptors (RyRs). We found that blocking IP_3-receptors with heparin blocked the dopaminergic facilitation of synaptic transmission, and that, interestingly, blocking RyRs in a separate group of neurons also fully blocked the dopaminergic facilitation. The dependence of the dopaminergic facilitation on both receptor types suggests that cross-talk between IP_3Rs and RyRs may be necessary to amplify the cytosolic calcium concentration via calcium-induced calcium release (CICR). Calcium-induced calcium release can also be triggered by activation of VGCCs, or nicotinic, and metabotropic glutamate receptors (Abdul-Ghani et al., 1996), and IP_3 and RyR receptors may collaborate in propagating CICR in entorhinal neurons. Ryanodine and IP_3 receptors are typically segregated in clusters by receptor type, but they remain located proximally to each other (Sharp et al., 1993; Martone etal., 1993; Pozzan et al., 1994), thus it is possible that calcium release via IP_3Rs can activate nearby IP_3Rs and RyR channels (Horne & Meyer, 1997; Verkhratsky & Shmigol, 1996; Leite, Burgstahler, & Nathanson, 2002). This may coordinate small, localized calcium signals and amplify them into larger calcium waves via positive feedback, and increase activation of Ca^{2+}-dependent kinases (e.g. (Berridge, Lipp, & Bootman, 2000; Jaffe & Brown, 1994). Additionally, CICR has also been shown to play a role in mechanisms related to long-lasting synaptic plasticity (Emptage, Reid, & Fine, 2001; Rose & Konnerth, 2001). In addition, the phosphorylation of IP_3 and/or RyR by PKA may contribute to enhancement of release from internal stores, since IP_3Rs contain phosphorylation sites for PKA (Soulsby & Wojcikiewicz, 2005; Furuichi et al., 1989;

Wojcikiewicz & Luo, 1998; Meissner, 2002), which can increase calcium-binding to IP_3Rs (Joseph & Ryan, 1993), and potentiate IP_3-induced calcium flux (Rose & Konnerth, 2001; Nakade et al., 1994). Our data suggest that CICR regulated by both IP_3Rs and RyR may play a role in the transient regulation of synaptic strength in entorhinal neurons, although further evidence is needed to understand the detailed mechanism of action.

Activation of PI-linked D_1-like receptors which stimulate PLC activity triggers increases in IP_3 and calcium release from internal stores (Zhen et al., 2005; Jin et al., 2003; Hernández-López et al, 1997; Lezcano & Bergson, 2002) and we therefore tested the involvement of this receptor subtype using application of the PI-linked D_1-receptor agonist SKF83959. SKF83959 lead to a significant increase in EPSCs, and this provides the first evidence for the presence of PI-linked dopamine receptors in the entorhinal cortex. In addition, both the dopaminergic facilitation, and the facilitation induced by SKF83959, was blocked by the PLC inhibitor U-73122 consistent with PI-linked D_1-like receptor activation of PLC and resulting IP_3-mediated calcium release. Similar findings in the prefrontal cortex, striatum and hippocampus, show that high levels of PI-linked D_1-like receptor-mediated activation of PLC induce internal Ca^{2+}release (Undie et al., 1994; Lezcano & Bergson, 2002) and this provides a direct signaling pathway through which dopamine may increase cytosolic calcium levels.

Calcium-Dependent Enhancement of AMPA Receptor Function

Increases in cytosolic calcium can increase the activities of the calcium-dependent protein kinases CaMKII and PKC. Because CaMKII activity is known to modulate glutamatergic synaptic transmission and plasticity (Mammen et al., 1997; Derkach et al., 1999; Lee et al., 2003; Gonzalez-Islas & Hablitz, 2002) we examined the effect of the CaMKII blocker KN-93 on the dopamine-induced facilitation of glutamatergic EPSCs. Blocking CaMKII did not block the dopamine-induced facilitation, indicating that CaMKII is not required. In contrast, our findings indicate that inhibition of PKC activity with PKC(19–36) results in a complete block of the dopamine-mediated enhancement of synaptic transmission in lateral entorhinal neurons. Our results that blocking PKC activity blocks the dopamine-induced enhancement of glutamatergic EPSCs are consistent with previously reported data in the nucleus accumbens (Chergui & Lacey, 1999), and with in vitro experiments that have demonstrated that, similar to CaMKII, PKC phosphorylates the AMPA-receptor subunit GluR1 at Ser^{845} (Mammen et al., 1997; Derkach et al., 1999; Lee et al., 2003; Wang et al., 1994). Activation of PI-linked D_1-like receptors and PLC can enhance PKC via two routes: PLC leads to production of diacylglycerol (DAG) which directly activates PKC, and PLC also increases production of IP_3 to enhance cytosolic Ca^{2+} that also activates PKC (Figure 3.5; Ming et al., 2006; Zhen et al., 2005; Jin et al., 2003). Therefore, activation of PI-linked dopamine receptors and PLC may effectively activate PKC through both DAG and Ca^{2+}, and this provides a major mechanism through which dopamine may enhance AMPA-mediated synaptic responses in the lateral entorhinal cortex.

Combined Role of PKA- and PKC-Dependent Signaling

Our work has shown that dopamine leads to a facilitation of synaptic responses in the lateral entorhinal cortex through the combined effect of two major signaling pathways. We have previously shown that the facilitation is dependent on ‘classical’ D_1-like receptors that stimulate the cAMP-PKA pathway and

lead to inhibition of PP-1 which is known to dephosphorylate the Ser^{845} residue on the AMPA receptor (Glovaci, Caruana, & Chapman, 2014). Inhibition of PP-1 can increase EPSCs by promoting the phosphorylation of GluR1 at Ser^{845} and enhancing the effects PKA which phosphorylates GluR1 at this same residue (Yan et al., 1999). The present results also indicate that the facilitation is dependent on activation of PI-linked D_1-like receptors that increase activity of PLC and PKC. Protein kinase C promotes synaptic transmission by phosphorylating the Ser^{831} residue on the GluR1 subunit, and the combined effects of both PKA and PKC on both Ser^{845} and Ser^{831}residues provides a mechanism for significant changes mediated by increases in both AMPA receptor open-time probability and channel conductance (Mammen et al., 1997; Derkach et al., 1999; Lee et al., 2003).

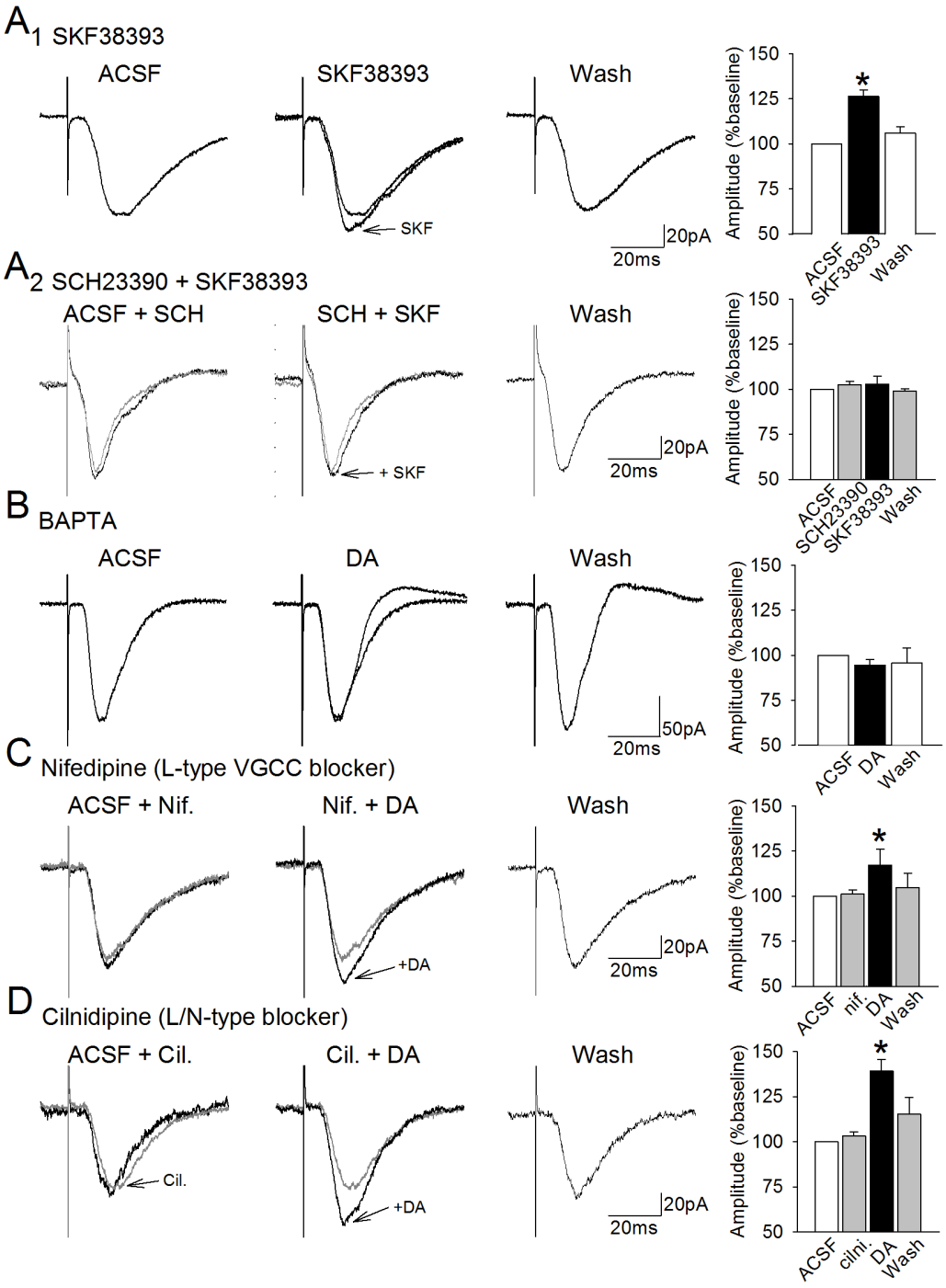
It is not clear why blocking elements in either signaling pathway can fully block the dopaminergic facilitation effect, and we do not currently have data to directly assess possible points of interaction between the pathways. Future studies using protein assays may be useful to determine the precise level of activation and possible sites of interaction between these pathways. The site of interaction must be dependent upon activation of both D_1-like receptor subtypes and be targeted by both signaling pathways. We believe that a probable site for the integration of both signals is adenylyl cyclase. Activation of 'classical' $G_{s/olf}$-linked D_1-like-receptors enhances adenylyl cyclase and cAMP-PKA activity, which could be further enhanced by activation of PI-linked D_1-like receptors via consequent rises in intracellular calcium. This idea is supported by findings showing that the increases in cytosolic calcium induced by PLC are similar to the concentrations of calcium required to elicit increases in adenylyl cyclase activity in vitro (Cali et al., 1994). In this scenario, activation of the PLC pathway alone might induce only partial PKC-dependent phosphorylation of the AMPA GluR1 receptor subunit, but increased Ca^{2+} might allow for enhanced cAMP-PKA-mediated phosphorylation of the AMPA receptor. Although IP_3R- and RyR-mediated increases in Ca^{2+} provide a possible mechanism for cooperative interactions between PLC and PKA signaling, it remains unclear why blocking elements of either pathway, such as PKC (Figure 3.4) or PKA and protein phosphatase 1 (Glovaci, Caruana, & Chapman, 2014) could result in an apparent inhibitory effect on activities in the complementary pathway to result in a full block of the facilitation effect.

Of the nine isoforms of adenylyl cyclase, calcium stimulates production of cAMP in isoforms AC1 and AC8. AC1 is found in the entorhinal cortex and throughout the hippocampal formation (Ferguson & Storm, 2004; Kumar et al., 2001), and similarly, AC8 is expressed abundantly in the entorhinal cortex, piriform cortex and hippocampus (Cali et al., 1994). In the hippocampus, mice lacking either isoform show greatly reduced calcium-induced adenylyl cyclase activity, which is linked to impairments in spatial memory and deficits in lasting synaptic plasticity (Wu et al., 1995; Vilacres et al., 1995; Wang et al., 2003). Half-maximal activation of AC1 by cytosolic calcium requires a four-fold lower concentration compared to AC8 (150–200 nM vs 800 nM), thus AC1 may be more sensitive to rises in cytosolic calcium, and subsequent production of cAMP. Although the exact adenylyl cyclase isoform involved in the dopaminergic facilitation remains to be determined, AC1 could be well-suited to act as a "coincidence detector" for the combined activation of G_s-coupled receptors and increased intracellular calcium because

these factors have a synergistic effect on synthesis of cAMP by AC1 that is not displayed by AC8 (Wayman, Wei, Wong, & Storm, 1996; Nielsen, Chan, Poser, & Storm, 1996).

Conclusions

The interplay between the two signaling cascades that we describe here indicates that the dopaminergic facilitation of glutamate-mediated EPSCs in layer II entorhinal neurons does not rely solely on classical D_1-like receptors and activation of the cAMP-PKA pathway, but that it also depends on PI-linked dopamine receptors that activate a PLC-dependent signaling cascade. The two pathways may interact synergistically via IP_3- and calcium-dependent enhancement of cAMP production. Since the dopaminergic facilitation of synaptic transmission described here likely serves to enhance processing of reward-relevant sensory information, the dependence of the facilitation upon the coordinated activity of two signaling cascades may serve to gate or limit increases in synaptic transmission to instances of intense and/or prolonged release of dopamine that could provide sufficient activation of both pathways. Further, it is also possible that the complexity of the signaling mechanisms that mediate the dopaminergic facilitation of synaptic transmission may allow for multiple points in the signaling process that may be modulated by activation of other neuromodulatory transmitter receptors as observed in other brain regions (Lezcano & Bergson, 2002; Partridge et al., 2002; Li & Rainnie, 2014; Navakkode, Sajikumar, & Frey, 2007).


