

Role of Muscarinic Acetylcholine Receptors in Adult Neurogenesis and Cholinergic Seizures

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Abstract

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Muscarinic acetylcholine receptors (mAChRs) are G protein-coupled receptors (GPCRs) that mediate important functions in the periphery and in the central nervous systems. In the brain these receptors modulate many processes including learning, locomotion, pain, and reward behaviors. In this work we investigated the role of mAChRs in adult neurogenesis and further clarified the regulation of muscarinic agonist-induced seizures.

We first investigated the role of mAChRs in adult neurogenesis in the subventricular zone (SVZ) and the subgranular zone (SGZ). We were unable to detect any modulation of adult neurogenesis by mAChRs. Administration of muscarinic agonists or antagonists did not alter proliferation or viability of adult neural progenitor cells (aNPCs) *in vitro*. Similarly, muscarinic agonists did not alter proliferation or survival of new adult cells *in vivo*. Loss of the predominant mAChR subtype in the forebrain, the M₁ receptor, also caused no alterations in adult neurogenesis *in vitro* or *in vivo*, indicating that the M₁ receptor does not mediate the actions of endogenous acetylcholine on adult neurogenesis.

We also investigated the interaction between mAChRs and cannabinoid receptor 1 (CB₁) in muscarinic agonist pilocarpine-induced seizures. Previous work showed that pilocarpine-induced seizures require activation of M₁ receptors; activation of these receptors also increases release of endocannabinoids which act at CB₁ receptors to reduce excitatory transmission. Using submaximal doses of pilocarpine, we found that pilocarpine-induced seizures were more severe in CB₁ KO mice compared to WT mice. Similarly, pretreatment with CB₁ antagonists also increased pilocarpine seizure severity. In contrast, pretreatment with CB₁ agonists had no effect on pilocarpine seizure severity. These results support the hypothesis that endocannabinoids act at CB₁ receptors to reduce sensitivity to pilocarpine seizures and that endocannabinoid activity following pilocarpine administration is maximal at CB₁ receptors, so exogenous CB₁ receptor agonists cannot further modulate sensitivity to pilocarpine seizures.

Because organophosphates require mAChR activity in order to induce seizures, we investigated whether seizures induced by the organophosphate paraoxon also shared the same regulation by M₁ receptors and CB₁ receptors as pilocarpine seizures. We compared seizure behaviors induced by paraoxon in WT and M₁ KO or CB₁ KO mice. We found that, in contrast to pilocarpine seizures, loss of M₁ or CB₁ receptors had no effect on paraoxon seizure severity. CB₁ antagonist or agonist pretreatment also had no effect on paraoxon-induced seizure severity, indicating that CB₁ receptors do not regulate paraoxon seizures. To further explore the difference in M₁ regulation of pilocarpine and paraoxon seizures, we compared the ability of pilocarpine and paraoxon to induce seizure-independent ERK activation in the hippocampus of WT and M₁ KO mice. Seizure-independent activation of ERK by pilocarpine in the hippocampus was dependent on M₁ receptors. However, paraoxon did not activate ERK in the hippocampus when seizure activity was blocked in WT or M₁ KO mice. This suggests that paraoxon administration,

unlike pilocarpine, does not strongly activate M_1 receptors in the hippocampus. Altogether these results indicate that, despite a shared requirement for mAChRs to induce seizures, there are significant differences in the regulation of pilocarpine and paraoxon seizures.

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LIST OF ABBREVIATIONS

2-PAM	pyridine-2-aldoxime methochloride
AC	adenylyl cyclase
ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
aNPC	adult neural progenitor cell
bFGF	basic fibroblast growth factor
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CaM	calmodulin
cAMP	cyclic AMP
CB ₁	cannabinoid receptor 1
CCh	carbachol
ChAT	choline acetyltransferase
CK1 α	casein kinase 1 α
COPD	chronic obstructive pulmonary disease
CP	CP55940
DAG	diacylglycerol
DCX	doublecortin
DSI	depolarization-induced suppression of inhibition
eCB	endocannabinoid
ECL	extracellular loop
EGF	epidermal growth factor
ERK	extracellular signal regulated kinase
FAAH	fatty acid amide hydrolase
GIRK	G protein-activated inward rectifier K ⁺ channel
GPCR	G-protein coupled receptor
GRK	G-protein receptor kinase
HBSS	Hank's balanced salt solution
HRP	horseradish peroxidase
ICL	intracellular loop
IP	intraperitoneal
IP3	inositol triphosphate
JNK	c-Jun NH ₂ -terminal kinase
KO	knockout
M ₁ -M ₅	muscarinic acetylcholine receptor subtypes 1-5
mAChR	muscarinic acetylcholine receptor
MAGL	monoacylglycerol lipase
MAPK	mitogen-activated protein kinase
MTT	thiazoyl blue tetrazolium bromide
NMS	N-methyl scopolamine
OAB	overactive bladder
OxoM	oxotremorine M
PBS	phosphate-buffered saline

PBST	PBS containing 2% Triton X-100
PCNA	proliferating cell nuclear antigen
PDE	phosphodiesterase
PI3K	phosphatidylinositol 3-kinase
PIP ₂	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA ₂	phospholipase A ₂
PLC	phospholipase C
PLD	phospholipase D
QNB	quinuclidinyl benzilate
SDS	sodium dodecyl sulfate
SE	<i>status epilepticus</i>
SGZ	subgranular zone
sIPSC	spontaneous inhibitory postsynaptic currents
SR1	SR141716
SVZ	subventricular zone
TM	transmembrane domain
TNB	Tris-NaCl blocking buffer
TSA	tyramide signal amplification
VACHT	vesicular ACh transporter
VTA	ventral tegmental area
WT	wild-type

CHAPTER 1

INTRODUCTION

Pioneering studies by Dale (1914) and Loewi (1921) identified acetylcholine (ACh) as an important signaling molecule. ACh is an ancient signaling molecule seen in bacteria, fungi, lichens, plants, lower invertebrates, as well as vertebrates (Horiuchi et al., 2003). In vertebrates ACh acts both as a neurotransmitter as well as in an autocrine fashion.

ACh is synthesized primarily by choline acetyltransferase (ChAT) from coenzyme A and choline (see review by Wessler and Kirkpatrick, 2008). ChAT is expressed in cholinergic neurons as well as many non-neuronal cells such as endothelial cells, immune cells, and epithelial cells of the airways, skin, eye, and placenta. ACh can also be synthesized by carnitine acetyltransferase, which is expressed at low levels in the heart and skeletal muscle (Tuček, 1982). In neurons, ACh is transported into vesicles by the vesicular ACh transporter (VACHT). Interestingly, in mammals, the entire coding sequence for VACHT is contained in the first intron of the ChAT gene (Bejanin et al., 1994; Erickson et al., 1994). In nonneuronal cells, the mechanisms for ACh release have not been clarified but studies have demonstrated that organic cation transporters can mediate release to varying degrees (Wessler et al., 2001; Schlereth et al., 2006). ACh is broken down by acetylcholinesterase (AChE), which is expressed in most tissues, and butyrylcholinesterase (also called plasma cholinesterase or pseudocholinesterase), which is synthesized by the liver and released into the blood stream (Giacobini, 2004). Therefore ACh is confined to its area of synthesis and release and does not act as a circulating hormone.

In vertebrates, ACh is an agonist at two types of receptors: nicotinic and muscarinic receptors. Nicotinic receptors are ligand-gated cation channels while muscarinic receptors are G-protein coupled receptors (GPCRs, Caulfield and Birdsall, 1998; Lindstrom, 2003). Nicotinic receptors mediate the actions of ACh at the neuromuscular junction while muscarinic receptors mediate the actions of ACh released from parasympathetic ganglia. Both types of receptors mediate the actions of ACh in the central and autonomic nervous systems and nonneuronal ACh in the periphery.

Classification and Structural Features of Muscarinic Receptors

Muscarinic receptors were initially characterized into two types based on their sensitivity to the muscarinic antagonist pirenzepine (Caulfield and Birdsall, 1998). Eventually 5 receptor subtypes, encoded by 5 different genes, were identified by molecular cloning (Kubo et al., 1986; Peralta et al., 1987; Bonner et al., 1988). All muscarinic subtypes are members of the G-protein coupled receptor (GPCR) superfamily and interact preferentially with one of the classes of heterotrimeric GTP-binding proteins (G-proteins). M_1 , M_3 , and M_5 receptors preferentially couple to $G_{q/11}$ and M_2 and M_4 receptors preferentially couple to $G_{i/o}$.

Like all GPCRs, muscarinic receptors contain 7 transmembrane (TM) domains connected by 3 extracellular loops (ECL) and 3 intracellular loops (ICL), with the N-terminus on the extracellular side and C-terminus on the intracellular side. Site-directed mutagenesis and recent crystal structures of the M_2 and M_3 receptors have identified roles for different domains and specific amino acids in receptor expression and function. The N-terminus is normally glycosylated; however, glycosylation was not necessary for M_2 receptor expression (van Koppen and Nathanson, 1990). ICL3 and the C-terminus contain multiple phosphorylation sites that are

important for agonist-induced receptor desensitization, internalization, and downregulation (Uchiyama et al., 1990; Moro et al., 1993; Haga et al., 1996). There are 2 conserved cysteine residues that form a disulfide bond between ECL1 and ECL2; mutations of these cysteines affect receptor expression (Kurtenbach et al., 1990; Savarese et al., 1992). Crystal structures have identified a pronounced outward bend in TM4 of M₂ and M₃ receptors not seen in other GPCRs crystallized so far (Kruse et al., 2012). A hydrogen bond between a glutamine (Gln207 in the M₃ receptor) and the backbone carbonyl of a nearby lysine (Lys204) stabilizes this bend. This bend appears important for receptor activation, as mutation of conserved amino acids in this region reduced the ability of the muscarinic agonist carbachol to activate M₃ receptors expressed in COS-7 cells (Scarselli et al., 2007).

ACh binds in an “aromatic cage” formed by conserved amino acids located on the outer half of TM3, TM5, TM6, and TM7 (Spalding and Burstein, 2006; Haga et al., 2012; Kruse et al., 2012). This binding site is part of a long hydrophilic channel extending approximately 33 Å from ECL2 (Haga et al., 2012). The choline head group interacts with an aspartate residue in TM3 (Asp 103 in the M₂ receptor) while the ester interacts with tyrosine and asparagine residues (Tyr 403, Asn 404) in TM6. The binding pocket is blocked on the extracellular side by three tyrosines (Tyr148, Tyr506, Tyr529 in the M₃ receptor) and by a set of conserved hydrophobic amino acids (Leu65 in TM2, Leu 114 in TM4, and Ile 392 in TM6 of the M₂ receptor) on the cytoplasmic side. The only difference in the binding pocket amino acids is seen in ECL2 where a phenylalanine (Phe 181) in the M₂ receptor replaces the leucine seen in all other subtypes. Comparison of the ligand binding site in crystal structures of M₂ and M₃ receptors suggests that the change from leucine to phenylalanine eliminates a small pocket in the ligand binding site. Competitive antagonists like N-methyl scopolamine (NMS) and quinuclidinyl benzilate (QNB)

also interact with many of the same residues that ACh does; however, they show differential sensitivity to mutations of the tyrosine and asparagine residues (Tyr 403, Asn 404 in the M₂ receptor) in TM6 (Blüml et al., 1994; Ward et al., 1999). In addition, other residues, such as an asparagine in TM3 (Asn 104 in the M₂ receptor), have been shown to affect binding of antagonists but not ACh.

The G-protein interacts with intracellular regions of muscarinic receptors, specifically with regions of TM3, TM5, TM6, ICL2, and ICL3 (Spalding and Burstein, 2006). Mutations in these regions can alter the level of constitutive receptor activity. There is a conserved DRY sequence in the C-terminal end of TM3 seen in most rhodopsin-like GPCRs (Caulfield and Birdsall, 1998). Interestingly, only mutation of the arginine residue in M₁ receptors reduced G-protein binding and activation (Jones et al., 1995). Mutation of the asparagine and tyrosine residues did not alter G-protein activation but instead significantly lowered M₁ receptor expression (Lu et al., 1997).

These regions also contain sequences that confer G-protein coupling specificity. Exchanging the ICL3 loop of M₂ receptors with that of M₃ receptors switched the M₂ receptor's G-protein preference from G_{i/o} to G_q and vice versa (Wess et al., 1990). Further studies identified two 4 amino acid motifs important for G-protein selectivity of muscarinic receptors. "SRRR" in ICL2 and "AALS" in ICL3 of M₁, M₃, and M₅ receptors are replaced by "CKPM" and "VTIL/F" in M₂ and M₄ receptors. Replacing the "AALS" motif of M₃ receptors with the "VTIL" motif seen in M₂ receptors reduced functional coupling of mutant M₃ receptors to G_q but allowed them to couple to G_{i/o} (Liu et al., 1995). However, replacing only the "VTIL" motif of M₂ receptors with "AALS" removed the ability of M₂ receptors to couple to G_{i/o} but did not allow them to activate G_q (Blin et al., 1995; Liu et al., 1995). Even exchange of ICL3 loops between M₂ and

M₃ receptors alone did not allow the chimeric receptors to activate G-proteins to the same extent as WT receptors (Wess et al., 1990). Mutant M₂ receptors preferentially coupled to G_q only when they contained the “SRRR” motif, the “AALS” motif, and the N-terminal region of ICL3 (Blin et al., 1995).

Crystal structures of the M₂ and M₃ receptors indicated that the position of the cytoplasmic end of TM5 relative to TM3 and TM6 was different between the two receptors (Kruse et al., 2012). These distances were similar to other GPCRs that were G_{i/o}-coupled and G_{q/11}-coupled respectively, suggesting that differences in overall structure determine the G-protein coupling specificity.

Muscarinic Receptor Signaling Pathways

Activation of muscarinic receptors leads to many changes including changes in second messenger levels and activity of kinases, phospholipases, ion channels, and other membrane receptors (see reviews by Caulfield, 1993; Felder, 1995; Nathanson, 2000). These changes are mediated primarily through G-protein dependent signaling pathways. Functional responses to activation of muscarinic receptors are highly cell-type specific and can include depolarization or hyperpolarization of cell membranes and changes in gene transcription, secretion of other signaling molecules, and cell proliferation.

The M₁, M₃, and M₅ receptors preferentially couple to G_{q/11}. G α_q activates phospholipase C β 1 (PLC β 1), producing diacylglycerol (DAG) and inositol triphosphate (IP3) from phosphatidylinositol-4,5-bisphosphate (PIP₂). DAG activates protein kinase C (PKC) and IP3 stimulates IP3 receptors, leading to an increase in intracellular calcium. The M₂ and M₄ receptors

preferentially couple to $G_{i/o}$. $G_{\alpha_{i/o}}$ inhibits adenylyl cyclase (AC), reducing production of cyclic AMP (cAMP) and subsequent protein kinase A (PKA) activity.

Muscarinic receptors also activate $G\beta\gamma$ -dependent signaling pathways. $G\beta\gamma$ mediates the ability of muscarinic receptors to activate mitogen-activated protein kinases (MAPKs) like extracellular signal regulated kinases (ERKs) and c-Jun NH₂-terminal kinase (JNK) via activation of phosphatidylinositol 3-kinase (PI3K) or Rho family GTP-binding proteins like Rac1, Ras, and Raf-1 (Crespo et al., 1994; Coso et al., 1996; Hawes et al., 1996). $G\beta\gamma$ can also mediate increases in DAG and IP₃ by $G_{i/o}$ -coupled receptors via activation of PLC β 2 (Camps et al., 1992; Katz et al., 1992). Other targets of $G\beta\gamma$ include G protein-activated inward rectifier K⁺ (GIRK) channels, AC types 2 and 4, G-protein receptor kinases (GRKs), and phospholipase A₂ (see review by Clapham and Neer, 1997).

Because so many potential signaling pathways can be activated downstream of muscarinic receptors, the end result is highly cell-type specific. For example, cAMP levels can be increased or decreased by muscarinic receptor activation. Muscarinic receptors can decrease cAMP not only via activation of $G_{\alpha_{i/o}}$ but also by activating cAMP-degrading phosphodiesterases (PDEs) that are activated by calcium-calmodulin or by cyclic GMP (cGMP, Hansen et al., 2000). Muscarinic receptors can increase cAMP by stimulating the calcium-calmodulin and PKC activated ACs (types 1 and 3) or the $G\beta\gamma$ activated ACs (types 2 and 4, Simonds, 1999). For instance, M₁ receptors were shown to increase cAMP in Rat1a cells in a calcium-dependent manner and M₄ receptors were shown to increase cAMP in the olfactory bulb in a $G\beta\gamma$ -dependent manner (Russell et al., 1994; Olianias et al., 1998). Finally, muscarinic receptors can couple to G_s ectopically, as shown in one study where overexpression of M₁ or M₄ receptors increased cAMP levels in JEG-3 cells (Migeon and Nathanson, 1994).

Muscarinic receptor-modulation of intracellular calcium is also dependent on cell type. As mentioned earlier, muscarinic receptors can increase calcium by enhancing production of DAG and IP3 by PLC. Muscarinic receptors can also modulate the activity of calcium channels. Muscarinic receptors can modulate the activity of the voltage-insensitive N and P/Q calcium channels by altering the levels of lipids like PIP₂ and arachidonic acid in the membrane and/or activating Gβγ (Herlitz et al., 1996; Suh and Hille, 2002; Wu et al., 2002; Liu and Rittenhouse, 2003; Gamper et al., 2004; Liu et al., 2008). Muscarinic receptors can modulate the activity of voltage-sensitive calcium channels in three major ways. First, they can modulate the membrane potential by altering the activity of potassium channels. Modulation of potassium channel activity affects the membrane potential; increased activity hyperpolarizes the membrane potential and reduces the probability that voltage-sensitive calcium channels will open and allow calcium into the cell. Some of the potassium channels that muscarinic receptors modulate include the GIRK channels expressed in the heart, which are activated by M₂ receptors in a Gβγ-dependent manner, and the M channels expressed in sympathetic ganglia, which are inhibited by M₁ receptors in a PIP₂-depletion dependent manner (Logothetis et al., 1987; Suh and Hille, 2002). Second, muscarinic receptors can modulate the phosphorylation state of voltage-sensitive calcium channels by altering the activity of PKA and PKC. For example, M₂ receptor activation decreased cAMP levels and PKA activity, leading to reduced calcium channel currents in frog hearts (Fischmeister and Hartzell, 1986). M₁ receptors decreased L-type calcium channel activity via activation of PKC in neostriatal neurons (Perez-Burgos et al., 2008). Finally, muscarinic receptors can also regulate channel function by affecting their trafficking (Cayouette et al., 2004; Ishikawa et al., 2004).

Muscarinic receptors can also activate phospholipase A₂ (PLA₂) and phospholipase D. PLA₂ increases the release of arachidonic acid while PLD generates phosphatidic acid. Muscarinic receptor activation of PLA₂ required PKC activity and calcium influx for M₁ and M₃ receptors expressed in A9L cells and M₅ receptors expressed in CHO cells (Conklin et al., 1988; Felder et al., 1990). However, muscarinic agonists were able to activate PLA₂ in the absence of extracellular calcium in M₃ receptor-expressing 1321N1 astrocytoma cells and in a PKC-independent manner in neonatal rat cervical ganglion neurons (Bayon et al., 1997; Liu and Rittenhouse, 2003). Instead, muscarinic receptor activation of PLA₂ was partially mediated by PLC (Liu and Rittenhouse, 2003). PLD activation by M₃ receptors in HEK293 cells was mediated by Rho family GTPases and not by PKC, even though PKC agonists could activate PLD in these cells (Schmidt et al., 1998).

Muscarinic receptors can activate ERKs, JNK, and p38 kinases in a few ways. Muscarinic receptors can activate ERKs and JNK via Gβγ-dependent activation of Ras and Rac1 (Coso et al., 1996; Kiyono et al., 1999). Muscarinic receptors can also activate ERKs via activation of tyrosine kinase receptors like EGF receptors or via β-arrestins (Luttrell et al., 1997; Daaka et al., 1998; Luttrell et al., 2001). Muscarinic activation of MAPKs causes changes in transcription of genes such as c-jun, MEF2A, and MEF2C (Marinissen et al., 1999).