A1 SKF38393
ACSF
SKF38393
Wash
SKF
20pA
20ms
Amplitude (%baseline)
150
125
100
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50
*
ACSF
SKF38393
Wash
A2 SCH23390 + SKF38393
ACSF + SCH
SCH + SKF
Wash
+ SKF
20pA
20ms
ACSF
SCH23390
SKF38393
Wash
B BAPTA
ACSF
DA
Wash
50pA
20ms
ACSF
DA
Wash
C Nifedipine (L-type VGCC blocker)
ACSF + Nif.
Nif. + DA
Wash
+DA
20pA
20ms
ACSF
nif.
DA
Wash
D Cilnidipine (L/N-type blocker)
ACSF + Cil.
Cil. + DA
Wash
Cil.
+DA
20pA
20ms
ACSF
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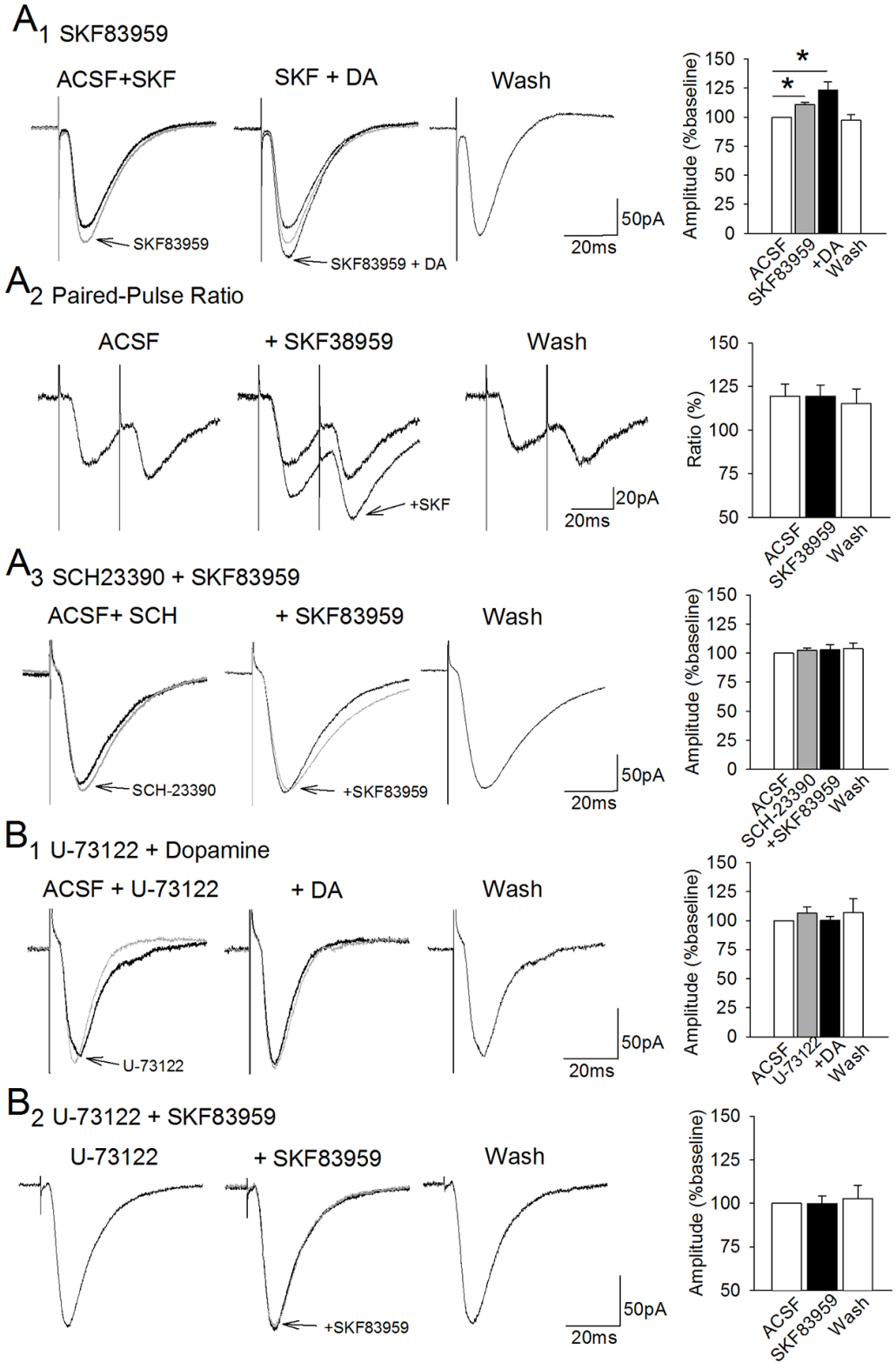
DA
Wash

Figure 3.1. Dopaminergic facilitation of EPSCs is dependent on intracellular calcium, but not L- or N-type VGCCs. **(A)** Bath application of the dopamine D_1-like receptor agonist SKF38393 (10 μM) induces a reversible facilitation of the amplitudes of glutamate-mediated excitatory postsynaptic currents in layer II lateral entorhinal cortex neurons **(A_1)**. Traces show averaged EPSCs for a neuron before, during, and after 5-min application of SKF38893. The histogram at right shows mean EPSC amplitudes for the group of cells. Bars indicate ± SEM and the asterisk indicates $p < 0.05$. In addition, the facilitation induced by SKF38393 is blocked in the presence of the D_1-like receptor blocker SCH23990 **(A_2)**. **(B)** Amplitudes of EPSCs recorded from cells filled with the Ca^{2+} chelator BAPTA (10 μM) remained stable during dopamine (DA) application. **(C, D)** Bath-application of the L-type voltage-gated calcium channel (VGCC) blocker nifedipine (10 μM; **C**) or of the L/N-type VGCC channel blocker cilnidipine (10μM; **D**) failed to block the dopaminergic facilitation of EPSCs (*, $p < 0.001$). Traces for the baseline and VGCCs blockers are superimposed.


A1 SKF83959
ACSF+SKF
SKF + DA
Wash
SKF83959
SKF83959 + DA
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Amplitude (%baseline)
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ACSF
SKF83959
+DA
Wash
A2 Paired-Pulse Ratio
ACSF
+ SKF38959
Wash
+SKF
20pA
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Ratio (%)
150
125
100
75
50
ACSF
SKF38959
Wash
A3 SCH23390 + SKF83959
ACSF+ SCH
+ SKF83959
Wash
SCH-23390
+SKF83959
50pA
20ms
Amplitude (%baseline)
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0
ACSF
SCH-23390
+SKF83959
Wash
B1 U-73122 + Dopamine
ACSF + U-73122
+ DA
Wash
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U-73122
+DA
Wash
B2 U-73122 + SKF83959
U-73122
+ SKF83959
Wash
+SKF83959
50pA
20ms
Amplitude (%baseline)
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125
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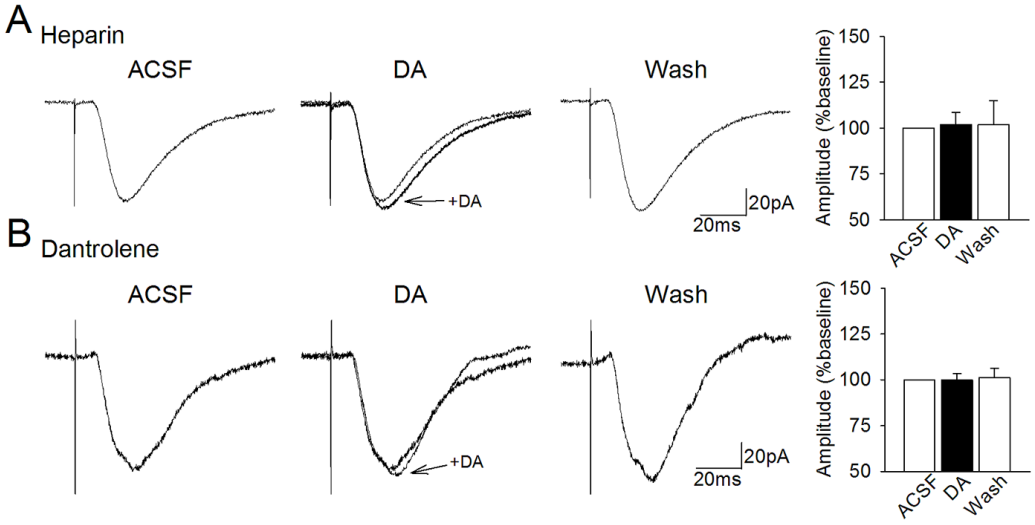
ACSF
SKF83959
Wash

Figure 3.2. The facilitation of EPSCs induced by the D_1-like receptor agonist SKF83959 is dependent upon PLC activation. **(A)** Bath application of the selective PI-linked D_1-like receptor agonist SKF83959 (5 or 10 μM) resulted in a significant enhancement of EPSCs (**A_1**, superimposed traces at left; *, $p < 0.05$). Subsequent addition of dopamine was followed by a further non-significant increase in mean EPSC amplitude (middle traces; $p = .06$). The facilitation of EPSC amplitude by SKF38959 was not associated with a change in paired-pulse facilitation ratio **(A_2)**. Application of the D_1-like receptor antagonist SCH23390 (10 μM; **A_3**) blocked the facilitation induced by SKF83959. **(B)** The PLC inhibitor U-73122 (10 μM) had no effect on baseline EPSCs (**B_1**, traces at left), but blocked the facilitation of EPSC amplitudes induced by dopamine (middle traces). Including U-73122 in the recording solution also blocked the facilitation of EPSC amplitudes induced by SKF83959 **(B_2)**.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Heparin
ACSF
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Wash
+DA
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20ms
Amplitude (%baseline)
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B
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Wash
+DA
20pA
20ms
Amplitude (%baseline)
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125
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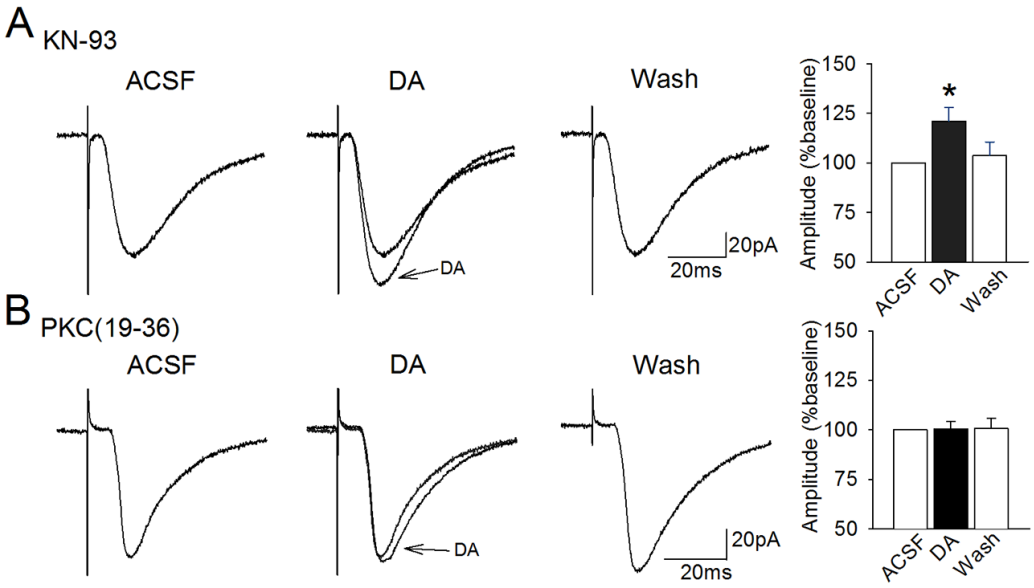
ACSF
DA
Wash

Figure 3.3. The DA-induced facilitation is dependent upon activation of both IP_3 and ryanodine receptors. **(A.)** Including the IP_3-receptor blocker heparin (1 mM) in the intracellular recording solution blocked the dopaminergic facilitation of EPSCs. **(B.)** Including the ryanodine receptor blocker dantrolene (20 μM) in the recording solution also blocked the facilitation of EPSCs by dopamine.


A
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20pA
20ms
Amplitude (%baseline)
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*
ACSF
DA
Wash
B
PKC(19-36)
ACSF
DA
Wash
DA
20pA
20ms
Amplitude (%baseline)
150
125
100
75
50
ACSF
DA
Wash

Figure 3.4. The dopaminergic facilitation is dependent on PKC but not on CaMKII. **(A)** The Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) can enhance AMPA receptor function but we found that intracellular application of the CaMKII inhibitor KN-93 (5 μM) did not prevent the dopamine-induced facilitation of EPSCs (*, $p < 0.05$). **(B)** In contrast, intracellular application of the PKC inhibitor PKC(19–36) reliably blocked the dopaminergic facilitation of EPSCs.

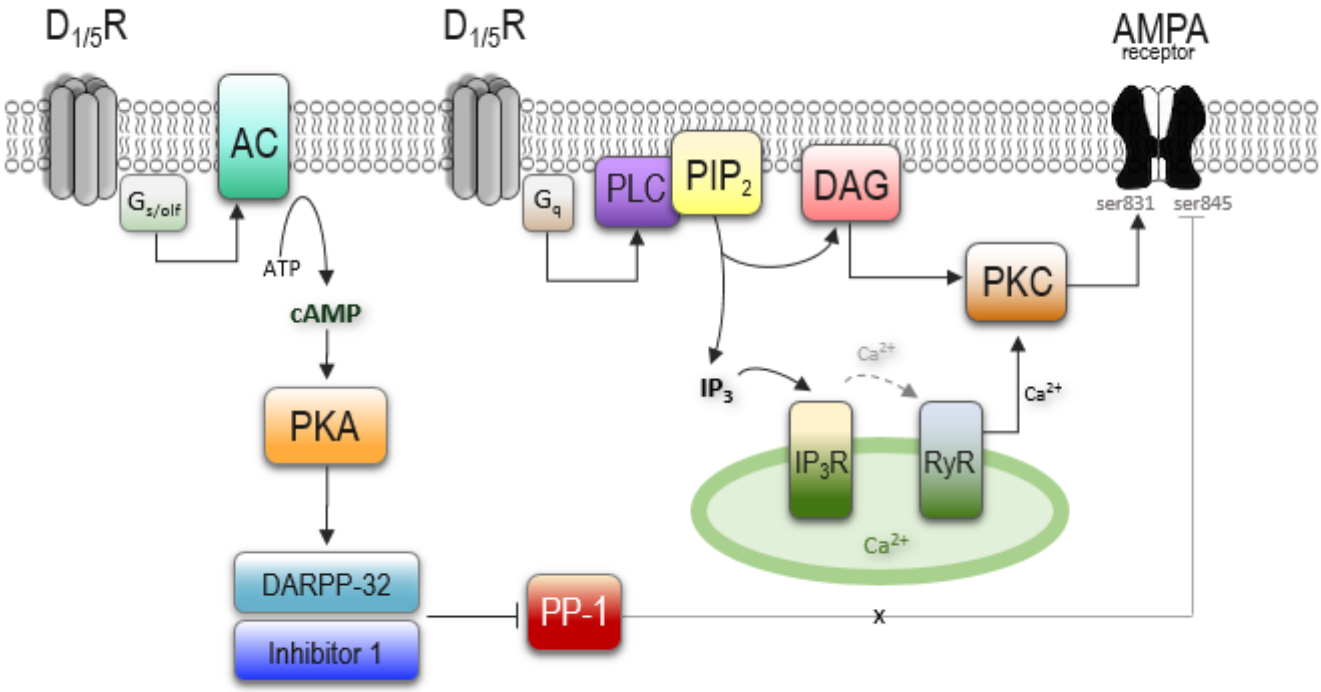

D1/5R
D1/5R
AMPA
receptor
AC
Gs/olf
ATP
cAMP
PKA
DARPP-32
Inhibitor 1
PP-1
Gq
PLC
PIP2
DAG
PKC
ser831
ser845
IP3
Ca2+
IP3R
RyR
Ca2+
Ca2+
x

Figure 3.5. Proposed signaling pathways that govern the dopaminergic facilitation of AMPA-mediated EPSCs. Classical D_1-like receptors are coupled to adenylyl cyclase (AC) via $G_{s/olf}$ proteins, and we have shown previously that the dopamine-induced facilitation is dependent upon the activity of both protein kinase A (PKA) and protein phosphatase 1 (PP-1) (Glovaci, Caruana, & Chapman, 2014). The PKA-mediated activation of inhibitor 1 (I-1) or of DARPP-32 (dopamine and cyclic AMP regulated phosphoprotein 32) at Thr^{34} may inhibit activity of protein phosphatase 1 (PP-1) and thereby potentiate EPSCs by reducing the dephosphorylation of AMPA GluR1 receptor subunits at Ser^{845}. The present results indicate that activation of D_1-like-receptors coupled to phospholipase C (PLC) via G_q proteins is also required for the facilitation of EPSCs. PLC leads to production of diacylglycerol (DAG) and IP_3 from PIP_2. IP_3 triggers Ca^{2+} release from internal stores via IP_3 receptor (IP_3R) activation, and may also trigger Ca^{2+} induced Ca^{2+} release via ryanodine receptors (RyR). Both increased cytosolic Ca^{2+} and DAG can activate protein kinase C (PKC) that can enhance AMPA receptor function via phosphorylation at Ser^{831}.