Muscarinic receptor signaling could be further complicated by the possibility of dimer or oligomer-specific signaling pathways. M₂ receptors have been isolated in multiple states including monomers, dimers, and trimers (Park and Wells, 2004). Coexpression of M₂ and M₃ receptors in JEG cells allowed the formation of heterodimers that displayed agonist-induced downregulation properties of M₂ receptors (Goin and Nathanson, 2006). While functional responses mediated by muscarinic receptor heterodimers have not been identified,

heterodimerization may explain unusual antagonist binding properties seen in some tissues (Caulfield and Birdsall, 1998).

Regulation of Muscarinic Receptor Expression

Muscarinic receptors, like other membrane proteins, are translated on ribosomes attached to the endoplasmic reticulum, transported to the Golgi, and eventually inserted into the plasma membrane (see review by Nathanson, 2008). Distribution of these receptors can be uniform or asymmetric in polarized cells. Studies in MDCK cells have indicated that muscarinic receptors can be sorted by preferential insertion into specific membrane domains or by transcytosis (Nadler et al., 2001; Iverson et al., 2005; Chmelar and Nathanson, 2006)

Once at the cell surface, muscarinic receptors can undergo agonist-induced or agonist-independent desensitization. The regulation of these processes differs depending on receptor subtype and the cell type studied (see reviews by van Koppen and Kaiser, 2003; Nathanson, 2008). Phosphorylation of residues in ICL3 and the C-terminus reduces muscarinic receptor activity even when agonist is bound. PKC and casein kinase 1 α (CK1 α) have been shown to phosphorylate M₁ and M₃ receptors independent of agonist-binding, while GRKs preferentially phosphorylate agonist-occupied receptors (Richardson and Hosey, 1990; Debburman et al., 1995; Haga et al., 1996; Schlador and Nathanson, 1997; Budd et al., 2000; Willets et al., 2005). Phosphorylation by GRKs allows β -arrestins to bind (Ferguson et al., 1996; Gurevich et al., 1997). GRKs can also desensitize receptors without phosphorylating them by sterically interfering with the binding of G-proteins to the receptor, as seen in hippocampal neurons where catalytically inactive GRK2 still caused agonist-induced desensitization of M₁ receptors (Willets et al., 2004).

Desensitized muscarinic receptors can be resensitized at the plasma membrane by dephosphorylation or internalized. Binding of β -arrestins following GRK-mediated receptor phosphorylation allows the recruitment of clathrin (Goodman et al., 1996), which is necessary for internalization of M_1 , M_3 , and M_4 receptors in multiple cell types (Claing et al., 2000). On the other hand, internalization of M_2 receptors can be independent of β -arrestin and clathrin in multiple cell types. Internalized muscarinic receptors can then be recycled back to the plasma membrane or degraded. While M_3 and M_4 receptors are recycled in multiple cell types, M_2 receptors are not recycled but only downregulated in HEK293 and IMR-32 cells (Krudewig et al., 2000).

If receptors are downregulated, their expression levels only recover after synthesis of new receptors. This can have significant effects on muscarinic agonist-induced activity. For example, activation of PLC by M_2 receptors expressed in HEK293 cells remained depressed even 16 hours after initial agonist exposure (Krudewig et al., 2000). Interestingly, newly synthesized receptors may not be fully functional at first. Hunter and Nathanson (1984) observed complete recovery of M_2 receptor expression after agonist-induced downregulation in the chick heart by 20 hours post agonist treatment but complete recovery of M_2 receptor functions only after 28 hours. The reason for the lag between recovery of protein expression on the cell surface and functional responses has not been determined but it was not due to differences in agonist or G-protein interactions with newly synthesized receptors.

Expression and Function of Muscarinic Receptors

Muscarinic receptors are expressed in many tissues within the body, mediating the effects of the parasympathetic nervous system, cholinergic neurons, and locally synthesized ACh (see

reviews by Wess, 2004; Eglén, 2006; Wess et al., 2007; Wessler and Kirkpatrick, 2008). Until recently there was a lack of subtype-specific agonists and antagonists, so the expression and functions of specific muscarinic receptor subtypes have mostly been determined by analyzing subtype-specific knockout (KO) animals.

Expression and function of muscarinic receptors in the periphery

All five muscarinic subtypes are expressed in the periphery, with M₂ and M₃ receptors being the most abundant (Ito et al., 2009). Administration of muscarinic agonists to the periphery commonly produces two effects: increased contraction (except for heart and vascular smooth muscle) and increased secretion of other compounds including other neurotransmitters.

Muscarinic agonist-induced contraction of smooth muscles in the lungs, stomach, bladder, gut, intestine, etc., is mediated by both M₂ and M₃ receptors (Chess-Williams et al., 2001; Matsui et al., 2002; Unno et al., 2005; Kitazawa et al., 2007). Interestingly, M₃ receptors mediate the majority of smooth muscle contraction in some tissues like the bladder even though M₂ receptor expression is significantly higher (Chess-Williams et al., 2001). M₂ and M₃ receptors can cause muscle contraction in a few ways such as altering calcium levels of the ileum and decreasing cAMP via activation of PDE4D in the airways (Hansen et al., 2000; Unno et al., 2005; Sakamoto et al., 2007; Tanahashi et al., 2009). In some tissues, M₂ and M₃ receptors modulate different aspects of contraction. For example, in the ileum, the M₂ receptor mediates synchronous contractions while the M₃ receptor regulates their periodicity (Tanahashi et al., 2013).

The M₃ receptor also primarily mediates contraction of the sphincter muscle of the eye, causing pupil constriction. Pupils are almost fully dilated in M₃ KO mice, indicating a normally high level of muscarinic receptor activation (Matsui et al., 2000; Bymaster et al., 2003). Application of the muscarinic antagonist atropine can still further increase pupil size, indicating

that other muscarinic receptors can increase pupil constriction. All 5 muscarinic subtypes have been shown to express in the eye, with M_3 receptors being the most prominent (Gil et al., 1997; Ishizaka et al., 1998). Unlike other smooth muscle tissues, the additional mediator of pupil constriction is not the M_2 receptor; instead, M_2 receptor activity appears to increase pupil dilation, as M_2/M_3 double KO mice had smaller pupils than M_3 KO mice (Bymaster et al., 2003). Additional pupil constriction may be mediated by M_5 receptors, as seen in a few species like the dog (Choppin and Eglen, 2001).

The heart and vasculature are the two tissues where muscarinic agonists cause muscle relaxation instead of contraction. In the heart, application of ACh causes a reduction in heart rate (bradycardia) mediated mostly by M_2 receptors, which activate GIRK channels that hyperpolarize the cells (Logothetis et al. 1987; Gomeza et al., 1999a; Stengel et al., 2000). However, when M_2 receptor activity was removed, muscarinic agonist carbachol increased the frequency of left atrial contractions in a M_3 -receptor dependent manner (Kitazawa et al., 2009). Vascular smooth muscle, on the other hand, is not innervated by cholinergic neurons; instead, pharmacologically administered ACh produced vasodilation by acting on vascular endothelial cells to increase nitric oxide production, which then diffuses into vascular smooth muscle to cause relaxation (Furchgott and Vanhoutte, 1989). Removal of these endothelial cells reverses the ACh response in vascular smooth muscle, causing them to contract (Furchgott and Zawadzki, 1980). In most arteries, vascular smooth muscle relaxation is primarily M_3 receptor-dependent but M_1 receptors contribute in human pulmonary arteries, indicating some region and species variability (Norel et al., 1996; Gericke et al., 2011).

The subtypes of muscarinic receptors that mediate muscarinic agonist-induced secretion are tissue-dependent, though M_2 and/or M_3 receptors are commonly involved. M_2 receptors are

the primary mediator of ACh-induced noradrenaline release in the heart, urinary bladder, and vas deferens, with M₃ receptors contributing a small portion in the heart and M₃ and M₄ receptors contributing in the vas deferens (Trendelenburg et al., 2003; Trendelenburg et al., 2005). M₃ and M₅ receptors mediate ACh-dependent increases in gastric acid and histamine release in the stomach (Aihara et al., 2003; Aihara et al., 2005). M₃ receptors also mediate ACh-stimulated mucus glycoprotein and electrolyte secretion from tracheal submucosal glands and insulin secretion from pancreatic beta cells (Ishihara et al. 1992; Ramnarine et al., 1996; Gautam et al., 2006). Muscarinic agonist-induced salivation is mediated mostly by M₃ receptors, with M₁ and M₄ receptors having a minor contribution (Matsui et al., 2000; Yamada et al., 2001a; Bymaster et al., 2003; Nakamura et al., 2004). Muscarinic receptors, primarily M₂ receptors but occasionally M₄ receptors, also regulate the release of ACh; activation of these receptors reduces ACh release (Slutsky et al., 2003; Takeuchi et al., 2005; Takeuchi et al., 2008).

Non-neuronal ACh acts on muscarinic receptors in both parasympathetic-innervated tissues and non-innervated tissues. In keratinocytes, roles have been identified for M₁, M₃, and M₄ receptors. Activation of M₁ receptors reduced the permeability of keratinocyte monolayers by reducing phosphorylation of β -catenin and p120-catenin (Chernyavsky et al., 2008). Loss of M₄ receptors decreased migration of human keratinocytes while loss of M₃ receptors caused increased migration, cell shrinkage, and acantholysis (Chernyavsky et al., 2003; Nguyen et al., 2003; Chernyavsky et al., 2004).

Activation of muscarinic receptors also leads to the release of cytokines and increased inflammation from immune and epithelial cells (see reviews by Kawashima et al., 2012; Kistemaker et al., 2012). So far, M₃ receptors appear to mediate a large portion of the inflammatory response in the airways while M₁ and M₅ receptors mediate muscarinic agonist

responses in immune cells. M₃ receptor activation increased the secretion of substances including interleukin-8, a cytokine which acts as a chemotactic agent for neutrophils and monocytes, from bronchial epithelial and airway smooth muscle cells (Koyama et al., 1992; Profita et al., 2008; Gosens et al., 2009; Oenema et al., 2010). M₃ receptor activation also increased the secretion of similar fluids from alveolar macrophages (Sato et al., 1998). M₁ and M₅ receptors modulated interleukin-6 secretion from spleen cells (Fujii et al., 2007). M₁ receptors also regulated the lytic activity of CD8+ T cells, though it may be due to regulation of CD8+ T cell differentiation (Zimring et al., 2005).

Finally, muscarinic receptors also regulate the proliferation, differentiation, and survival of many cells including cancerous cells, fibroblasts, and immune cells (see reviews by Resende and Adhikari, 2009; Shah et al., 2009; Kawashima et al., 2012; Kistemaker et al., 2012).

Muscarinic receptors can modulate proliferation, differentiation, and survival by activation of MAPKs, PI3K, etc. (Resende and Adhikari, 2009). The receptors involved and the pathways activated are cell-type specific. For example, M₁ and M₃ receptor activation increased proliferation of human fibroblasts in a PLC- and calmodulin (CaM)-dependent manner, while in the airways, muscarinic agonists increased proliferation of lung fibroblasts was G_{i/o}-dependent, suggesting a role for M₂ receptors in proliferation of these cells (Casanova et al., 2006; Matthiesen et al., 2006). Changes in muscarinic receptor expression seen in cancerous cells may allow them to be more responsive to muscarinic agonists and increase their proliferation and/or survival (Resende and Adhikari, 2009; Shah et al., 2009).

Expression and function of muscarinic receptors in the brain

All five muscarinic receptor subtypes are expressed in the brain and mediate a multitude of functions including regulation of learning and memory, cholinergic seizures, locomotion,

body temperature, body weight, and nociception (see reviews by Wess, 2004; Eglen, 2006; Wess et al., 2007). A combination of radioligand binding assays in subtype-specific KO mice and immunohistochemistry has been used to determine the expression and localization of muscarinic receptor subtypes in different brain regions (Levey et al., 1991; Mrzljak et al., 1993; Rouse et al., 1998; Rouse et al., 2000; Fukudome et al., 2004; Oki et al., 2005). The M₁ receptor is the predominant subtype in the cortex and the hippocampus; M₂, M₃, and M₄ receptors are also expressed at lower levels (Oki et al., 2005). The M₂ receptor is the predominant subtype in hindbrain regions including the midbrain, pons, cerebellum, and spinal cord. The diencephalon and hypothalamus mostly express M₂, M₃, and M₄ receptors. The primary muscarinic receptors in the striatum are M₁ and M₄ receptors but there is also significant M₂ receptor expression. The M₅ receptor is expressed at very low levels and is not detected by radioligand binding assays in most brain regions but has specialized localization to cerebral arteries and dopaminergic neurons in the substantia nigra and ventral tegmental area (VTA, Vilaró et al., 1990; Weiner et al., 1990; Yamada et al., 2001b).

Within brain regions, muscarinic receptor subtypes are expressed differently. The M₁ and M₃ receptors are generally expressed postsynaptically, while the M₂ and M₄ receptors are generally expressed presynaptically (Levey et al., 1991; Mrzljak et al., 1993; Rouse et al., 1998). In the cortex, M₁ receptors are expressed in layers II-VI on dendritic spines or shafts (Levey et al., 1991; Mrzljak et al., 1993). M₂ receptors are expressed in layer IV and the V/IV border, mostly on presynaptic axons but also postsynaptically in spines and smaller dendrites. M₄ receptors are in cortical layers II/III and patches of layers IV and V. In the hippocampus, M₁ receptors are expressed in the CA1 and dentate gyrus granule cells while M₃ receptors are in the granule cell layer and molecular layer (Rouse et al., 1998). M₂ receptors are expressed in the

CA2 and CA4 and are presynaptic in the granule cell region (Levey et al., 1991; Rouse et al., 1998). They have been found to colocalize with VACHT or with GABAergic neuronal markers (Rouse et al., 2000; Fukudome et al., 2004). M₄ receptors are localized to the middle third of the molecular layer (Levey et al., 1991). In the striatum, M₁ receptors are in the perikarya and dendrites of D1 or D2 dopamine receptor-positive medium spiny neurons (Narushima et al., 2007). M₄ receptors are also expressed in medium spiny neurons as well as striatal interneurons (Hersch et al., 1994). While M₁ and M₄ receptor expression will sometimes overlap, M₂ receptor expression appeared specific to large striatal interneurons including cholinergic interneurons.

There has been great interest in the regulation of learning and memory by muscarinic receptors. While non-specific muscarinic receptor antagonists produce significant deficits in learning and memory, the role of different muscarinic receptor subtypes in learning and memory is still unclear. Behavior studies have suggested possible roles for M₁, M₂, M₃, and M₅ receptors in learning and memory. Deficits in non-matching to sample working memory, context, and cued memory in M₁ KO mice or by M₁ antagonists have been seen in some studies but not others (Miyakawa et al., 2001; Anagnostaras et al., 2003; Sheffler et al., 2009). Interestingly, one study saw no difference in learning and memory between 9-month-old M₁ KO and WT mice but saw significant deficits in spatial, context, and social memory of M₁ KO mice at 18 months (Medeiros et al., 2011). Studies with M₃ KO mice have also been mixed. Yamada et al. (2001b) saw no effect of M₃ KO on context or spatial memory, while Poulin et al. (2010) saw a deficit in context memory that was accompanied by a loss of c-Fos expression following learning-dependent receptor phosphorylation. Studies with M₂ KO mice showed that they had a deficit in aversive memory, behavioral flexibility, and working memory (Tzavara et al., 2003; Seeger et al.,

2004). Finally, M₅ KO mice showed a deficiency in novel object recognition and spatial memory (Araya et al., 2006).

The hippocampus and cortex are important regions for learning and memory (Gale et al., 2001; Tinsley et al., 2011; Pergola and Suchan, 2013). Thus many studies have looked into the role of muscarinic receptor subtypes in modulating electrophysiological properties of cortical and hippocampal neurons. M₁ receptor activity is responsible for almost all of the G_q activation, PI hydrolysis, and MAPK activation induced by muscarinic agonists in the cortex and hippocampus (Berkeley et al., 2001; Hamilton and Nathanson, 2001, Porter et al., 2002; Bymaster et al., 2003). Despite the dominance of M₁ receptor activity in muscarinic agonist-induced biochemical measures, roles for M₂, M₃, and M₄ have also been identified in the cortex and hippocampus. Both M₂ and M₄ receptors regulate the concentration of ACh in the hippocampus and cortex (Zhang et al., 2002a; Tzavara et al., 2003; Kuczewski et al., 2005). Meanwhile, M₃ receptors mediate actions similar to M₁ receptors in the cortex and hippocampus, albeit at a smaller magnitude, as complete abolishment of ACh-induced extracellular field potential depression in the cortex, muscarinic agonist-induced endocannabinoid release in the hippocampus, and agonist-induced spike acceleration of basket cells in the hippocampus was only seen in M₁/M₃ double KO and not M₁ KO mice (Kuczewski et al., 2005; Cea-del Rio et al., 2010). While M₅ receptor protein expression has not been identified in the hippocampus, M₅ KO mice had reduced numbers of dendritic spines on CA2, CA3, and dentate gyrus neurons (Araya et al., 2006).

Long-term potentiation (LTP) and long-term depression (LTD) of neurons in the hippocampus, which are commonly used as predictors for learning and memory (Vitureira and Goda, 2013), are regulated by M₁ and M₂ receptors. Muscarine-induced LTP is mediated by M₂

receptors (Seeger et al., 2004). Muscarinic regulation of LTP following theta burst stimulation (TBS) was diminished by loss of M₂ receptors and possibly M₁ receptors (Anagnostaras et al., 2003; Seeger et al., 2004; Kamsler et al., 2010). The ability of M₂ receptors to facilitate LTP was blocked by GABA_A antagonists, indicating that M₂ receptors were acting presynaptically to inhibit firing of GABAergic inhibitory neurons (Seeger et al., 2004). M₁ receptors also potentiate NMDA-induced LTP and metabotropic glutamate receptor-induced LTD (Marino et al., 1998; Kamsler et al., 2010).

The hippocampus and cortex are also the first areas affected by pilocarpine-induced seizures (Turski et al., 1984). Pilocarpine-induced seizures require activation of M₁ receptors (Hamilton et al., 1997; Bymaster et al., 2003). However, as shown in Chapter 4, the M₁ receptor is not the only muscarinic subtype that can mediate seizure activity. Organophosphate-induced seizures also require muscarinic receptor activity (Shih et al., 1991), but in Chapter 4 we show that paraoxon, an organophosphorus inhibitor of AChE, can cause seizures even in M₁ KO mice.

Muscarinic receptors also modulate locomotion. Locomotor activity is primarily controlled by the striatum (Pisani et al., 2007). Muscarinic agonists cause tremors mediated by M₂ receptors that are believed to be expressed in the striatum (Gomez et al., 1999a; Bymaster et al., 2003). KO mice studies have implicated both M₁ and M₄ receptors in the regulation of basal locomotor activity. Loss of M₄ receptors caused a significant increase in basal locomotor activity (Gomez et al., 1999b). Loss of M₁ receptors also increased basal locomotor activity in certain cases, interfering with the ability of behavioral studies to determine changes in learning and memory (Gerber et al., 2001; Miyakawa et al., 2001; Anagnostaras et al., 2003; Gullledge et al., 2009; Woolley et al., 2009). Muscarinic receptors can alter locomotor activity through the modulation of dopamine signaling. Muscarinic agonists cause an increase in dopamine release in the striatum

mediated primarily by M₄ receptors (Zhang et al., 2002b). M₁ receptor activity increased dopamine release in one study but had no effect in another (Gerber et al., 2001; Zhang et al., 2002b). M₅ receptor activation also increased dopamine release, while M₃ receptor activation decreased dopamine release (Yamada et al., 2001b; Zhang et al., 2002b). Modulation of dopamine release by M₃ and M₄ receptors was via modulation of GABAergic neuron activity; M₃ receptors increased GABAergic activity while M₄ receptors decreased it (Zhang et al., 2002b). Specifically, M₄ receptors expressed in D1 dopamine receptor-expressing GABAergic neurons were able to reduce hyperactivity induced by amphetamine and apomorphine (Dencker et al., 2011). In addition, M₄ receptors decrease ACh levels in the striatum, despite not localizing to cholinergic neurons (Zhang et al., 2002a).

Muscarinic receptor activation also regulates the electrophysiological properties of striatal neurons. M₁ receptors have been shown to mediate muscarinic agonist-induced decreases in KCNQ currents and Kir2 currents of medium spiny neurons by depleting PIP₂, leading to increased excitability of these cells (Shen et al., 2005; Shen et al., 2007). M₁ receptors also mediate depolarization-induced suppression of inhibition (DSI) of medium spiny neurons via increasing endocannabinoid levels (Narushima et al., 2007).