CHAPTER 4

DOPAMINE INDUCES TRANSIENT INCREASES IN INTRACELLULAR CALCIUM IN LAYER II LATERAL ENTORHINAL CORTEX FAN CELLS

ABSTRACT

The lateral entorhinal cortex (LEC) plays an important role in the sensory and mnemonic functions of the medial temporal lobe, particularly in regard to contextual information and object recognition processing. Therefore, modulation of synaptic input to the LEC may have important implications for learning and memory processes. Midbrain dopaminergic inputs terminate in layer II of the LEC, and we have shown previously that dopamine modulates synaptic responses in layer II LEC neurons in a dose-dependent manner, wherein high concentrations of dopamine (DA; 50-100 μM) suppress excitatory synaptic transmission, and lower concentrations (1 μM) facilitate synaptic transmission through a signalling cascade dependent upon activation of D_1-like receptors. The dopaminergic facilitation of glutamatergic synaptic transmission in layer II LEC neurons was dependent on activation of the classical D_1-cAMP-PKA pathway as well as on activation of PI-linked-D_1-like receptors that result in an increase in PLC, IP_3, PKC, and intracellular calcium. In the present study, we combined electrophysiological recordings of evoked EPSCs with fluorescent imaging of intracellular calcium using the indicator fluo-4 to monitor calcium transients evoked by dopamine in LEC fan and pyramidal cells. Bath application of dopamine (1 μM), or of the PI-linked D_1-like receptor agonist SKF83959 (5 μM), induced reliable and reversible increases in fluorescence in fan cells. However, no significant changes in fluorescence were observed during application of the classical D_1-like receptor agonist SKF38893 (10 μM). Inclusion of either heparin (1 mM) or dantrolene (20 μM) in the intracellular recording solution to block Ca^{2+} release from intracellular stores abolished both the Ca^{2+} transients and facilitation of EPSCs induced by dopamine. Calcium transients were also observed in the absence of extracellular calcium, further indicating that they are dependent on release from internal stores. Taken together, results indicate an important role for calcium signalling in the dopaminergic facilitation of synaptic transmission in layer II lateral entorhinal cells.

The entorhinal cortex is positioned as an interface between cortical sensory and associational areas and the hippocampal formation (Burwell, 2000) and it is thought to play an important role in spatial navigation, sensory processing, and mnemonic function (Hargreaves et al. 2005; McNaughton et al. 2006; Albasser et al. 2010; Yoganarasimha et al. 2010; Witter & Moser, 2006). Glutamatergic inputs carrying multimodal sensory information project from the neocortex to the superficial layers of the entorhinal cortex (Burwell & Amaral, 1998). In turn, principal neurons from these layers project via the perforant and temporoammonic paths to the dentate gyrus, CA3, CA1, and subicular regions of the hippocampal formation (Witter et al., 1989). The activity of layer II and III entorhinal cells that project to the hippocampal formation is regulated by several neuromodulatory transmitters, including dopamine (Mingote et al., 2015; Mitchell et al., 1982; Köhler et al., 1980; Palkovits et al., 1979). Mesocorticolimbic dopamine inputs play an important role in the processing of salience and reward-relevant stimuli, contribute to hippocampal and cortical mechanisms of learning and memory (Bandyopadhyay, Gonzalez-Islas, & Hablitz, 2005; Berridge, 2007; Bromberg-Martin et al., 2010), and could contribute to the mnemonic functions of the entorhinal cortex through the modulation of excitatory transmission within the superficial layers of the entorhinal cortex.

Dopaminergic cells in the ventral tegmental area and substantia nigra project heavily to the superficial layers of the LEC, and synapse directly onto clusters of LEC cell 'islands' (Clark et al., 2012; Mingote et al. 2015). In layer II neurons of the lateral entorhinal cortex, high concentrations of dopamine transiently suppress excitatory synaptic transmission via a D_2-like receptor-dependent suppression of glutamate release (Caruana & Chapman, 2008), but lower concentrations of dopamine enhance excitatory synaptic transmission post-synaptically via D_1-like receptor-mediated signalling (Caruana et al., 2006; Glovaci & Chapman, 2015). Physiological release of dopamine in the lateral entorhinal cortex may therefore promote excitatory synaptic activation of entorhinal neurons, and enhance transmission of salient information to the hippocampal formation during processes contributing to working memory and long-term memory formation.

We have found that the dopaminergic facilitation of glutamate-mediated synaptic responses in layer II principal cells occurs via two intracellular signalling cascades that lead to a rapid and reversible increase in AMPA receptor-mediated currents (Glovaci et al., 2014; Glovaci & Chapman, 2015). The increase in AMPA-mediated-currents induced by dopamine was blocked by inhibition of PKA, suggesting that the facilitation was due to activation of 'classical', protein kinase A (PKA)-linked D_1-like receptors, and inhibition of PP-1-dependent dephosphorylation of AMPA receptors (Snyder, Fienberg, Huganir, & Greengard, 1998; Kemp & Bashir, 2001; Malenka & Bear, 2004). Activation of atypical phosphatidylinositol (PI)-linked D_1-like receptors has also been found to lead to enhancement of AMPA currents, via increased activity of phospholipase C (PLC), production of diacylglycerol (DAG) and IP_3, and subsequent release of calcium from internal stores (Jin et al., 2003; Zhen et al., 2005). Similarly, we found that the facilitation induced by dopamine in the LEC was prevented by chelation of intracellular calcium using BAPTA, or by blocking either IP_3 or ryanodine receptors, which are known to control release of calcium from internal stores (Glovaci & Chapman, 2015). These results mirror similar findings linking

increases in cytosolic calcium subsequent to D_1-like receptor activation to enhanced synaptic transmission in the striatum (Flores-Hernandez et al., 2002), neocortex (Cepeda et al., 1992), prefrontal cortex (Tseng & O'Donnell, 2004), and hippocampus (Yang, 2000). The dopaminergic facilitation of AMPA currents observed in the lateral entorhinal cortex was also dependent upon protein kinase C (PKC), which can be activated by DAG as well as by increases in intracellular calcium (Glovaci & Chapman, 2015). Enhanced activation of PKC can increase phosphorylation of AMPA receptors at $Ser^{831/818}$, a mechanism known to potentiate AMPA-receptor currents in hippocampal neurons by increasing channel conductance (Barria et al., 1997; Mammen et al., 1997). Together, these findings suggest that dopamine facilitates synaptic transmission in layer II LEC neurons by increasing AMPA-receptor-mediated currents both via classical D_1-like receptors, and by activation of PI-linked D_1-like receptors that increase intracellular calcium signalling.

Both IP_3 and RyR receptors appear to contribute to the dopamine-induced facilitation of synaptic responses in layer II entorhinal neurons. Activation of dopamine receptors is known to modulate calcium entry via voltage-gated calcium channels (VGCC; Malgaroli et al. 1987; Surmeier et al. 1995; Young & Yang, 2004; Bender et al. 2010), but we have previously reported that blocking L/N-type VGCCs did not decrease the dopaminergic enhancement of synaptic currents in LEC neurons (Glovaci & Chapman, 2015). However, blocking activation of either IP_3 or RyR receptors, which control calcium release from internal stores, abolished the facilitation induced by dopamine (Glovaci & Chapman, 2015). IP_3Rs are abundant in the hippocampal and parahippocampal areas (Sharp et al., 1993; Evstratova & Toth, 2011; Kapur, Yeckel, & Johnson, 2001), and moderate levels of ryanodine type II receptor protein are also expressed in the entorhinal cortex, with the highest density being concentrated in the superficial layers (Zhao, Meiri, Xu, et al., 2000). IP_3Rs are activated by a number of Ca^{2+}-binding proteins, and by IP_3 which is produced by PLC (Berridge, 1993; Yang et al. 2002). In contrast, RyRs are activated by caffeine or increases in intracellular Ca^{2+} which are mediated by IP_3R, VGCCs, or NMDA glutamate receptors (Trafford et al. 1995). We have previously demonstrated that Ca^{2+} signalling is required for the dopaminergic facilitation of AMPA currents in LEC layer II neurons, but the time-course and size of the increases in calcium concentrations during dopamine application have not been described.

Here, we combined whole-cell patch-clamp recordings and fluorescence imaging of intracellular calcium concentration with the indicator fluo-4 to track changes in synaptic transmission and cytosolic calcium concentration during application of dopamine to layer II entorhinal principal cells. The classical D_1-receptor agonist SKF383893 and the PI-linked D_1-like receptor agonist SKF83959 were also applied to assess the effects of activating either pathway on cytosolic calcium concentration (Glovaci & Chapman, 2015). The source of calcium transients was also assessed using recordings in the absence of external calcium to minimize entry of extracellular calcium, and by including either IP_3 or ryanodine receptor blockers in the intracellular recording solution. Layer II entorhinal cortex fan cells, which show a marked sag in voltage response to strong hyperpolarizing current injections and firing patterns that include oscillatory periods, consistently show synaptic facilitation during application of dopamine, while layer II pyramidal cells, which generally show no substantial sag response and regular firing patterns, did not

show the facilitation reliably. The current experiments allowed us to assess wether differences in the facilitation effects in fan and pyramidal cells are associated with the presence or absence of changes in intracellular calcium during dopamine application. Our findings indicate that, in responsive neurons, dopamine or the PI-linked D_1-like receptor agonist SKF83959 both result in moderate elevations in intracellular calcium that are dependent on the activity of both IP_3R and RyR.

METHOD

In Vitro Slice Preparation

Brain slices (300 µm) were obtained from 4 to 9-week-old male Long Evans rats (Charles River). Animals were anesthetised with isoflurane, decapitated, and brains were quickly extracted and submerged into ice cold, high-sucrose artificial cerebrospinal fluid (ACSF; saturated with 95% O_2 and 5% CO_2, pH ≈7.3) containing (in mM) 250 sucrose, 2 KCl, 1.25 NaH_2PO_4, 7 $MgCl_2$, 26 $NaHCO_3$, 0.5 $CaCl_2$ and 10 dextrose. All drugs were obtained from Sigma–Aldrich or Tocris Life Sciences, unless indicated otherwise. Horizontal slices containing the entorhinal region were obtained using a vibratome (300 µm thick; WPI, Vibroslice, Sarasota, USA). There was a recovery period of at least one hour in normal ACSF containing (in mM) 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 dextrose (pH ≈7.4; 300-310 mOsm; ~22 ºC).

Electrophysiological Recordings

During recordings, individual slices were maintained in a recording chamber and superfused with ACSF at room temperature (2-4 ml/min). Individual slices were visualized with an upright microscope using differential interference contrast optics and a water-immersion objective equipped with a 40x objective (Leica, DM-LFS). Layer II of the lateral entorhinal cortex was distinguished from layers I and III based on the presence of cell islands (Amaral & Witter, 1989). Borosilicate glass recording pipettes (1.0 mm OD; 3.2 to 4.8 MΩ) were filled with (in mM) 140 K-gluconate, 10 HEPES, 5 NaCl, 2 $MgCl_2$, 2 ATP-Tris, 0.4 GTP-Tris, and 0.15 fluo-4 (Molecular Probes, OR). A bipolar stimulating electrode made from two tungsten electrodes (~1 MΩ, FHC Inc.) was placed in layer I of the lateral entorhinal cortex to evoke excitatory postsynaptic currents in layer II cells. Patch-clamp recordings were obtained using an Axopatch 200B amplifier, filtered at 5-10 kHz, and digitized at 20 kHz (Molecular Devices, Digidata 1322A).

After obtaining tight seals (1-3 GΩ) and whole-cell configuration in voltage clamp mode, electrophysiological characteristics and firing properties of entorhinal neurons were characterized in current-clamp mode to distinguish fan and pyramidal neurons as previously described (Glovaci, Caruana, & Chapman; Glovaci & Chapman, 2015; Tahvildari & Alonso, 2005). Membrane potential responses were recorded in response to 500 ms duration current pulse injections, ranging from -200 to +60 pA. Responses were analyzed using the Clampfit 8.2 software package (Molecular Devices). Inward rectification was quantified as the ratio between peak and steady-state input resistances in response to -200 pA hyperpolarizing current pulses. Action potential waveform was assessed using the first action potential evoked in response to positive current injection. Action potential amplitude was calculated from

resting membrane potential and action potential duration and afterhyperpolarization were measured from action potential threshold.

Excitatory postsynaptic currents were recorded at a holding potential of -60 mV and were evoked by 0.1 ms-duration constant current pulses delivered using a stimulus timer and isolation unit (WPI, Models A300 and A360). Stimulus intensity (92.7 ± 13.2 μA) was adjusted to evoke responses of roughly 70% maximal amplitude. Synaptic responses were recorded once every 15 sec and 10 traces were obtained in each recording condition (see below). Input resistance, access resistance and capacitance were monitored using responses to -10 mV, 50 ms voltage steps delivered following each evoked synaptic response, and recordings were discontinued if values changed by >15%.

Fluorescence Imaging

The Ca^{2+} indicator probe fluo-4 (excitation: 494 nM, emission: 516 nM) was allowed to diffuse into the cell from the recording electrode for a minimum of 20 min prior to the start of fluorescence recordings. Fluorescence illumination was controlled via a Lambda 10-2 optical filter changer (Sutter Instruments, CA) and fluorescence filter cube (Chroma, C6892, ET480/40x and ET535/50m filters). Fluorescence images were acquired using a CCD camera (Sensicam QE; Optikon, ON) controlled using Axon Imaging Workbench 4.0. (Axon Instruments, CA). Images were collected using 150 ms exposure times (8 bit/pixel, 8 x12 binning) once every 4 sec during a 5-10 min baseline period, a 5-10 min drug application period, and a 10-20 min washout period.

Pharmacological Testing

After recording baseline electrophysiological responses, fluorescence calcium signals were monitored during a 5 to 15 min baseline period, and during 5 to 10 min bath application of either dopamine (1 μM, Sigma), the classical D_1-like receptor agonist SKF38393 (10 μM, Sigma), or the PI-linked agonist SKF83959 (5 μM). Oxidation of dopamine was inhibited by co-application of sodium metabisulfite (50 μM), and there was a wash-out period of up to 20 min to assess the reversibility of drug effects.

In additional tests, the IP_3 receptor blocker heparin (1 mM; Sigma) or the ryanodine receptor blocker dantrolene (1 μM; Tocris) were included in the intracellular solution to test the effects of blocking release of calcium from intracellular stores during dopamine application. To determine if calcium transients were reliant on influx of extracellular calcium, dopamine was applied in tests conducted during constant bath application of a low-calcium ACSF. Electrophysiological responses were recorded at the end of the drug-application period, and at the end of the washout period in all cases, except when synaptic transmission was blocked during for recordings during exposure to low-calcium ACSF.

Data Analysis

Fluorescence signals in each cell were quantified by measuring the mean intensity value of a manually selected region of interest within the soma that did not include the electrode. Prolonged, strong depolarization of neurons to elevate intracellular calcium due to entry via voltage-gated calcium channels resulted in much larger fluorescent signals than induced by dopamine application (not shown), indicating that the calcium transients recorded were well below saturation levels for fluo-4. Fluorescence levels were quantified using Axon Imaging Workbench 4.0. Fluorescence data were first corrected for background

fluorescence by subtracting the mean value within a region distant from the recorded cell and any visible dendrites. Changes in Ca^{2+}-dependent fluorescence were then expressed as a percentage change in fluorescence relative to the mean baseline value.

To assess drug effects on fluorescence, the mean intensity during the last 5 min of the baseline period, the last minute of the drug application period, and the last minute of the washout period were obtained for each cell, and averaged across cells. Differences between treatment conditions in both fluorescence and electrophysiological measures were assessed using one-way repeated measures ANOVAS, followed by post-hoc Student-Newman-Keuls pairwise comparisons, using an alpha level of .05.

RESULTS

Dopamine Leads to Increased Calcium Signals in LEC Fan Neurons

Consistent with our previous findings that low doses of dopamine facilitate synaptic responses in layer II LEC neurons via a calcium-dependent intracellular signalling cascade (Glovaci & Chapman, 2015), we found that bath application of 1 μM dopamine led to increased calcium fluorescence during dopamine application (Figure 4.1A). Increases in somatic fluorescence were observed within approximately 2 min of dopamine application, and generally reached near-maximum levels after 5 min of dopamine application (110.6 ± 3.98 %; $n = 6$; $F_{(5,17)} = 5.9$, $p = .02$). The increased fluorescence reversed towards baseline levels during 10 to 20 min washout in ACSF (102.1 ± 2.47 %).

Since we previously demonstrated that fan and pyramidal cells can be modulated differentially by dopamine, such that fan cells exhibit reliable increases in synaptic transmission during dopamine application, but pyramidal cells vary in their responses (Glovaci & Chapman, 2015), we investigated how elevations in calcium levels may differ by cell type. Three of the cells exposed to dopamine were identified as fan cells, and 3 were identified as pyramidal neurons, based largely on electrophysiological properties. Fan cells consistently showed large increases in calcium fluorescence when exposed to 5 min dopamine (118.6 ± 3.8 % during dopamine vs 106.1 ± 3.0 % at washout; $F_{(2,8)} = 9.6$, $p = .03$). In contrast, pyramidal cells did not show a significant increase in calcium fluorescence in the presence of dopamine (102.7 ± 1.4 % during dopamine vs. 98.1 ± 2.3 % at washout; $F_{(2,8)} = 3.1$, $p = .15$).

Increased fluorescence signals were paired with increases in evoked EPSCs, such that fan cells that showed increased glutamatergic EPSCs during dopamine application also showed elevated fluorescence signals (Figure 4.1B). Across all fan and pyramidal neurons tested, EPSC amplitude was increased during dopamine to 122.5 ± 8.2 % of baseline ($n = 6$; $F_{(5,17)} = 6.08$, $p = .02$; *SNK:* $p = .017$ for baseline vs dopamine) and reversed to control levels during washout (*SNK:* $p = .79$ for baseline versus washout conditions). However, the facilitation of EPSCs was dependent on celltype, and correlated with increases in intracellular calcium. There were reliable and reversible increases in EPSC amplitudes in fan cells (138.4 ± 8.3 % of baseline; $F_{(2,8)} = 24.3$, $p = .006$; *SNK:* $p = .007$ for baseline vs dopamine, and $p = .665$ between baseline and washout conditions), but EPSC amplitudes in pyramidal cells did not increase substantially or significantly during dopamine application (106.6 ± 2.8 % of baseline; $F_{(2,8)} = 1.13$, $p = .41$).

Activation of Classical versus PI-linked D_1-like Receptors

The atypical D_1-like receptor agonist SKF83959 induced changes in calcium-dependent fluorescence and in EPSCs amplitude similar to those induced by dopamine. A 5 min bath application of SKF83959 increased fluorescence signals (115.2 ± 3.1% during dopamine; n = 8; $F_{(7,22)}$ = 17.57, p < .001; see Figure 4.2) in a reversible manner (104.0 ± 1.7 % after washout; p = .003). As observed with dopamine, this effect was dependent on increased fluorescence in fan cells (n = 5; 119.7 ± 3.3 % during dopamine vs 106.6 ± 2.19 % at washout; $F_{(4,13)}$ = 20.7, p = .001) as compared to smaller changes in fluorescence observed in pyramidal cells (n = 3; 107.6 ± 2.5 % during dopamine vs 100.6 ± 1.2 % at washout; $F_{(2,8)}$ = 4.9, p = .08). Similar to our previous results (Glovaci & Chapman, 2015), SKF83959 induced reliable increases in EPSC amplitude in fan cells (119.0 ± 4.0 % of baseline; $F_{(2,14)}$ = 19.17, p < .001; *SNK:* p = .002 for baseline vs dopamine; p =.70 between baseline and washout conditions), but did not induce significant increases in EPSC amplitude in pyramidal neurons (101.6 ± 4.7 % of baseline; $F_{(2,8)}$ = 0.2, p = 0.82). One pyramidal cell showed a moderate increase in EPSCs (109.8 %), consistent with a small increase in fluorescence in this cell (109.3%).

Application of the classical D_1-like receptor agonist SKF38393 (10 μM), however, did not result in correlated changes in calcium fluorescence and EPSC amplitudes. SKF38393 resulted in a significant facilitation of EPSC amplitudes to 118.1 ± 4.1% of baseline values (n = 6 including 5 fan and 1 pyramidal cell; $F_{(5, 10)}$ = 17.93, p < .001; *SNK:* p = .001 for baseline vs dopamine, p = .77 for baseline vs washout conditions), but it did not significantly affect calcium fluorescence (99.2 ± 1.2 % during dopamine and 99.9 ± 0.5 % during washout; $F_{(5, 15)}$ = 0.46, p = .65; Figure 4.3). This suggests that activation of classical D_1-like receptors does not play a significant role in calcium transients during dopamine application, and emphasizes the importance of the atypical PI-linked D_1-like receptors in triggering calcium transients in LEC neurons during dopamine application.

It has been previously suggested that PKA can increase PLC activity, and thereby enhance the PLC-DAG-IP_3 pathway (Yu, Eisner, Yamaguchi, et al., 1996). To determine if this might impact calcium transients induced by dopamine receptors in layer II neurons, two fan cells were continually exposed to the classical D_1-like-receptor agonist SKF38393, and the effect of 5 min co-application of PI-linked agonist SKF83959 on calcium signals was assessed. Results demonstrated that while SKF38393 alone did not change fluorescence signals from baseline levels (application in all cells 102.8 ± 2.1 %, p = .879, subsequent co-application of SKF83959 significantly increased cytosolic calcium ($F_{(2, 7)}$ = 25.03, p = .038; *SNK:* p = .027, from 102.3 ± 2.1 % in SKF38393 to 114.2 ± 3.4 % in both agonists, data not shown). This increase is similar to the increased fluorescence observed during application of SKF83959 alone (see Figure 4.2), suggesting that increases in cytosolic calcium arising from activation of PI-linked receptor and PLC-DAG-IP_3 pathway are not substantially enhanced by concurrent activation of PKA via classical D_1-like receptors.

Increases in Intracellular Calcium during Dopamine Application Depend on IP_3R and RyRs

Our previous electrophysiological recordings demonstrated that blocking calcium release from internal stores via IP_3R or RyR, but not blocking voltage-gated-calcium channels, prevented the

dopaminergic facilitation of EPSCs (Glovaci & Chapman, 2015). Results obtained here demonstrate that blocking IP_3 receptors or RyR abolishes the increase in fluorescence induced during application of dopamine, and also prevents the facilitation of synaptic responses. In cells loaded with the IP_3R-blocker heparin (1 mM), fluorescence signals remained stable during dopamine application (n = 4 including 3 fan cells and 1 pyramidal cell; 102.0 ± 1.6 % during dopamine vs 98.7 ± 2.9 % at washout; $F_{(3,10)}$ = 1.65, p = .28), and there was also no significant increase in EPSCs induced by application of dopamine (102.0% ± 6.7% in dopamine, Figure 4.4A-B). Similarly, cells filled with the RyR blocker dantrolene (1 μM) did not show significant increases in either fluo-4 fluorescence (n = 6 including 4 fan cells and 2 pyramidal cells; 102.7 ± 1.9 % during dopamine vs 102.0 ± 0.0 % at washout; $F_{(3,8)}$ = 1.05, p = .45), or EPSC amplitudes (99.8% ± 3.7% during dopamine, Figure 4.4C-D). These data indicate that activation of both IP_3 receptors and RyR is required for the dopamine-induced release of calcium from internal stores, and are consistent with the previous finding that blocking increases in intracellular calcium prevents the dopaminergic facilitation of synaptic responses induced by dopamine in layer II LEC neurons(Glovaci & Chapman, 2015).

To further determine whether release from internal stores alone underlies dopamine-induced calcium transients, we also conducted recordings in calcium-free ACSF, to inhibit entry of extracellular calcium via voltage-gated calcium channels or NMDA receptors. Slices were incubated in Ca^{2+}-free ACSF containing 2 mM of the calcium chelator EGTA for a minimum of 10 min prior to application of dopamine. In Ca^{2+}-free ACSF (Figure 4.5), application of dopamine significantly elevated fluorescence levels (n = 6 including 4 fan cells and 2 pyramidal cells; 117.6 ± 5.0 % in dopamine to 99.9 ± 1.1 % at washout; $F_{(5, 13)}$ = 7.44, p = .02). The increases in calcium fluorescence that were observed in Ca^{2+}-free ACSF were similar to those observed in normal ACSF, suggesting that intracellular calcium transients induced by dopamine do not depend substantially on influx of extracellular calcium.

DISCUSSION

We report here that application of either dopamine or the PI-linked D_1-like receptor agonist SKF83959 leads to transient increases in the concentration of intracellular Ca^{2+} in layer II principal cells of the lateral entorhinal cortex. These increases in intracellular calcium were reliably observed in fan, but not pyramidal, cells, even in the absence of extracellular calcium, suggesting that the mechanism of action is dependent on internal calcium stores. This result is confirmed by our findings that the dopamine-induced increases in intracellular calcium were dependent on activation of both IP_3 and ryanodine receptors. This suggests that dopamine acts to increase intracellular calcium in fan cells through activation of non-classical D_1-like receptors that are linked to activation of PLC (Panchalingam & Undie, 2001; Lezcano & Bergson, 2002). In contrast, we did not observe any changes in intracellular calcium during application of SKF38393, an agonist that activates conventional D_1-like receptors linked to increases in cAMP production and PKA activity.

Our results indicate that dopamine exerts differential effects in fan and pyramidal cells of layer II of the LEC. During the presence of dopamine or its agonists, we did not observe a reliable increase in

intracellular calcium in pyramidal neurons. As such, the results suggest that, as opposed to mechanisms present in fan cells, dopamine does not activate signalling cascades leading to increased intracellular calcium in pyramidal neurons. This points to the likelihood that fan and pyramidal cells differ in their contributions to computational functions in the processing of salient stimuli that may be encountered during activation of dopamine neurons. Fan and pyramidal neurons differ in terms of morphology, especially in their dendritic arborization patterns (Tahvildari & Alonso, 2005), and it has been recently suggested that layer II LEC neurons appear to loosely cluster together into sublayers that are generally composed of different cell types (Kobro-Flatmoen & Witter, 2017). This suggests possible functional differences between the cell types. At present, however, it is unclear how cell-type-specific increases in intracellular calcium in response to dopamine may impact entorhinal cortex function.