Muscarinic agonists induce analgesia via M₂ and M₄ receptors (Gomez et al., 1999a and 1999b; Duttaroy et al., 2002). M₂ receptors are the most abundant muscarinic subtype in the spinal cord, followed by M₃ and M₄ receptors (Oki et al., 2005). M₂ receptors localize to lamina II and the outer surface of motor neurons in lamina IX (Duttaroy et al., 2002). Muscarinic agonists decrease spontaneous inhibitory postsynaptic currents (sIPSCs) of a majority but not all glycinergic neurons in lamina II (Zhang et al., 2006; Zhang et al., 2007). Muscarinic agonist-induced decreases in sIPSCs are mediated by M₂ and M₄ receptors, while muscarinic agonist-

induced increases in sIPSCs, only seen when M₂ receptor activity is absent, are mediated by M₃ receptors.

Muscarinic receptors in the hypothalamus regulate normal body functions such as hormone levels, body temperature, and eating. Activation of M₂ receptors leads to an increase in serum corticosterone levels (Hemrick-Luecke et al., 2002). M₂ receptors also mediate muscarinic agonist-induced hypothermia (Gomez et al., 1999a; Bymaster et al., 2003). M₃ receptors regulate eating. M₃ KO mice are hypophagic and lean due to eating less food (Matsui et al., 2000; Yamada et al., 2001a). These mice have reduced serum levels of leptin and triglycerides, and AGRP does not stimulate eating in these mice (Yamada et al., 2001a).

Activation of M₅ receptors in the cerebrovasculature causes arterial dilation (Yamada et al., 2001b). Like the M₃ receptor in the peripheral vasculature, the M₅ receptor is expressed in the endothelium of cerebral arteries (Tayebati et al., 2003). Araya et al. (2006) speculate that the reduced blood flow to areas such as the hippocampus in M₅ KO mice caused the reduction of dendritic spines and dendritic tree size, leading to the observed learning deficits.

The M₅ receptor is also the only receptor expressed in dopaminergic neurons of the VTA (Vilaró et al., 1990; Weiner et al., 1990). M₅ KO mice lost the third, prolonged phase of dopamine release evoked by stimulation of dopaminergic neurons in the VTA (Forster et al., 2002). Loss M₅ receptors also reduced the reward effects of morphine and cocaine (Basile et al., 2002; Fink-Jensen et al., 2003). Behaviorally, M₅ KO mice showed reduced conditioned place preference and decreased withdrawal symptoms for morphine and cocaine. Biochemically this was reflected in the reduced dopamine release and Fos-B expression by morphine seen in the accumbens of M₅ KO mice (Basile et al., 2002).

Muscarinic regulation of APP processing and tau phosphorylation is mediated by the M₁ receptor (Davis et al., 2010; Medeiros et al., 2011). Muscarinic agonists increased processing of APP to APPs α instead of A β without increasing levels of APP processing enzymes. Loss of M₁ receptor activity in a transgenic Alzheimer's disease mouse line caused increased A β in the vasculature and S-positive fibrillar A β deposits (Medeiros et al., 2011). The increase in A β due to loss of M₁ receptor activity correlated with upregulation of interleukin-1 β (IL-1 β), tumor necrosis factor α (TNF α), and increased numbers of astrocytes and microglia.

Finally, studies with embryonic neural progenitor cells have indicated that activation of muscarinic receptors increases neuronal cell proliferation and differentiation *in vitro* (Ma et al., 2000; Li et al., 2001; Zhou et al., 2004; Resende and Adhikari, 2009). These cells expressed significant levels of M₂, M₃, and M₄ receptors but not M₁ and M₅ receptors (Ma et al., 2000). The role of muscarinic receptors in adult neural progenitor cell proliferation and differentiation is explored in Chapter 2. While some studies suggested that activation of muscarinic receptors could also increase proliferation and differentiation of adult neural progenitor cells (Kotani et al., 2006; Van Kampen and Eckman, 2010), we did not observe any effect of muscarinic agonists on adult neural progenitor cell proliferation and survival.

Therapeutic Uses for Muscarinic Agonists and Antagonists

Muscarinic agonists and antagonists have many potential therapeutic uses for treatment of diseases (see reviews by Eglen, 2006; Wess et al., 2007). However, due to many unwanted side-effects, non-selective muscarinic agonists or antagonists are most commonly used for short-term therapy or in low doses, limiting their therapeutic potential. The development of subtype-

selective agonists, antagonists, and allosteric modulators is necessary for muscarinic receptor-based therapies to truly be useful clinically.

Muscarinic agonists could be useful for treating cognitive deficits including those seen in Alzheimer's disease (AD), dementia, and schizophrenia (see reviews by Terry and Buccafusco, 2003; Conn et al., 2009; Jones et al., 2012). Cholinergic agonists, mostly AChE inhibitors, have been used clinically for treating Alzheimer's disease but toxicity limited their long-term use and dose (Conn et al., 2009). AChE inhibitors appeared to increase cognitive performance even in normal, non-AD patients. Animal studies (reviewed above) have shown that M₁, M₂, M₃, and M₅ receptors positively modulate learning and memory and could be ideal targets for these diseases. M₁ receptor agonists are especially attractive drugs for AD, as they may not only improve cognition but they decrease A β and tau phosphorylation, abnormalities seen in brains of AD patients.

There has been a lot of interest in the use of M₁, M₄, and M₁/M₄ agonists in treating symptoms of schizophrenia. M₁ and M₄ receptor expression levels are reduced in schizophrenic patients (Jones et al., 2012). The M₄ receptor also modulates anxiety behavior (Degroot and Nomikos, 2006). The M₁/M₄ agonist xanomeline caused a reduction in vocal outbursts, suspiciousness, delusions, and other signs of psychosis in AD patients (Bodick et al., 1997). These behavioral improvements would also be beneficial for schizophrenia.

M₂/M₄ agonists could be useful as analgesics. While M₂ receptors mediate most of the analgesia induced by non-selective muscarinic agonists, M₄-preferring agonists can also induce significant analgesia (Duttaroy et al., 2002). M₄-selective agonists may be more ideal than M₂ agonists, as M₂ agonists would have cardiac side effects.

M₃ agonists could be useful in treating Type 2 diabetes. Even though M₃ KO mice appeared to have increased insulin sensitivity, loss of M₃ receptors specifically in pancreatic β cells increased serum glucose levels while overexpression of M₃ receptors or expression of constitutively active M₃ receptors in pancreatic β cells reduced serum glucose levels (Yamada et al., 2001a; Gautam et al., 2006; Gautam et al., 2010). Mice with increased M₃ receptor expression or constitutively active M₃ receptors in beta cells also had improved glucose tolerance and maintained normal levels of serum glucose even when fed a high-fat diet (Gautam et al., 2006; Gautam et al., 2010). The increased sensitivity to insulin in whole body M₃ KO is most likely due to the fact that those mice are hypophagic and lean (Yamada et al., 2001a).

M₃ agonists could also be useful for treating dry mouth and peptic ulcers because they are the primary mediators of ACh-mediated salivation and gastric acid secretion (Matsui et al., 2000; Yamada et al., 2001a; Aihara et al., 2003). For dry mouth, M₁ agonism could also be beneficial as M₁ receptors also mediate a smaller but significant amount of muscarinic agonist-induced salivation (Nakamura et al., 2004). Indeed, the muscarinic agonist cevimeline, which is used to relieve dry mouth and other symptoms of Sjögren's syndrome, primarily acts at M₁ and M₃ receptors (see review by Fox et al., 2001). For peptic ulcers, M₅ agonism could also be beneficial as M₅ receptors also increase gastric acid secretion (Aihara et al., 2005).

Muscarinic antagonists, particularly ones that target M₃ receptors, are useful for relieving excessive contraction of smooth muscles in diseases such as overactive bladder (OAB), chronic obstructive pulmonary disease (COPD), and asthma (see reviews by Abrahms et al., 2006; Kistemaker et al., 2012). M₃ receptors are the primary mediators of muscarinic agonist-induced contractions in smooth muscles, with M₂ receptors playing a smaller but significant role (Matsui et al., 2002; Unno et al., 2005; Kitazawa et al., 2007). In the bladder, M₂ receptors only play a

minor role in contraction, so M₃-selective antagonists would be ideal (Chess-Williams et al., 2001). However, excessive blockade of M₃ receptors causes side effects such as dry mouth due to inhibition of salivation (Abrams et al., 2006). Therefore, many OAB drugs target muscarinic receptors as well as calcium or potassium channels.

While muscarinic antagonists have been used clinically primarily to relax bronchial smooth muscles, muscarinic antagonism may be additionally beneficial in COPD to reduce inflammatory responses and airway remodeling (see review by Kistemaker et al., 2012). Cigarette smoke extract was shown to increase the expression of M₂ receptors, M₃ receptors, and ChAT, and it worked synergistically with muscarinic agonists to increase production of LTB₄ (Profita et al., 2011). Increased muscarinic activity can also synergistically increase the upregulation of inflammatory genes caused by bronchial smooth muscle stretching (Kanefsky et al., 2006). The M₃ muscarinic antagonist tiotropium, which was shown to increase the lung function of COPD patients during a 4-year clinical trial (Tashkin et al., 2008), was able to significantly reduce the inflammation caused by cigarette smoke; the number of neutrophils that accumulated in the lungs of mice was reduced 60% and the concentrations of many inflammatory markers in the bronchial fluid was reduced 50-90% (Wollin and Pieper, 2010). However tiotropium did not reduce the number of neutrophils or the increase in inflammatory factors in bronchial fluid induced by LPS, suggesting that muscarinic antagonism may be effective only in certain conditions.

M₅-specific antagonists could be used to treat drug addiction. M₅ KO mice have reduced reward and withdrawal symptoms from morphine and cocaine (Basile et al., 2002; Fink-Jensen et al., 2003). Unlike M₄ receptors, whose activation can reduce dopamine release from VTA neurons, M₅ receptor activation specifically increase dopamine signals involved in reward and

not locomotion. M₅ antagonists may be especially useful in the case of morphine and other opiates used to treat pain, since M₅ KO mice still experienced morphine-induced analgesia (Basile et al., 2002). In addition, M₅ receptors only play minor roles in the periphery and therefore M₅ antagonists would have little peripheral side-effects.