Relationship of Increased Intracellular Calcium to the Facilitation of Synaptic Responses

The increases in intracellular calcium in fan cells induced by dopamine and by SKF83959 were associated with concurrent increases in the amplitude of EPSCs. Cells showing increases in fluo-4 fluorescence in the presence of dopamine also showed a facilitation of evoked excitatory postsynaptic currents, and both increases in calcium fluorescence and EPSC amplitude were reversed after removal of dopamine from the ACSF. We observed previously that the dopaminergic facilitation of EPSCs was blocked by the calcium chelator BAPTA, or by blocking IP_3R or RyR (Glovaci & Chapman, 2015), and the present results indicate that blocking calcium release via IP_3R or RyR blocks the facilitation of EPSCs induced by dopamine. This suggests that intracellular calcium is a critical component of intracellular signalling mediating the effects of D_1-like receptor activation on AMPA-mediated synaptic currents (Glovaci, Caruana, & Chapman, 2014). Facilitation of synaptic responses by dopamine in the prefrontal cortex, striatum, and hippocampus (Flores-Hernandez et al., 2002; Cepeda et al., 1992; Tseng & O'Donnell, 2004; Yang, 2000) are also dependent on increases in intracellular Ca^{2+}, but the mechanisms linking increased calcium to increases in synaptic responses in these regions are not well understood.

Increases in the PLC-DAG-IP_3 pathway induced by SKF83959 can lead to increased intracellular calcium and subsequent increased activation of calcium-dependent kinases (e.g. Zhen et al. 2005). We previously showed that the facilitation of synaptic responses by dopamine in the entorhinal cortex is dependent upon PKC, but not CaMKII (Glovaci & Chapman, 2015), which can phosphorylate GluR1 AMPA-receptor subunits at Ser^{831}, thereby increasing channel conductance (Lee et al., 2000; Derkach, Barria, & Soderling, 1999). Therefore, the facilitation of synaptic responses induced by dopamine and by SKF83959 is likely mediated in part by PKC-dependent strengthening of AMPA-receptor-mediated synaptic transmission. Consistent with this is the suggestion that PKC can be a stronger catalyst of AMPA-receptor phosphorylation in hippocampal tissue when compared to CaMKII (Tan et al., 1994), although this may be restricted to cultured tissue (McGlade-McCulloh et al., 1993).

A surprising finding observed here was that application of the classical D_1-like receptor agonist SKF38393, although increasing the amplitude of synaptic responses as has been observed before (Glovaci & Chapman, 2015), was not associated with increased calcium fluorescence. Activation of classical D_1-like receptors is well known to lead to increased cAMP production and increases in PKA

activity. Both IP_3 and ryanodine receptors contain phosphorylation sites for PKA (Soulsby & Wojcikiewicz, 2005; Furuichi et al., 1989; Meissner, 2002), and phosphorylation of these sites leads to increases in calcium-binding to IP_3Rs (Joseph & Ryan, 1993), which can potentiate calcium flux (Nakade et al., 1994). This would have provided a possible pathway through which classical D_1-like receptor activation could have contributed to increased intracellular calcium as an intermediate step in the facilitation of AMPA-receptor-mediated responses. However, we did not observe any increases in fluo-4 fluorescence during application of the classical D_1-like agonist SKF38393. This suggests that PKA-mediated phosphorylation of IP_3R and RyR on internal stores is not sufficient for dopamine-induced increases in cytosolic calcium in layer II lateral entorhinal cortex neurons.

The present results, combined with our previous observations that blocking PKA activation also blocks the dopaminergic facilitation of synaptic responses (Glovaci, Caruana, & Chapman, 2014) suggest that activation of classical D_1-like receptors leads to a PKA-dependent facilitation of AMPA-receptor-mediated responses that is not related to marked increases in intracellular calcium. This process is likely mediated by PKA-dependent conversion of the dopamine- and cAMP-regulated phosphoprotein, 32 kDa, (DARPP-32) into a potent inhibitor of protein phosphatase 1 (PP-1). Since under basal conditions, PP-1 dephosphorylates GluR1 subunits of the AMPA receptors (Yan et al., 1999; Song & Huganir, 2002), reducing PP-1 activity could lead to increased AMPA-receptor phosphorylation, and enhanced synaptic currents, and this could mediate the SKF38393-induced facilitation of AMPA-mediated synaptic transmission that we observed here.

The Combined Roles of IP_3 and RyRs

We show here that dopamine-dependent increases in intracellular calcium in layer II fan neurons are due to internal release, rather than influx of extracellular calcium. First, the dopaminergic facilitation was blocked in the absence of extracellular calcium. Further, both the dopamine-induced increases in fluorescence and synaptic responses were blocked by intracellular application of either heparin or dantrolene to block IP_3 or ryanodine receptors, which are known to control calcium release from internal stores (Berridge, 1998). The hippocampal and parahippocampal regions are rich in both receptor types (Sharp et al., 1993; Evstratova & Toth, 2011; Kapur et al., 2011; Zhao, Meiri, Xu, et al., 2000). It is interesting that activation of both IP_3R and RyR is required for dopamine-induced increases in intracellular calcium. This effect might be linked to their different distribution within cells. While IP_3Rs are widely distributed within spines, dendrites, soma and synaptic terminals (Mignery et al. 1989; Otsu et al., 1990; Takei et al. 1992), RyRs are predominantly located in apical dendrites (Sharp et al., 1993). IP_3Rs and RyR also have different signalling mechanisms, with IP_3R-mediated calcium release being dependent on activation of G-proteins-coupled receptors (Niswender & Conn, 2010; Berridge, 1993), and RyRs being primarily activated by elevations in cytosolic calcium and caffeine (e.g. Tsien & Tsien, 1990; Hernández-Cruz et al., 1997). It is possible that the increases in observed fluorescence signals reflect calcium release initiated by IP_3 receptor activation that is further sustained by ryanodine receptor activation. Calcium-induced-calcium-release has been observed in a number of other brain regions (CICR; Kano et al., 1995; Verkhratsky & Shmigol, 1996), with the amplitude of the calcium transients varying across cell types.

However, the sizes of the calcium transients observed here are relatively modest as compared to the size of calcium transients observed during classical CICR, and we did not observe rhythmic peaks in fluorescence signals that are typical of calcium-induced calcium release (Berridge, 2000; Jaffe & Brown, 1994), suggesting that a typical CICR mechanism is unlikely to underlie the dopaminergic facilitation. In a different study, Ming and colleagues (2006) also found that SKF83959 application resulted in sustained elevations in cytosolic calcium in hippocampal neurons. The increase in calcium was detectable within three minutes of drug application, peaked within 5 min, and remained at a stable plateau for at least 20 min during drug application. Previously, sustained elevations in intracellular calcium have been suggested to modulate processes of neuroplasticity and gene expression, suggesting that in addition to transiently enhancing synaptic transmission, dopamine D_1-like receptor activation could potentially contribute to changes in long-term synaptic transmission in this area (e.g. Otmakhova & Lisman, 1996).

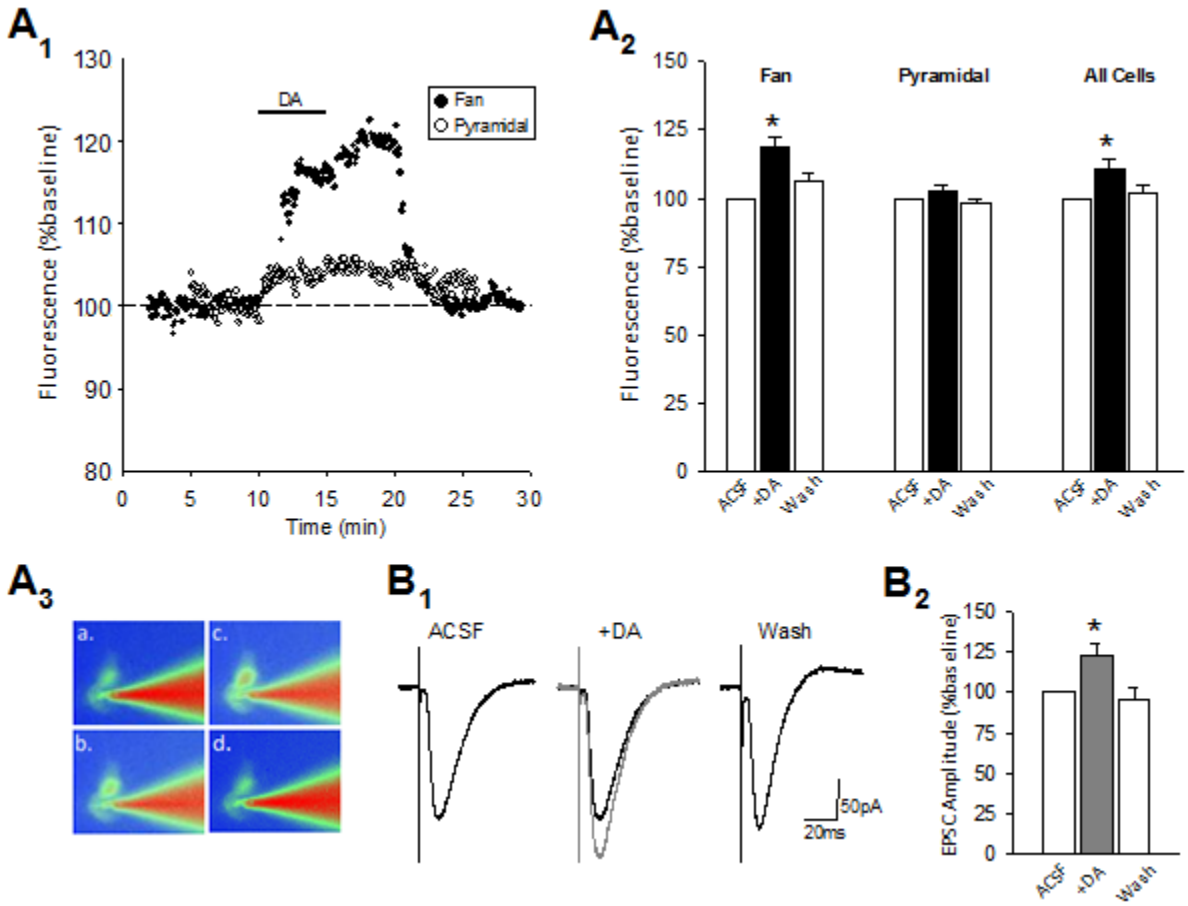
The present results reflect modest increases in intracellular calcium during dopamine, and are consistent with a milder form of cooperativity between IP_3R and RyR. Dopamine may recruit both IP_3Rs and RyRs to induce relatively small but *sustained* elevations in intracellular calcium. Because calcium can promote activation of both IP_3 and RyRs, calcium itself may contribute to modest amplification of calcium release from internal stores via both IP_3 receptors and RyR (Larkum et al., 2003; Nakamura et al., 1999), and a threshold level of mutually reinforcing release from both IP_3R and RyR may be required to induce substantial increases in calcium fluorescence signals, as well as increases in synaptic responses. Our data describe changes in calcium fluorescence localized to the soma, and the resolution of the imaging methods used here does not allow us to distinguish between calcium signals in subcompartments of the soma, the dendritic tree, or within dendritic cellular subcompartments, within which calcium dynamics can vary considerably (Berridge & Bootman, 2000). Since elevations in calcium can be highly localized (Martone et al., 1993; Sah et al., 1994), future studies should investigate whether dopamine application correlates with localized changes in intracellular calcium.

Linkage of Dopamine Receptors to PLC

Our results demonstrate that application of the D_1-like receptor agonist SKF83959 leads to a reliable increase in cytosolic calcium in fan cells, and this is consistent with a contribution of phosphatidylinositol-linked D_1-like receptors and activation of the PLC-DAG-IP_3 pathway. It has been previously proposed that SKF83959 may not be entirely selective for PI-linked D_1-like receptors (Hasbi et al., 2010), or that SKF83959 may lead to increased activation of PLC through initial activation of classical D_1 receptors and PKA (Lee, Kant, Blake, et al., 2014). However, our results indicate that the two agonists have distinct effects on intracellular signalling in layer II entorhinal neurons, providing evidence that they are functionally different compounds. We show here that application of the D_1-like agonist SKF38393, which is known to increase PKA activity, does not lead to increases in intracellular calcium as application of SKF83959 did. Therefore, it is unlikely that the effects of the putative PI-linked agonist are mediated via activation of conventional D_1-like receptors and PKA-dependent phosphorylation of PLC, as has been previously proposed (Lee et al., 2014; Yu et al., 1996). Our findings are more in line with other reports pointing to a role for PI-linked receptors in increasing intracellular calcium in neurons (Liu et al., 2009,

Ming et al., 2006; Jin et al., 2003) and astrocytes (Liu et al., 2009). Studies related to the use of SKF83959 have been conducted using a variety of brain regions, cell types, and experimental techniques, and there is not a clear consensus regarding SKF83959 as a selective PI-linked D_1-like receptor antagonist. Our current study, however, has shown the first direct evidence that, in contrast with the classical D_1-like receptor agonist SKF38393, PI-linked SKF83959 leads to transient increases in intracellular calcium in fan cells of layer II of the lateral entorhinal cortex that are similar to those induced by low doses of dopamine. This finding, together with our previous finding that the D_1-like receptor blocker SCH23390 blocks the facilitation of synaptic responses induced by SKF83959 (Glovaci & Chapman, 2015), strongly suggests that SKF83959 acts via a mechanism involving activation of PI-linked D_1-like receptors.

It has been proposed that D_1-D_2 receptor heteromers can be activated by SKF83959, which could underlie the activation of the PLC-DAG-IP_3 pathway (Rashid et al., 2007; Hasbi et al., 2010; Perreault et al., 2012), but the involvement of D_1-D_2 receptor heteromers in the dopaminergic facilitation of synaptic responses in entorhinal fan cells is unlikely. Activation of this pathway by D_1-D_2 heterodimers requires concurrent activation of both receptor subtypes, and we have previously demonstrated that blocking D_2-like receptors does not diminish the dopaminergic facilitation of synaptic transmission in layer II LEC neurons (Glovaci, Caruana, & Chapman, 2014). In the LEC, therefore, dopamine is more likely to lead to transient increases in intracellular calcium via atypical D_1-like receptors that activate the PLC-DAG-IP_3 pathway.