Finally, muscarinic agonists and antagonists may be useful for treating different cancers. Muscarinic receptor expression has been reported in many cancerous cell types, including brain, breast, colon, skin, lung, and prostate cancer (see review by Shah et al., 2009). In many of these cell types, activation of M₁, M₂, and/or M₃ receptors causes an increase in proliferation, but muscarinic receptor activation can also cause cell cycle arrest and decreased proliferation. In addition, muscarinic receptor subtypes may have opposing actions, making a subtype-selective agonist or antagonist more ideal to enhance the desired effect. For example, in skin cells, M₃ agonism and M₄ antagonism would be ideal to reduce skin cell migration (Chernyavsky et al., 2003; Nguyen et al., 2003; Chernyavsky et al., 2004). Muscarinic agonist and antagonist treatment of cancer, therefore, would need to be specific to the type of cancer.

In conclusion, the 5 subtypes of muscarinic receptors are structurally similar GPCRs expressed throughout the body. The M₁, M₃, and M₅ receptors couple to G_{q/11} and the M₂ and M₄ receptors couple to G_{i/o}. Through activation of G-proteins, muscarinic receptors can activate a plethora of signaling pathways including regulation of second messengers, other neurotransmitters, and ionic currents. These changes allow muscarinic receptors to modulate many functional responses in the periphery and the brain. Subtype-specific KO mice have clarified the roles of muscarinic receptor subtypes in various tissues and have identified both synergistic and antagonistic interactions between coexpressed muscarinic receptors. Despite a relative lack of subtype-selective muscarinic agonists and antagonists, muscarinic receptors are

still an attractive therapeutic target for the regulation of many diseases like Alzheimer's and Parkinson's disease, asthma and COPD, irritable bowel syndrome, etc. The development of additional subtype-selective muscarinic agonists and antagonists will only increase the therapeutic potential of muscarinic receptors.

CHAPTER 2

THE ROLE OF MUSCARINIC ACETYLCHOLINE RECEPTORS IN ADULT NEUROGENESIS

Introduction

Since the discovery of a low but persistent birth of neurons (neurogenesis) in adult mammalian brain, there has been increasing interest in the possible roles of adult neurogenesis in normal and pathological brain functions. Under normal conditions, adult neurogenesis has been proposed to regulate learning and memory and mood (Deng et al., 2010; David et al., 2010). Many neuronal diseases alter the levels of adult neurogenesis. For instance, adult neurogenesis is increased after ischemia or seizures and decreased in many models of neurodegeneration and depression (Ming and Song, 2005). A multitude of factors including age, genetic background, environmental factors, hormones, and neurotransmitters have been shown to regulate adult neurogenesis (Suh et al., 2009).

Adult neurogenesis in mammals normally occurs in two specific regions: the subventricular zone (SVZ) that lines the lateral ventricles and the subgranular zone (SGZ) that lines the innermost layer of the dentate gyrus (Suh et al., 2009). Over the course of 4-7 weeks, new cells in the SVZ and SGZ are born from neuronal stem cells, differentiate, and migrate to the olfactory bulb and dentate gyrus respectively. While a small but significant number of cells are born each day, a large percentage of these newborn cells will not be incorporated into mature circuits. At 2-3 weeks of age, new cells that have not been integrated into olfactory bulb or dentate gyrus circuits undergo apoptosis.

Many studies have suggested that acetylcholine (ACh) is a positive regulator of adult neurogenesis with differential regulation of SVZ-olfactory bulb and dentate gyrus neurogenesis. Lesioning of cholinergic fibers caused a significant decrease in the number of proliferating cells in the SGZ with no effect on the SVZ (Mohapel et al., 2005). Numerous studies have used acetylcholinesterase (AChE) inhibitors to examine the effect of ACh signaling on proliferation and survival of new cells in the SVZ-olfactory bulb and dentate gyrus (Mohapel et al., 2005; Kaneko et al., 2006; Kotani et al., 2006; Itou et al., 2011). While these studies give conflicting results on whether increasing endogenous ACh levels caused an increase in cell proliferation in the SGZ, they all demonstrated an increase in the number of new neurons integrating into the olfactory bulb and the dentate gyrus, indicating a positive effect of increased endogenous ACh on adult neurogenesis.

The involvement of muscarinic receptors in adult neurogenesis was first reported after pilocarpine-induced seizures in rats (Parent et al., 1997; Parent et al., 2002). Increased cell proliferation in the SGZ and SVZ were observed for a period of up to two weeks following pilocarpine status-epilepticus, leading to an increase in newborn cells incorporated into the dentate gyrus and olfactory bulb respectively. There is also evidence that activation of muscarinic receptors may be able to increase adult neurogenesis in a seizure-independent manner. Van Kampen and Eckman (2010) found that administration of the muscarinic agonist oxotremorine increased cell proliferation in the hippocampus of rats, an effect blocked by coadministration of the muscarinic antagonist pirenzepine. Kotani et al. (2006) found that administration of the muscarinic antagonist scopolamine decreased the number of newborn cells that lived to 4 weeks of age in the dentate gyrus of rats, though it was unclear whether this was due to an effect on proliferation and/or survival of new cells.

In vitro studies suggest that activation of muscarinic receptors expressed by neural progenitor cells themselves could lead to an increase in their proliferation and/or survival. Studies with embryonic neural progenitor cells have shown that activation of muscarinic receptors increased cell proliferation and differentiation in a calcium-dependent manner (Ma et al., 2000; Li et al., 2001; Zhou et al., 2004). While similar *in vitro* studies have yet to be done in adult neural progenitor cells, ACh and muscarine caused calcium responses in hippocampal adult neural stem cells, indicating that these cells express functional muscarinic receptors (Itou et al., 2011).

The goals of this study were to confirm a role of muscarinic receptors in adult neurogenesis, determine which processes they regulated, and identify the signaling pathways that may be involved. Instead we found no evidence that muscarinic receptor activity significantly affected adult neurogenesis. Short-term activation of muscarinic receptors *in vivo* using muscarinic agonists did not increase cell proliferation in a seizure-independent manner. *In vitro* studies demonstrated that direct modulation of muscarinic receptors expressed on adult neural progenitor cells did not change their growth rate or viability. In addition, loss of the M₁ subtype, which is the most abundant muscarinic receptor subtype in the hippocampus and forebrain (Oki et al., 2005), did not affect adult neurogenesis.

Materials and Methods

Animals

C57Bl/6 male mice were obtained from Charles River and used at 8-12 weeks of age except for one experiment where 6-month-old mice were used. WT and M₁ knockout (KO) mice were age-matched and used at 8-10 weeks of age. Mice were prehandled for at least 2 days prior

to use in experiments. All procedures involving animals were approved by the University of Washington Institutional Animal Care and Use Committee.

Chemicals and Drugs

Pilocarpine hydrochloride, oxotremorine sesquifumarate, oxotremorine M, 5-Bromo-2'-deoxyuridine (BrdU), atropine sulfate, carbamylcholine chloride (carbachol), and thiazolyl blue tetrazolium bromide (MTT) were all purchased from Sigma. Diazepam (Hospira), phenobarbitol (West-Ward), ketamine hydrochloride (Ketaset), and xylazine (AnaSed) were purchased as stock solutions dissolved in 0.9% saline from the University of Washington Medical Center Pharmacy. Advanced DMEM/F12, Pen Strep, L-Glutamine (200 mM), N-2 supplement, B-27 supplement, 0.05% trypsin-EDTA, and trypsin inhibitor (soybean) were all purchased from Invitrogen. EGF was purchased from EMD and bFGF was purchased from Millipore. Stock solutions of EGF and bFGF were made to 100 ng/ μ L in PBS + 1% BSA and stored at -80°C.

In vitro neurogenesis

For comparisons of seizure-dependent vs. seizure-independent neurogenesis, mice were divided into 3 groups: control, seizure-prevented, and seizure-permitted mice. For the seizure-permitted group, mice were injected intraperitoneally (IP) with 325 mg/kg pilocarpine. After 2 hours of *status epilepticus* (SE) or 2 hours after pilocarpine injection if no seizures occurred, 4 mg/kg diazepam was given by IP injection to terminate seizure activity. Forty-five minutes to 1 hour later, 30 mg/kg phenobarbitol was given by IP injection to further block seizures. For the seizure-prevented group, diazepam was given 15 minutes prior to pilocarpine and phenobarbitol was given 3 hours after pilocarpine. Control mice were treated the same as seizure-permitted mice except an equal volume of saline was substituted for pilocarpine. BrdU was given one, three, or six days after drug treatments (see below). Mice were perfused the following day.

To examine seizure-independent neurogenesis more closely, a different scheme was used. 4 mg/kg diazepam was given 15 minutes prior to saline or 325 mg/kg pilocarpine, and no phenobarbital was used. BrdU was given two or three days later and mice were perfused the following day.

To examine the effect of extended mAChR activation on neurogenesis, 1 mg/kg oxotremorine was injected IP 3 times a day, 4 hours apart, for 3 days. BrdU was given the following day. Mice were perfused one day or four weeks after BrdU injection.

To compare basal neurogenesis levels between WT and M₁ KO mice, age-matched or WT and M₁ KO littermates were injected with BrdU at 8 weeks of age and perfused 4 weeks later.

BrdU labeling

Mice were injected IP with 200 mg/kg BrdU dissolved in 0.9% saline/0.007N NaOH twice, 6 hours apart.

Tissue processing

Mice were anaesthetized with 140 mg/kg ketamine/10 mg/kg xylazine by IP injection before cardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight. Brains were then soaked in 30% sucrose in PBS for 1-2 days before frozen on dry ice. Frozen brains were sectioned at 40 µm, and sections were stored at -20°C in a cryoprotectant solution (30% ethylene glycol, 30% glycerol, 0.1 M phosphate buffer, pH 7.4).