A1
130
120
110
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Fluorescence (%baseline)
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B1
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EPSC Amplitude (%baseline)
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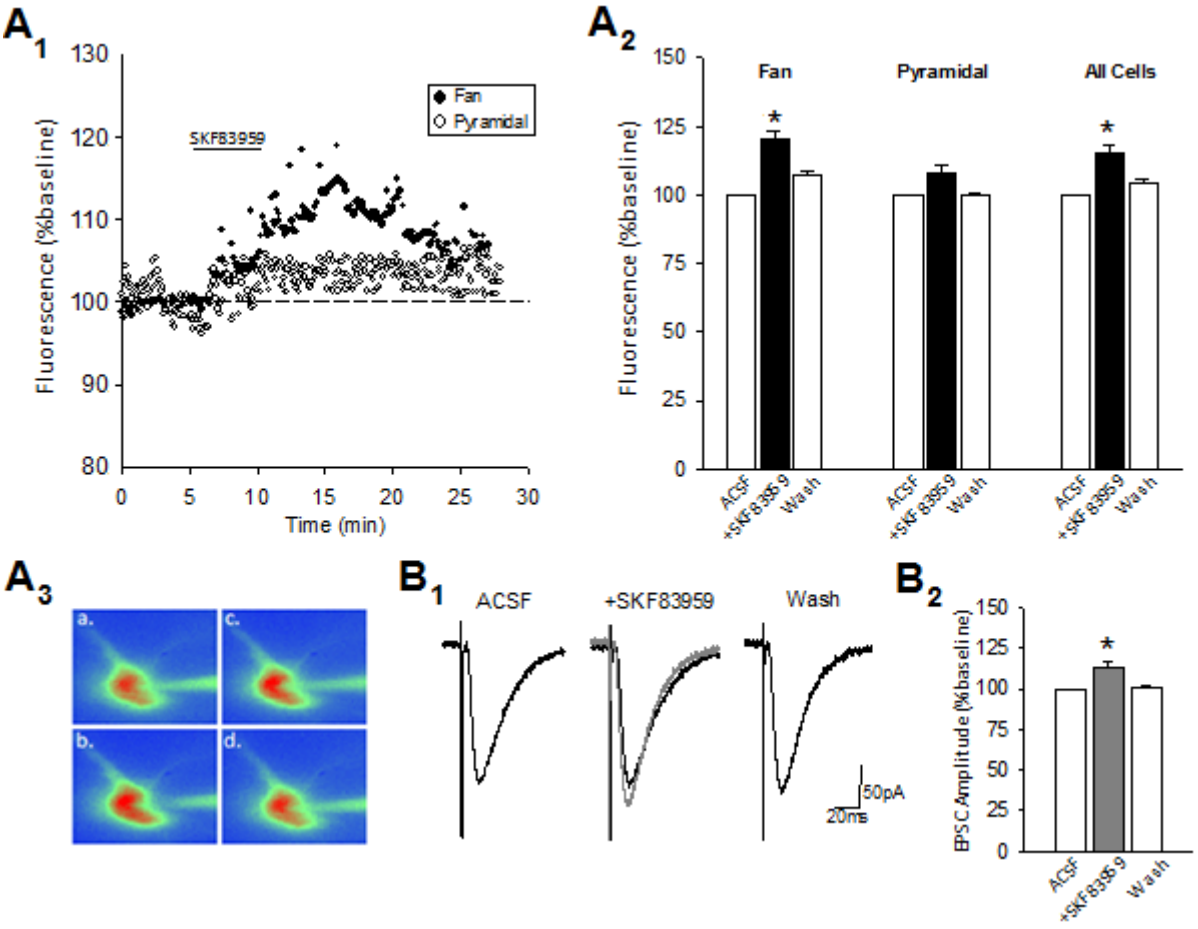
ACSF
+DA
Wash

Figure 4.1. Dopamine induces parallel and reversible increases in cytosolic calcium and EPSCs amplitude reliably in fan cells, but not in pyramidal, cells. **(A)** Changes in fluorescence emission in individual cells, expressed as a proportion of the baseline, show that dopamine increased calcium signals strongly in a fan cell (black circles; **A_1**), but not in pyramidal neurons (open circles). The histogram shows changes in mean fluorescence in fan, pyramidal, and combined cell-type groups. Bars indicate ± one SEM and the asterisk indicates $p < 0.05$ **(A_2)**. Sample fluorescence images obtained before (a), during (b, 1.5 min; c, 5 min), and after (d) bath application of 1 µm DA **(A_3)** are shown for the fan cell in A_1. **(B)** Sample traces show averaged EPSCs recorded before, during, and following bath-application of dopamine (**B_1**) in a representative neuron. The histogram indicates mean EPSC amplitudes for the group of cells (± one SEM**;** **B_2**).


A1
Fluorescence (%baseline)
Time (min)
SKF83959
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Pyramidal
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Fan
Pyramidal
All Cells
ACSF
+SKF83959
Wash
A3
a.
b.
c.
d.
B1
ACSF
+SKF83959
Wash
50pA
20ms
B2
EPSC Amplitude (%baseline)

Figure 4.2. Application of the PI-linked agonist SKF83959 induces reversible elevations in cytosolic calcium and EPSC amplitudes similar to those observed during application of 1 μm dopamine. **(A)** Changes in fluorescence emission in individual cells, expressed as a proportion of the baseline, show that SKF83959 causes a clear increase in calcium signals in a fan cell (black circles), but not in a pyramidal neuron (open circles; **A_1**). The histogram shows changes in mean fluorescence in fan, pyramidal, and combined cell-type groups **(A_2)**. Bars indicate ± one SEM, and the asterisk indicates $p < 0.05$. Sample fluorescence images obtained before (a), during (b, 1.5 min; c, 5 min), and after (d) bath application of SKF83959 are shown for the fan cell in A_1 **(A_3). (B)** Sample traces show averaged EPSCs recorded before, during, and following bath-application of the PI-linked D_1-like receptor agonist SKF83959, in a representative neuron **(B_1)**. The histogram **(B_2)** indicates mean EPSC amplitudes for the group of cells (± one SEM).

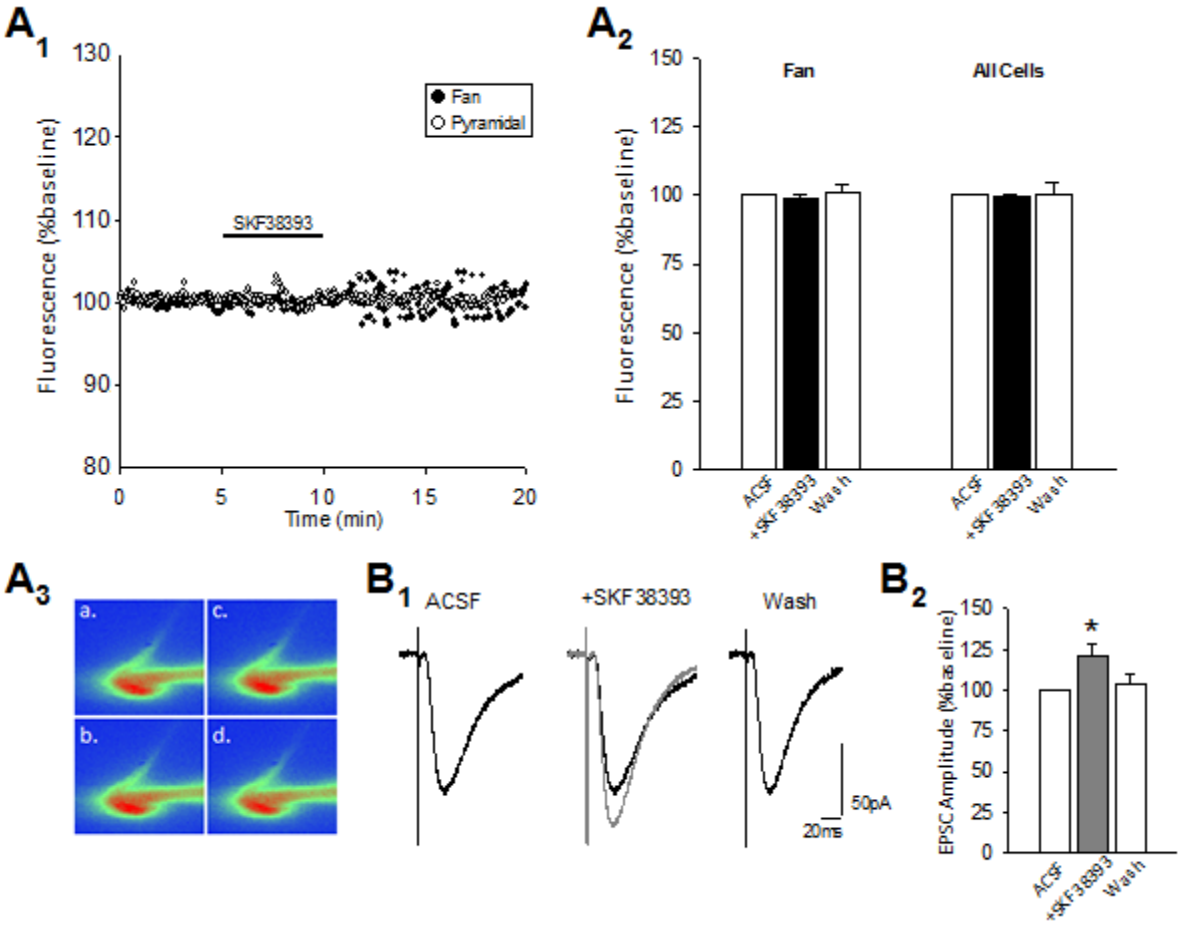
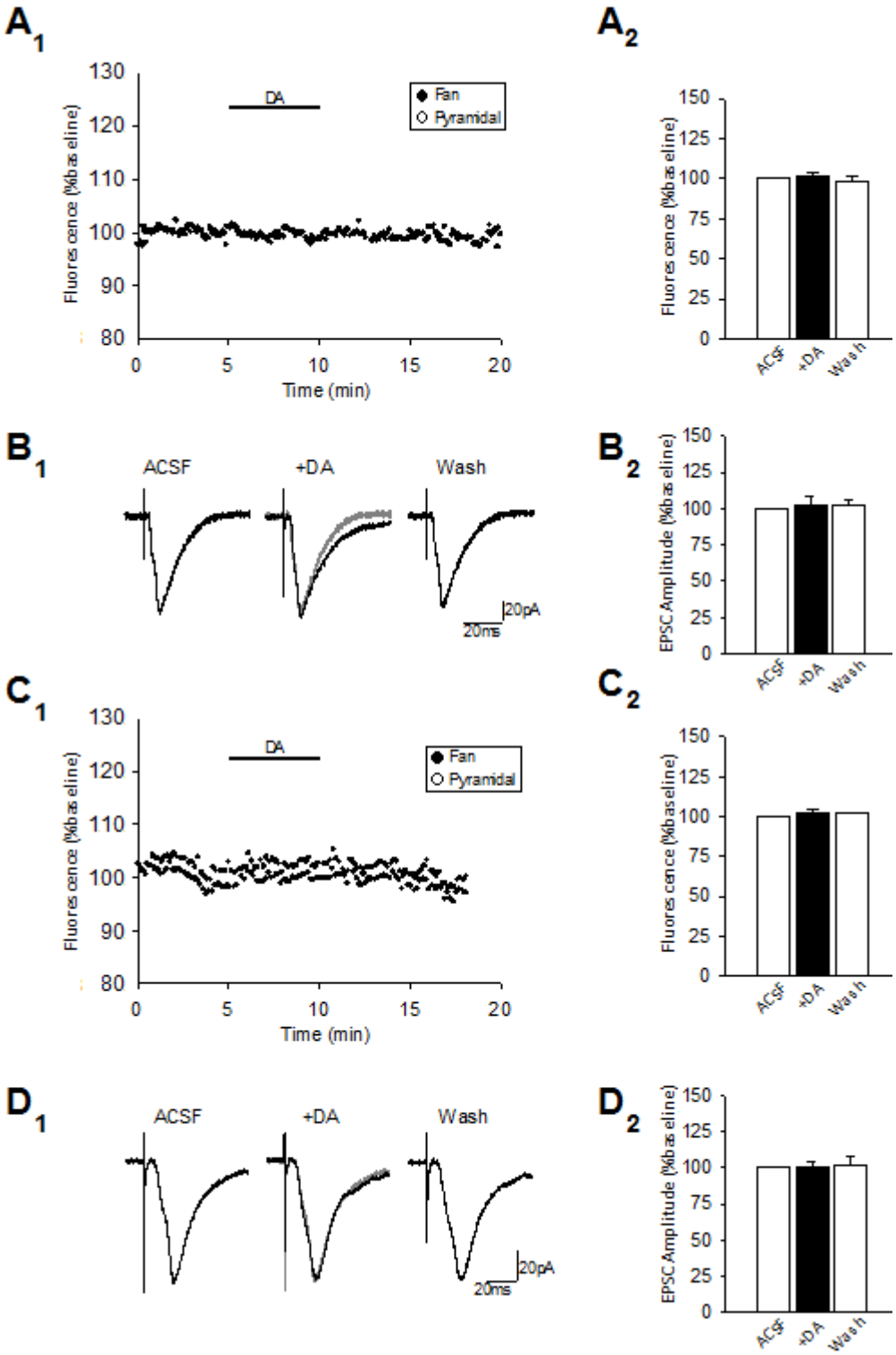

A1
Fluorescence (%baseline)
Time (min)
Fan
Pyramidal
SKF38393
A2
Fan
All Cells
Fluorescence (%baseline)
ACSF
+SKF38393
Wash
A3
a.
c.
b.
d.
B1
ACSF
+SKF38393
Wash
50pA
20ms
B2
EPSC Amplitude (%baseline)
*
ACSF
+SKF38393
Wash