Immunofluorescence

40 µm free-floating sections were washed in PBS and PBST (PBS containing 2% Triton X-100) before processing for BrdU or PCNA immunofluorescence. For BrdU

immunofluorescence, sections were treated with HCl before being blocked. They were washed for 5 minutes in water before they were incubated for 10 minutes at 4°C with 1 M HCl and 30 minutes at 37°C with 2 M HCl. Acid treatment was quenched with two 5-minute washes with 0.1 M sodium borate, pH 8.5. For PCNA immunofluorescence, antigen retrieval (10 minutes of boiling in 10 mM sodium citrate, pH 6) was performed prior to blocking. Blocking was done for 1 hour at room temperature in blocking buffer (2% bovine serum albumin, 0.1 M glycine, 0.05% sodium azide, 10% appropriate animal serum in PBST). Sections were then incubated in primary antibody solutions for 4 hours at room temperature or overnight at 4°C. Primary antibodies were diluted in blocking buffer at the following dilutions: 1:200 for rat anti-BrdU (AbD Serotec), 1:400 for donkey anti-doublecortin (Santa Cruz), 1:1000 for mouse anti-NeuN (Millipore), and 1:1000 for mouse anti-PCNA (Santa Cruz).

If double-immunofluorescence was performed, sections were reblocked and incubated with the second primary antibody solution. After primary antibody incubations were finished, sections were washed multiple times with PBST before incubated in secondary antibody solution for 3 hours at room temperature. Secondary antibodies (donkey anti-mouse IgG Alexa Fluor 488, donkey anti-rat IgG Alexa Fluor 488, goat anti-rat IgG Alexa Fluor 488, goat anti-mouse IgG Alexa Fluor 568, and/or donkey anti-goat IgG Alexa Fluor 568, all from Invitrogen) were diluted at 1:500 in blocking buffer. Sections were counterstained with 10 µM Hoechst 33342 in PBS for 5-10 minutes and mounted with Vectashield (Vector Laboratories).

Quantification of neurogenesis in vivo

Images of the dentate gyrus and the olfactory bulb were taken with a 10X objective on a Nikon Eclipse S600 equipped with a QImagine QIClick camera. Images of the subventricular zone were taken with a 40X objective on a Zeiss LSM510 META microscope. Positive cells

were counted from at least 8 sections per animal. Total positive cell counts for each region were normalized to the total region area measured per animal. Counting and area measurement was done blind to treatment using ImageJ (NIH).

Neurosphere culture

Tissue containing the subventricular zone or the dentate gyrus was dissected from brains of 4 or 5 8-10-week-old mice and collected in basal media (DMEM/F12 without glutamine containing N2 supplement, B27 supplement, 2 mM L-glutamine, Pen Strep, and 2 µg/ml heparin). The tissue was digested with 0.05% trypsin-EDTA in HBSS at 37°C for 10 minutes. The digestion was terminated by adding equal volume of 0.014% w/v trypsin inhibitor in HBSS. The tissue was collected by centrifugation and the pellet was resuspended in 1 mL of basal media plus growth factors (20 ng/mL EGF and 10 ng/mL bFGF). The cells were triturated with a p1000 tip until smooth before they were resuspended in a total volume of 10 mL of basal media containing growth factors. The suspension was filtered through a 40 µm sieve and plated in 60 mm petri dishes at 5 mL/dish.

After 7-14 days for primary spheres or 5-7 days for secondary spheres, spheres were passaged as follows. Spheres were collected by centrifugation, resuspended in 0.05% Trypsin-EDTA, and incubated for 5 minutes at room temperature. An equal volume of 0.014% w/v trypsin inhibitor in HBSS was added and cells were collected by centrifugation into a pellet. The pellet was resuspended in basal media containing growth factors and triturated with a p1000 pipette tip. Cells were replated at 10,000 cells/cm² in 60 mm petri dishes. Spheres were passaged up to 9 times.

MTT assay

Cells were plated in uncoated 96-well plates at a density of 10,000 cells/100 μ L/well. Oxotremorine M, carbachol, atropine, or saline was added to each well immediately after plating. 4 hours before the desired endpoint, 10 μ L of 5 μ M MTT in PBS was added to each well. The normally yellow MTT compound was converted to the reduced purple form by active mitochondria. After 4 hours, MTT reduction was halted by adding 100 μ L of 10% SDS/0.01 M HCl to each well. Absorbances at 570 nm were read with a microplate reader in order to measure the concentration of reduced MTT in each well.

Statistics

Data are expressed as means \pm S.E.M. For experiments containing only two groups, the Student's t-test was used to determine statistical significance. For experiments containing 3 or more groups, one-way ANOVA was used. P values of 0.05 or lower were considered statistically significant.

Results

Single high-dose of pilocarpine does not cause seizure-independent neurogenesis

Changes in neurogenesis following pilocarpine-induced seizures have been well-studied (Parent et al., 1997; Parent et al., 2002). Cell proliferation is increased for up to 2 weeks following pilocarpine administration in both the SVZ and SGZ, resulting in an increase in new cells incorporated into the olfactory bulb and dentate gyrus respectively. To investigate the possibility of muscarinic receptor activation increasing neurogenesis in a seizure-independent manner, we first compared cell proliferation levels between mice that experienced pilocarpine-induced seizures, mice that were given the same dose of pilocarpine but did not have seizures

due to diazepam pretreatment, and saline-treated controls. Since cell proliferation in the dentate gyrus was previously shown to be elevated up to 14 days following pilocarpine-induced *status epilepticus* (SE) in rats (Parent et al., 1997), we looked at cell proliferation in the dentate gyrus 1, 3, and 6 days after pilocarpine administration. We saw a ~45% increase in cell proliferation 6 days after pilocarpine-induced SE, confirming that the dose of pilocarpine we used caused the expected increase in cell proliferation following SE. In contrast, a ~36% increase in cell proliferation in diazepam-pretreated pilocarpine mice over saline controls was seen only at 3 days post pilocarpine treatment (data not shown).

In a follow-up experiment, we focused on comparing cell proliferation between diazepam-pretreated pilocarpine and saline mice. In contrast to the previous experiment, diazepam was given prior to pilocarpine or saline in both groups of mice. This ensured that any difference seen in cell proliferation was not due to differences in mouse handling. However, no difference in cell proliferation was seen 2 or 3 days following pilocarpine treatment (Fig. 1). This suggests that the increase in cell proliferation seen after pilocarpine-induced seizures is due only to seizure activity.

Administration of multiple doses of oxotremorine does not increase neurogenesis

Since a single injection of the muscarinic agonist pilocarpine did not increase cell proliferation when its seizure-evoking activity was blocked, we investigated whether multiple injections of a muscarinic agonist could increase neurogenesis in the absence of seizures. We injected mice 3 times a day for 3 days with 1 mg/kg of the muscarinic agonist oxotremorine (Fig. 2A). Oxotremorine has a longer half-life and is less likely to cause seizures compared to pilocarpine. With a dose of 1 mg/kg, we could give multiple injections of oxotremorine a day without needing diazepam to block potential seizure activity.

We used the DNA analog BrdU and proliferating cell nuclear antigen (PCNA) to mark for proliferating cells and found no change in cell proliferation in the SGZ or SVZ following 3 days of oxotremorine treatment (Fig. 2B-C). The percentage of cells born shortly following oxotremorine treatment that survived to 4 weeks of age also did not change (data not shown). Therefore repeated oxotremorine treatment did not increase the number of new cells born or their likelihood to survive.

Because adult neurogenesis is highest in young adult mice and decreases with age, we looked into the possibility that activation of muscarinic receptors would produce a greater and more detectable change in adult neurogenesis in older mice. We compared cell proliferation levels between oxotremorine- and saline-treated 6-month-old mice. We observed significantly lower levels of cell proliferation in 6-month-old mice compared to 10-week-old mice as expected, but oxotremorine treatment did not rescue this deficit (Fig. 2B-C). Therefore a high basal level of neurogenesis does not mask the effect of muscarinic agonists on adult neurogenesis.

Muscarinic agonists and antagonists do not affect neurogenesis in vitro

We used cultures of adult neural progenitor cells (aNPCs) to investigate the effects of muscarinic agonists and antagonists on adult neurogenesis *in vitro*. By using aNPC cultures we could investigate the effects of activating or blocking muscarinic receptors expressed by aNPCs. ANPCs obtained from the SVZ and the dentate gyrus were allowed to proliferate in the presence of different concentrations of the muscarinic agonist oxotremorine M, the ACh analog carbachol, and/or the muscarinic antagonist atropine. To measure changes in cell proliferation, we compared the number of viable cells in control and drug-treated wells on different days over the course of a week using MTT assays. These assays indirectly measure the number of viable cells

by measuring their mitochondrial activity. We found that the presence of muscarinic agonists and antagonists in the media had no effect on the proliferation of aNPCs (Fig. 3).

Since normally the media of *in vitro* cultures of aNPCs is supplemented with growth factors, we examined whether removal of these growth factors from the media would reveal a role for muscarinic receptors in promoting proliferation or viability of aNPCs. Muscarinic agonists and antagonists did not prevent or delay the death of aNPCs plated in media containing no growth factors (data not shown). They also did not prevent or delay the decrease in cell viability of aNPCs seen one week after plating when growth factors have been depleted from culture media (data not shown). Altogether these results confirmed that the activity of muscarinic receptor expressed on aNPCs did not affect aNPC growth or viability.

Loss of M₁ receptor does not affect basal neurogenesis

Since our previous results suggest that increasing muscarinic receptor activity did not affect adult neurogenesis *in vivo*, we examined whether loss of muscarinic receptor activity could affect neurogenesis *in vivo*. Cholinergic denervation had been shown to decrease neurogenesis in adult rats, suggesting that endogenous ACh activity positively affected neurogenesis (Mohapel et al., 2005). Because the M₁ muscarinic receptor is the most abundant muscarinic receptor subtype in the hippocampus and is also highly expressed in the olfactory bulb (Oki et al., 2005), we compared basal levels of neurogenesis in 12-week-old WT and M₁ KO mice. 4 weeks prior to examining neurogenesis in these mice, we injected them with BrdU in order to date a population of cells and determine how many of these new cells survived for 4 weeks.

Our initial use of age-matched mice that were not littermates led us to incorrectly conclude that loss of the M₁ receptor led to decreases in SGZ proliferation, in the number of immature neurons, and in the subsequent surviving new cells in the dentate gyrus, while only

affecting the survival of new olfactory bulb cells and not their proliferation in the SVZ (Fig. 4, exp. 1). Subsequent comparisons of neurogenesis levels between WT and M₁ KO littermates showed no significant differences in cell proliferation and the number of immature neurons in the SGZ and SVZ (Fig. 4A and 4C, exp. 2 and 3). There was also no difference in surviving new cells in the dentate gyrus or olfactory bulb (Fig. 4B and 4D, exp. 2 and 3). Instead, we saw greater differences in neurogenesis levels, both in the number of proliferating cells and immature neurons, between different litters of WT mice than between WT and M₁ KO littermates, indicating that natural variation between litters caused the initial result.