Figure 4.3. Activation of 'classical' D_1-like receptors does not cause an increase in cytosolic calcium in either fan or pyramidal cells. **(A)** No changes in fluorescence emission were observed in individual cells following application of SKF38893 in layer II lateral entorhinal principal cells **(A_1)**. The histogram shows changes in mean fluorescence in fan and combined cell-type groups **(A_2)**. Bars indicate ± one SEM. Data for the one pyramidal cell recorded are not shown separately. **(A_3)** Sample fluorescence images obtained before (a), during (b, 1.5 min; c, 5 min), and after (d) bath application of SKF38893 are shown for the fan cell in A_1. **(B)** Sample traces from a representative neuron show averaged EPSCs recorded before, during, and following bath-application of the classical D_1-like agonist **(B_1)**, and reflect a facilitation of EPSCs in the absence of increased calcium signalling. The histogram **(B_2)** indicates mean EPSC amplitudes for the group of cells (± one SEM), and the asterisk indicates $p < 0.05$.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+DA
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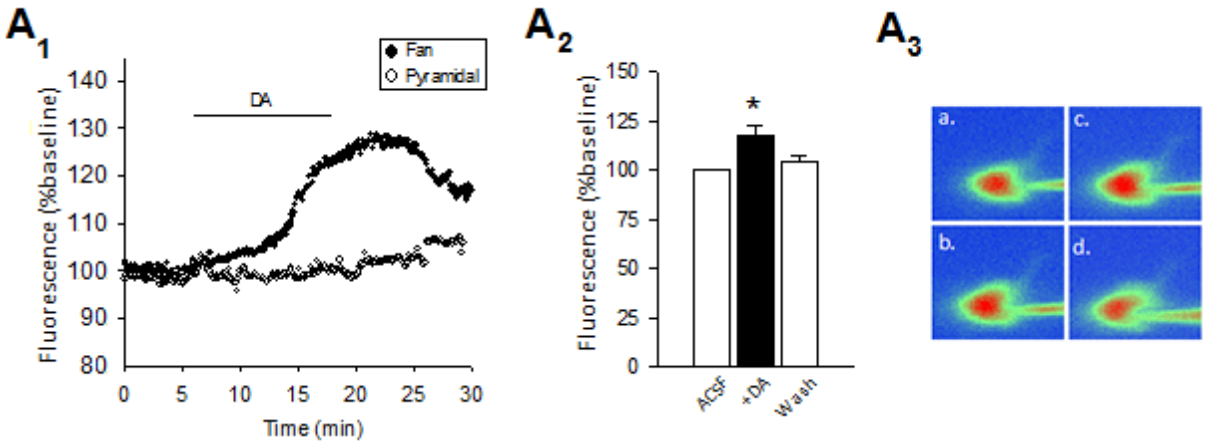
20ms
B2
EPSC Amplitude (%baseline)
C1
DA
Fan
Pyramidal
Fluorescence (%baseline)
Time (min)
C2
Fluorescence (%baseline)
D1
ACSF
+DA
Wash
20pA
20ms
D2
EPSC Amplitude (%baseline)

Figure 4.4. Blocking either IP_3 or ryanodine receptors blocks the dopaminergic facilitation of synaptic responses, as well as increases in cytosolic calcium, in layer II lateral entorhinal cells. **(A)** Representative recordings for a fan cell, wherein the IP_3 receptor blocker heparin was applied intracellularly. No marked changes in fluorescence emission were observed in either fan or pyramidal cells **(A_1)**. The histogram shows a lack of significant change in mean fluorescence in a combined group of fan and pyramidal cells **(A_2)**. Bars indicate ± one SEM. **(B)** Sample traces show averaged EPSCs recorded before, during, and following bath-application of dopamine in a cell containing the IP_3R blocker heparin **(B_1)**. The histogram indicates mean EPSC amplitudes for the group of cells (± one SEM; **B_2**). **(C)** Representative results of fluorescence recordings in a fan cell containing the ryanodine receptor blocker dantrolene **(C_1)**. The histogram shows a lack of significant change in mean fluorescence in the combined group of fan and pyramidal cells **(C_2)**. Bars indicate ± one SEM. Similar to heparin-containing cells, no marked changes in fluorescence were observed in dantrolene-containing cells. **(D)** Sample traces show averaged EPSCs recorded before, during, and following bath-application of dopamine **(D_1)**, with accompanying histogram indicating the mean group EPSC amplitudes **(D_2)**.


A1
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Time (min)
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Fluorescence (%baseline)
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Figure 4.5. The dopaminergic facilitation in layer II LEC cells persists in the absence of extracellular calcium. **(A)** Changes in fluorescence emission in individual cells, expressed as a proportion of the baseline show that dopamine markedly increased calcium signals in a fan cell (black circles) but not a pyramidal cell (open circles), even in the absence of extracellular calcium **(A_1)**. The histogram shows a significant increase in mean fluorescence in the combined group of fan and pyramidal cells **(A_2)**. Bars indicate ± one SEM and the asterisk indicates $p < 0.05$. **(C_1)** Sample fluorescence images obtained before (a), during (b, 1.5 min; c, 5 min), and after (d) bath application of 1 μm DA.

CHAPTER 5

GENERAL DISCUSSION

DISCUSSION

This thesis has shown that dopamine, at a concentration of 1 μM, can promote glutamatergic synaptic transmission in layer II lateral entorhinal cortex neurons via activation of D_1-like receptors and subsequent activation of a complex signalling cascade that involves activation of the cAMP-PKA and PLC-IP_3-DAG pathways. Additionally, the experiments presented here showed that dopaminergic modulation of glutamatergic synaptic transmission in layer II neurons differs by cell-type examined, with dopamine consistently inducing a reversible facilitation of excitatory postsynaptic potentials in fan cells, whereas the facilitation of synaptic transmission in pyramidal neurons was unreliable and infrequent.

Dopamine has been acknowledged as an important neuromodulator that plays a central role in learning and memory processes, as well as in behaviours related to rewarding or salient stimuli (Goldman-Rakic, 1996; Phillips, Ahn, & Floresco, 2004; Watanabe, Kodama, & Hikosake, 1997). As such, the observed dopamine-dependent increases in the strength of AMPA-mediated synaptic transmission in layer II cells is likely to be a major mechanism through which dopamine contributes to modulating the activity of the lateral entorhinal cortex. Dopaminergic modulation of synaptic activity in the LEC likely promotes synaptic processing of sensory, object-related, and contextual information, and may promote transmission from the LEC to the hippocampus during exposure to salient cues. Synaptic enhancements within the entorhinal cortex, and resulting enhancements in synaptic transmission in efferents to the hippocampus, may contribute to enhancing LEC-dependent cognitive processes such as working memory (Ramus & Eichenbaum, 2000; Young et al., 1997), and object processing (Van Cauter et al., 2012; Zhu et al., 1995). This thesis has contributed substantially to determining the intracellular signalling mechanisms through which dopamine enhances LEC glutamatergic transmission.

Dopamine Does Not Modulate Inhibitory Inputs in the LEC

In our experiments, low doses of dopamine did not appear to alter inhibitory synaptic transmission in layers I/II of the entorhinal cortex, as demonstrated by the lack of change in recorded inhibitory postsynaptic potentials (Chapter 2, Figure 2.1), or in excitatory inputs recorded in several intracellular recordings from layer II inhibitory interneurons (data not shown). These findings differ from findings in the prefrontal cortex, where dopamine, acting via D_1-like receptors, can reduce extracellular GABA concentrations (Brozoski et al., 1979; Grobin & Deutch, 1998) and cause a reduction in inhibitory postsynaptic potentials in pyramidal cells (Gonzales-Islas & Hablitz, 2001). This reduction in synaptic inhibition is thought to lower the inhibitory tone in the network, and to thereby promote the efficacy of excitatory synaptic transmission. The superficial layers of the entorhinal cortex are generally under tight, feedforward inhibitory control (e.g. Finch, Tan, & Isokawa-Akesson, 1988; Woodhall, Bailey, Thompson, Evans, & Jones, 2005; de Curtis & Paré, 2004), and it is possible that this inhibitory tone is necessary and beneficial to input discrimination processes in the LEC. Therefore, by selectively promoting excitatory synaptic transmission in principal LEC neurons without enhancing spontaneous background activity, dopamine likely enhances transmission of *salient* information, and promotes its further processing in the hippocampus.

Dopaminergic Facilitation of Synaptic Transmission

Chapter 2 of this thesis (Glovaci, Caruana, & Chapman, 2014) demonstrated that dopamine facilitates excitatory, glutamatergic synaptic transmission in layer II LEC neurons via a postsynaptic mechanism that relies on D_1-like receptor activation, cAMP production and PKA activity, reduction in PP-1 activity, and a subsequent enhancement of AMPA-, but not NMDA-, receptor-mediated currents. This 'classical' signalling mechanism has been shown to underlie facilitation of glutamatergic synaptic transmission in other brain areas including the striatum (Flores-Hernandez et al., 2002), neocortex (Cepeda et al., 1992), prefrontal cortex (Tseng & O'Donnell, 2004), and hippocampus (Yang, 2000), with some of the aforementioned studies also pointing to the role of calcium in the dopaminergic facilitation of synaptic transmission (Tseng & O'Donnell, 2004; Neve, Seamans & Trantham-Davidson, 2004; Yang, 2000). The present experiments were the first to demonstrate the importance of this signalling cascade in the lateral entorhinal cortex. Chapter 3 of this thesis (Glovaci & Chapman, 2015) demonstrated that in addition to the activation of the cAMP-PKA intracellular cascade, D_1-like receptor activation can lead to increased AMPA-receptor-mediated currents via a mechanism that is dependent on activation of the PLC-IP_3-DAG pathway, which induces release of calcium from internal stores and promotes activation of PKC, a kinase that is sensitive to both calcium and DAG.

The work outlined in this thesis has shown that dopamine induces effects dependent on both classical D_1-like and PI-linked signalling cascades, each of which result in an increased activation of second messenger-regulated kinases that are known to phosphorylate AMPA receptors. Protein kinase and phosphatase activity is an important mechanism for bidirectional control of ion channel activity (e.g. Smart, 1997), and their control of glutamatergic receptor activity is of particular interest, as it has been shown to underlie synaptic plasticity (e.g. Nicoll & Malenka,1995; Roche et al. 1996; Soderling & Derkach, 2000). In particular, cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and calcium/calmodulin-dependent protein kinase II (CaMKII) exert strong control over glutamate transmission, as previous reports have indicated that all three kinases are implicated in the phosphorylation of both AMPA and NMDA receptors in numerous brain regions, including the prefrontal cortex, hippocampus, and striatum (e.g. Greengard et al. 1991; McGlade-McCulloh et al. 1993; Roche et al. 1996; Wang et al., 1994). Specifically, AMPA receptors are phosphorylated by PKA on Ser^{845} of the GluR1 AMPA subunit, whereas PKC and CaMKII phosphorylate Ser^{831} of the same receptor subunit (Mammen et al. 1997; Roche et al. 1996; Derkach et al., 1999; Lee et al., 2000). Our results show that whilst blocking PKA or PKC blocked the dopamine-induced facilitation of synaptic responses in layer II LEC cells, blocking CaMKII did not. This finding is interesting considering that both PKC and CaMKII phosphorylate the same residue (Mammen, Kameyama, Roche, & Huganir, 1997; Barria, Derkach, & Soderling, 1997). Similar to PKC phosphorylation, CaMKII phosphorylation of Ser^{831} increases channel conductance of the AMPA receptor (Derkach, Barria, & Soderling, 1999), and this effect has been suggested to underlie long-term potentiation (Benke, Luthi, Isaac, & Collingridge, 1998). As such, it is still unclear why PKC, but not CaMKII, underlies the synaptic facilitation in LEC cells. Although both kinases typically show increased activation with increased intracellular calcium levels, it is possible that their

different selectivity, activation profiles, regulatory mechanisms, and intracellular distributions underlie their differential involvement.

In contrast to CaMKII, our results suggest that signalling involving both PKA or PKC can result in increased AMPA-receptor-mediated currents in layer II LEC cells. Previously, PKA phosphorylation of the GluR1 subunit has been shown to increase the opening frequency and mean opening time of AMPA receptors (Greengard, Jen, Nairn, & Stevens, 1991; Banke, Bowie, et al., 2000), whereas PKC phosphorylation potentiates AMPA-receptor conductance (Wang, Dudek, Browning, & MacDonald, 1994; Roche, O'Brien, Mammen, Bernhardt, & Huganir, 1996). These findings were supported by in vivo experiments which demonstrate that, in hippocampal slices, forskolin and phorbol esters increase phosphorylation of Ser_8^{45} and Ser^{831}, respectively (Mammen, Kameyama, Roche, & Huganir, 1997). Although we did not directly measure PKA and PKC-induced channel open time probability and conductance, it is likely that phosphorylation of AMPA receptors by either kinase in LEC cells leads to similar functional effects on EPSC amplitude. It is difficult to assess the functional significance as to why both PKA- and PKC-dependent signalling cascades are activated by D_1-like receptor binding that leads to the dopaminergic facilitation of synaptic responses in layer II entorhinal cells. One possibility is that the interaction between intracellular events is likely to result in a much more complex pattern of signalling within cells that may reflect the complexity of information integration processes. Further, in the present set of experiments, we have not assessed the direct level of phosphorylation of AMPA-receptor subunits by either kinase, but future experiments could be conducted to determine the degree of phosphorylation of AMPA receptor subunits induced by these kinases following activation of D_1-like receptors by dopamine, or classical and PI-linked agonists. It is nevertheless interesting to note that phosphorylation of GluR1 on Ser^{845} and Ser^{831}, by PKA and PKC respectively, seems to occur both during basal conditions (Mammen et al. 1997) and to be required during dopamine-induced facilitation of synaptic transmission, suggesting that dopamine serves to further 'boost' the activity of an ongoing intracellular process.

While binding to classical D_1-like receptors is known to increase PKA activity (e.g. Tseng & O'Donnell, 2004), activation of atypical phosphatidylinositol-linked (PI-linked) D_1-like receptors coupled to PLC leads to increases in intracellular calcium and PKC activity (Undie et al., 1994; Wang et al., 1995; Jin et al., 2003). Stimulation of PLC by activation of putative atypical D_1-like receptors has been observed in the adjacent brain areas of the hippocampus and the amygdala (Undie & Friedman, 1990; Leonard, Anderson, Lachowicz, & Schulz, 2003). Further, in neurons of the amygdala, which receive moderate dopaminergic innervation and express D_1-like receptors, activation of D_1-like receptors produces little increase in cAMP synthesis, but does result in increased PLC activity (Leonard et al., 2003; Kiltz et al., 1988). This indicates that PI-linked D_1-receptor activation may act independently of cAMP activation, but via PLC-dependent mechanisms, to promote synaptic function in other temporal lobe structures.

Chapter 4 of this thesis used combined electrophysiological recordings and fluorescence imaging of intracellular calcium to characterize transient increases in intracellular calcium associated with application of dopamine, the PI-linked D_1-like-receptor agonist SKF83959, and the classical D_1-like receptor agonist SKF38393. Chapter 3 also examined mechanisms mediating synaptic facilitation effects

induced by these agonists. Our results indicated that the increases in synaptic transmission in fan cells induced by dopamine or the PI-linked agonist SKF83959 occurred concurrently with transient increases in cytosolic calcium. In contrast, application of the classical D_1-like receptor agonist SKF38393 alone, while resulting in increased synaptic responses, did not result in increases in intracellular calcium levels. Interestingly, however, when BAPTA, or IP_3R or RyR blockers, were used to block increases in intracellular calcium, the facilitation of synaptic responses induced by dopamine was blocked. These data therefore suggest that increases in cytosolic calcium mediate the facilitation of synaptic responses induced by activation of PI-linked dopamine receptors.

The precise identity of dopamine receptors activated by SKF83959 is presently unknown. Previous reports have suggested that activation of either D_1-D_2 receptor heterodimers (Rashid et al., 2007) or D_5 receptors (Sahu et al., 2009; Furini et al., 2014) can lead to activation of PLC in other regions. Although it has been previously suggested that the effects exerted by SKF83959 may be due to effects at these sites, SKF83959 is the most potent and selective agonist presently available for stimulation of PI-linked D_1-like receptors, and was therefore the best compound for use in the current experiments. Further, it is unlikely that activation of D_1-D_2 heterodimers or of classical D_5 receptors underlies the dopaminergic facilitation of synaptic responses we observed here. The application of D_2-like receptor blockers should inhibit the activation of D_1-D_2 heterodimers, yet we did not observe a decrease in the dopaminergic facilitation of synaptic responses of LEC cells upon application the D_2-like receptor blocker sulpiride (Glovaci, Caruana, & Chapman, 2014). Further, if the increase in intracellular calcium levels would have been due D_5 receptor activation, we would expect to have seen an increase of fluorescence signals upon application of SKF38393, which activates both D_1 and D_5 receptors, yet this was not the case. In light of this, we believe that dopamine is more likely to lead to transient increases in intracellular calcium via activation of atypical D_1-like receptors that increase the activity of the PLC-DAG-IP_3 pathway.

Different Functional Roles for Fan and Pyramidal Cells

Cells in layer II of the LEC have been previously categorized based on their morphological and electrophysiological profiles, with the large majority of layer II LEC principal cells being categorized as either fan or pyramidal cells (Tahvildari & Alonso, 2005; Canto & Witter, 2012). Other reports also suggest that principal cells in both medial and lateral subdivisions of the rodent entorhinal cortex have two different chemical profiles, wherein they express either calbindin or reelin (Fujimaru & Kosaka, 1996; Nilssen et al., 2015). Whereas in layer II of the MEC the calbindin-positive (CB+) and reelin-positive (RE+) cells are grouped in clusters, layer II LEC neurons appear to be loosely segregated into two sublayers, with RE+ cells forming layer IIa and CB+ cells forming layer IIb (Fujimaru & Kosaka, 1996; Leitner et al., 2016). Furthermore, layer IIa appears to be mostly composed of RE+ neurons, with one report suggesting that approximately 98% of excitatory cells in layer IIa are RE+, whereas 70% of excitatory cells in layer IIb appear to be CB+ cells (Leitner et al., 2016). RE+ cells appear to be mostly fan cells, although some multiform, but not pyramidal, cells also express RE+ (Leitner et al., 2016). CB+ cells are generally pyramidal or multiform, as indicated by their morphological and electrophysiological profiles (Leitner et al., 2016). A number of GABAergic interneurons also expressed calbindin (Leitner et al., 2016). Therefore, it

is likely that layer IIa is mostly composed of fan cells and a small number of multiform cells, whereas layer IIb is likely more diverse, being composed of pyramidal, multiform, and possibly fan cells.

Interestingly, RE+ and CB+ neurons display different responses to stimuli, and appear to project to different areas, which suggests that they play different functional roles in memory processes. Functionally, RE+ neurons may play a larger role in odor discrimination, as they appear to be more odor-selective than CB+ neurons, and fire in a more specific manner in response to their "preferred" odor, whereas CB+ neurons respond to a wider range of odors (Leitner et al., 2016). Anatomically, experiments have shown that injections of a retrograde tracer into the dentate gyrus results in labeled neurons in layer IIa of the LEC, of which 98% were RE+ neurons (Leitner et al., 2016), suggesting that projections to the dentate gyrus from the LEC originate primarily from fan cells. In contrast, anterograde labelling of CB+ cells in layer II of the LEC determined that they project to the granule cells of the olfactory bulb, to the contralateral LEC, and, to a lesser degree, to the piriform cortex (Leitner et al., 2016). Approximately 12% of CB+ glutamatergic neurons project to *both* the OB and PIR (Leitner et al., 2016), although these findings remain controversial (Chapuis et al., 2013). Therefore, CB+ and RE+ appear to receive relatively similar sensory inputs, but give rise to different outputs.

Reelin-positive cells in layer II of the entorhinal cortex provide a strong source of excitatory input to the dentate gyrus from layer II of the LEC. Leitner and colleagues (2016) concluded that, on the basis of morphology and outputs to the hippocampus, "fan cells are identical with RE+ neurons described in (their) study", suggesting that the hippocampus-projecting feedforward RE+ cells in their study are likely to be fan cells. This proposal is interesting when viewed from the perspective of this thesis. Low levels of dopamine appear to activate a complex intracellular signalling cascade to preferentially enhance synaptic transmission in *fan* cells. Based on prior reports (Kesner, Hunsaker, & Ziegler, 2011; Weeden, Hu, Ho, & Kesner, 2014), it is likely that at least a part of the information conveyed by these fan cells is highly specific odor information, with different cells firing preferentially to a specific odor. Fan cells have direct projections to the dentate gyrus, and the dentate gyrus is known to be heavily involved in pattern separation and organization of incoming inputs and pattern separation (e.g. Hunsaker, Rosenberg & Kesner, 2008; Leutgeb, Leutgeb, Moser, & Moser, 2007; Neuneubel & Knierim, 2014; Treves & Rolls, 1994), as well as memory processes (e.g. Kesner, Hunsaker, & Ziegler, 2011). Intriguingly, RE+ cells of layer II LEC are the first to show severe loss of synapses during the early stages of Alzheimer's disease (Kobro-Flatmoaen, Nagelhus, & Witter, 2016), and impaired function in LEC neurons that project to the dentate gyrus may underlie some of the memory impairments observed during these stages. These findings argue that layer II LEC RE+ neurons, which have been characterized as putative fan cells, likely convey object-related and selective sensory information to the dentate gyrus, and thus contribute heavily to mnemonic processes. Dopamine, by enhancing synaptic transmission in these neurons, may promote memory processing by promoting discrimination between specific object-related cues, odors, and integration with other contextual sensory information.

If the presumed role of layer II LEC fan-cell activity, enhanced by dopamine, is to contribute to sensory and mnemonic function by enhancing the strength of inputs to the hippocampal formation, the

question arises as to the functional role of pyramidal cells in the LEC. Although the role of these cells remains unclear, some tentative speculations can be made based on their anatomical and functional connectivity. CB+ cells, of which approximately half were identified as pyramidal cells, appear to provide *feedback* information to olfaction-processing areas, as they project *back* to the olfactory bulb and the piriform cortex (Leitner et al., 2016). These cells also appear to have a broader response to a variety of odors (Leitner et al., 2016), suggesting that they may not be as heavily involved in pattern discrimination. We found here that pyramidal cells rarely show a facilitation of synaptic responses in the presence of dopamine. This suggests that dopamine plays a larger role in modulating sensory input to the hippocampal region during exposure to salient stimuli than it does in modulating the output of the LEC to sensory regions, but further studies are needed to differentiate between the roles of CB+ and RE+ neurons.

Functional Implications of Dopaminergic Facilitation in LEC Neurons

There is a variety of evidence related to the potential roles of the lateral entorhinal cortex in cognitive functions, but the specific computational functions of layer II neurons, and the functional effects of dopaminergic modulation of excitatory inputs to fan cells, is less clear. The entorhinal cortex is known to contribute to the encoding and retrieval of memories (Morrissey et al., 2012; Tannien et al., 2013), as well as to working memory function (Ramus & Eichenbaum, 2000; Young et al., 1997), with neurons in the superficial layers of the entorhinal cortex typically firing during transient events within specific contexts, suggesting that they contribute to differentiating between different experiences (Pilkiw, Insel, Cui, Finnery et al., 2017). The lateral division of the entorhinal cortex receives heavy sensory inputs (Burwell & Amaral, 1998; Beckstead, 1978; Kerr et al., 2007), which likely contribute to its role in object-related identification and information processing, which can underlie its proposed contribution to medial temporal lobe processes involved in the binding of physical, relational, and contextual information that shapes everyday experience (Eichenbaum, 2000; Morrisey & Takehara-Nishiuchi, 2014; Squire, 1992). Because the lateral entorhinal cortex is a major component of the parahippocampal region, and provides hippocampus with a large portion of its cortical sensory input, changes in synaptic activity in the LEC may have profound consequences for ongoing cognitive and memory functions mediated by the medial temporal lobe.

Layer II of the lateral entorhinal cortex receives strong inputs from sensory and associational cortices, and the dopaminergic modulation of these inputs may play a different role in cognitive processes depending on the intensity of the dopaminergic input. Dopamine may be a mechanism that selectively enhances and controls which information is conveyed from the lateral entorhinal cortex to the hippocampus, by increasing the salience of specific inputs. Interestingly, dopamine has been noted to have bidirectional effects on synaptic strength both in the LEC (Caruana, Sorge, Stewart, & Chapman, 2006) and in other brain areas (e.g. Seamans et al., 2001; Alberto, Trask, Quinln, & Hirasawa, 2006; Zheng, Zhang, Bunney, & Shi, 1999) wherein, while *low* concentrations of dopamine (1 μM) facilitate synaptic transmission, *larger* concentrations of dopamine (> 50 μM) decrease synaptic transmission. In layer II lateral entorhinal cortex, therefore, relatively small increases in the activity of dopaminergic inputs likely enhances the salience of important contextual cues and object representations. These moderate

increases in dopamine concentration are likely to occur during typical reward-related behaviours and exploratory activities, and contribute to ongoing cognitive and mnemonic processes. In contrast, much larger increases in dopamine release which are likely to suppress synaptic transmission and mnemonic processes (Tahvildari et al., 2007; Young et al., 1997) are more likely to be associated with atypical, excessive dopaminergic activation during episodes of acute stress (Arnsten & Goldman-Rakic, 1998).

The exact concentration of dopamine released in the LEC *in vivo* during learning and memory tasks is unknown. Further, it is unclear how closely the constant bath application of dopamine mimics physiological function. Generally, dopaminergic outputs from the VTA range from nanomolar to millimolar concentrations, and depend on the state of activation of dopaminergic neurons (Shultz, 2007). To better assess which dopamine receptors and subsequent intracellular cascades are activated during endogenous release of dopamine, it may be possible to use optical stimulation of VTA terminals in layer II of the LEC to mimic tonic versus phasic burst firing in dopamine cells (Schultz, 2007; Grace, 2000). Optogenetic stimulation of dopaminergic terminals could be evaluated in terms of its effects on both evoked synaptic responses, as well as on the amplitude of spontaneous synaptic responses. This experimental approach would provide valuable insights into the magnitude of effects of endogenously released dopamine on excitatory synaptic responses in the entorhinal cortex.

Although our experiments demonstrate that dopamine may induce transient changes in synaptic strength to affect the salience of sensory information processed by the lateral entorhinal cortex, there is no evidence that dopamine may contribute to lasting synaptic plasticity in the LEC. In the hippocampus, different experiments focusing on hippocampal-dependent synaptic plasticity (e.g., Otmakhova & Lisman 1996; Edelmann & Lessmann 2013) suggest a strong modulatory role for dopamine in synaptic mechanism of long-term memory. Dopamine signalling in the hippocampus appears to be important for learning in the context of novelty detection (e.g. Lisman & Grace, 2005; Moncada & Viola, 2007) and novel-object recognition (Yang et al., 2017). Dopamine-dependent strengthening of hippocampal synapses may therefore be required to help discriminate between old and novel stimuli. However, in the entorhinal cortex, long-term potentiation (LTP) is difficult to induce, and dopamine does not appear to facilitate the induction of LTP (S. Barrett and C.A. Chapman, personal communication). This suggests a critical difference between the entorhinal cortex and the hippocampus, in which long-term changes in synaptic strength are more easily induced (Malenka & Bear, 2004). It is therefore possible that the lateral entorhinal cortex may serve, in part, as a filter of sensory information which sends the strongest, and therefore likely most salient, inputs to the hippocampus. From this viewpoint, transient enhancement of synaptic transmission becomes crucial, and neuromodulators play a critical role in mnemonic processes by enhancing or reducing the strength of received signals.

Changes in synaptic strength within layer II of the lateral entorhinal cortex are likely to affect not only synaptic processing within the entorhinal area, but also spatial and mnemonic processes such as pattern separation and integration, which depend upon the dentate gyrus and other structures within the entire hippocampal region. Much of the cortical information about properties of objects in the environment is thought to reach the hippocampus via layers II/III of the LEC (e.g. Burwell & Amaral, 1998; Beckstead,

1978; Kerr et al., 2007). Enhancement of synaptic transmission by dopamine is likely to contribute in this context by increasing the likelihood of salient, physiologically relevant, or reward-relevant signals that are received by the entorhinal cortex being transmitted in a robust way to the hippocampus, and contribute there to processes leading to the encoding of new memory.

Synaptic inputs from the lateral entorhinal cortex, providing contextual information, may also be implicated in hippocampal spatial processing. It has been found that hippocampal place cells alter their firing rates (Leutgeb et al., 2007) in response to object and contextual information, such as floor texture (Young, Fox, & Eichenbaum, 1994) and odor (Wood, Dudchenko, & Eichenbaum, 1999; Komorowski, Manns, & Eichenbaum, 2009), and a subset of cells respond to *changes* in the properties of the environment, such variations in odors or colours. As such, changes in synaptic transmission in the lateral entorhinal cortex may play a role in the modifications that take place in the activity of hippocampal place cells.

Similarly, although experimental evidence shows that lesions of the LEC do not alter the basic spatial firing properties of place cells, which retain their precise place fields (Lu et al., 2013), rate remapping is impaired by entorhinal lesions, especially when the lesions extensively affect the superficial layers II/III (Lu et al., 2013) which project to the hippocampus (Witter, Groenewegen, & Da Silva, 1989). Rate remapping refers to the way in which sensory inputs can modulate the firing rate of hippocampal place cells without altering their field locations, and this mechanism is believed to arise from the interaction between LEC-driven inputs, which primarily provide contextual information, and the MEC-driven inputs, which provide spatial information (Hargreaves et al., 2005). It has been previously postulated that changes in firing rates of place cells are a way for the hippocampus to represent non-spatial characteristics in the environment (Hampson et al., 1993; Wood et al., 1999). Even moderate environmental changes, such as shifting the shape of a previously-familiar environment from a square to a circle, can lead to changes in rate remapping (Leutgeb et al., 2005). As such, it is possible that inputs from the lateral entorhnial cortex may modulate rate remapping, and therefore contribute to discrimination processing between different events or contexts, thereby aiding in memory formation. Therefore, dopaminergic modulation of LEC neurons may have profound, and numerous, effects on episodic memory processes.

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