In vitro studies demonstrated that loss of M₁ or M₃ did not affect aNPC proliferation or viability. Growth rates of dentate gyrus-derived aNPCs isolated from M₁ KO and M₃ KO mice were similar to those from WT mice in the presence or absence of growth factors (Fig. 5). In addition, oxotremorine M had no effect on proliferation of dentate gyrus-derived M₁ and M₃ KO aNPCs (data not shown). SVZ-derived aNPCs from M₃ KO mice were also similar to WT mice (data not shown), suggesting that loss of M₃ activity also does not affect adult neurogenesis.

Discussion

While previous literature suggested that muscarinic receptor activation positively affected adult neurogenesis, we did not observe any effect of muscarinic receptor activation on adult neurogenesis *in vivo* or *in vitro*. A single high dose of pilocarpine, multiple doses of oxotremorine, or the genetic deletion of the M₁ receptor did not affect adult neurogenesis *in vivo*, while muscarinic agonists, muscarinic antagonists, or the absence of the M₁ or M₃ receptor did not affect the proliferation or viability of adult neural progenitor cells (aNPCs) *in vitro*. A high basal level of neurogenesis in controls did not mask the effects of muscarinic receptor activity, as

using older mice or removing growth factors from culture media affected levels of neurogenesis equally in controls and muscarinic agonist- and antagonist-treated mice and aNPCs.

The ability of muscarinic receptor-specific agonists to increase adult neurogenesis in a seizure-independent way, specifically proliferation, had been investigated in two previous studies. Van Kampen and Eckman (2010) observed a significant increase in cell proliferation in the dentate gyrus even at their lowest dose of oxotremorine, while Veena et al. (2011) only saw an increase in cell proliferation after oxotremorine treatment in mice that had been previously subjected to restraint stress. It is possible that the method in which oxotremorine was administered affected whether an increase in cell proliferation was observed. Our treatment scheme of 9 x 1 mg/kg IP injections of oxotremorine over 3 days was more similar to that used by Veena et al. (2011), who gave 0.2 mg/kg of oxotremorine IP daily for 10 days. Meanwhile, Van Kampen and Eckman (2010) infused oxotremorine intracerebroventricularly at a rate of 1, 5, or 30 µg/hour for 10 days. Because the method of oxotremorine delivery was different, it is difficult to determine whether Van Kampen and Eckman (2010) observed an increase in cell proliferation after oxotremorine treatment because they achieved the optimal peak concentration of oxotremorine in the dentate gyrus or the optimal cumulative dose of oxotremorine delivered to the dentate gyrus.

Conflicting results regarding the ability of AChE inhibitors to increase adult neurogenesis also support the idea that the treatment method determines the experimental outcome. Mohapel et al. (2005), Van Kampen and Eckman (2010), and Itou et al. (2011) used infusion pumps or multiple injections in a short period of time in order to keep a sustained concentration of AChE inhibitor, and they all observed an increase in cell proliferation after AChE treatment in the dentate gyrus. On the other hand, Kaneko et al. (2006) only injected AChE inhibitors once a day

and did not observe an increase in cell proliferation. Instead, Kaneko et al. (2006) observed an increase in surviving cells when AChE inhibitors were given for a 4-week period during the entire process from proliferation to survival, a result supported by Kotani et al. (2006), who only observed an increase in the number of 4 week-old cells but not 2-week-old cells with AChE treatment. A similar study has not been performed with muscarinic agonists, so it is possible that a similar treatment scheme using oxotremorine could increase the survival of new cells.

If muscarinic receptor activation does increase adult neurogenesis, then it is not due to direct activation of muscarinic receptors on aNPCs. Muscarinic agonists and antagonists did not increase proliferation or viability of aNPCs, even when growth factors were depleted or removed. This is in contrast to studies done with embryonic neural stem cells (Ma et al., 2000; Li et al., 2001; Zhou et al., 2004). The lack of proliferative effect is unlikely due to a lack of muscarinic receptor expression on neural progenitor cells, as muscarinic agonists have been shown to increase calcium in adult neural stem cells of hippocampal slices (Itou et al., 2011).

Previous studies had speculated that the M_1 receptor would primarily mediate muscarinic effects on adult neurogenesis, since it is the most abundant muscarinic receptor subtype in the neurogenic regions (Mohapel et al., 2005; Van Kampen and Eckman, 2010). We found that genetic deletion of the M_1 receptor did not change levels of cell proliferation or the number of surviving new cells in both neurogenic pathways. The fact that the M_1 KO mice lack expression of the M_1 receptor from birth could mean that other mechanisms rescued the deficiency in adult neurogenesis due to the loss of M_1 receptor activity. However, studies done with other knockout mice such as $\alpha 7$ nicotinic receptor KO mice, $\beta 2$ nicotinic receptor KO mice, and dopamine D3 receptor KO mice have shown that changes in adult neurogenesis are still present even if the receptor is missing since birth, making this possibility less likely (Harrist et al., 2004; Campbell

et al., 2010; Egeland et al., 2012). Interestingly, β 2 nicotinic receptor KO mice only showed decreased neurogenesis compared to WT mice at 7-10 months and not at earlier or later ages (Harrist et al., 2004). Therefore we cannot rule out the possibility that loss of the M_1 receptor would affect adult neurogenesis levels only at a certain age.

Our study also does not rule out the possibility that other muscarinic receptors are involved in regulating adult neurogenesis. While the M_1 receptor is the predominant muscarinic receptor subtype in the hippocampus and forebrain, there is also expression of M_2 , M_3 , and M_4 receptors (Oki et al., 2005). Comparison of adult neurogenesis in WT and other muscarinic receptor knockout or double knockout mice may reveal a role for different or a combination of muscarinic receptors in modulating adult neurogenesis.

In conclusion, we have found no evidence that muscarinic receptor activity regulates adult neurogenesis. However, differences in treatment schemes may explain why we did not observe any changes in adult neurogenesis due to muscarinic receptor activity while others have (Kotani et al., 2006; Van Kampfen and Eckman, 2010). Nevertheless we saw no evidence that muscarinic agonists, muscarinic antagonists, or genetic deletion of the M_1 or M_3 receptor affected neurogenesis *in vitro*, indicating that direct activation of muscarinic receptors expressed on adult neural progenitor cells is not the mechanism in which muscarinic receptor activity increases adult neurogenesis. While our results suggest that muscarinic receptor activity in general does not alter adult neurogenesis, further studies with other muscarinic receptor subtype-specific KO mice or double KO mice may still uncover a role of muscarinic receptors in adult neurogenesis.

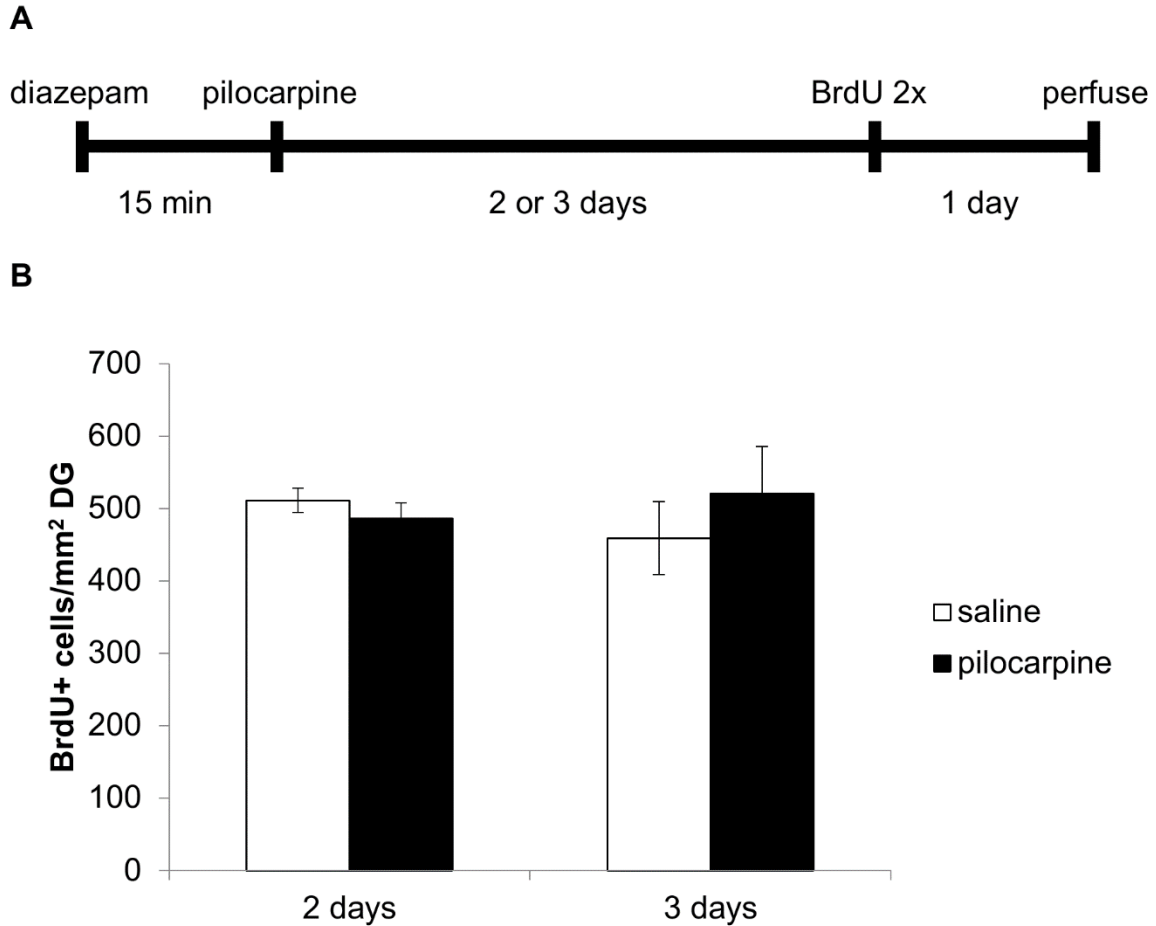


Fig. 1. Pilocarpine administration does not increase cell proliferation in the dentate gyrus in a seizure-independent manner. **A.** Experimental scheme. 4 mg/kg diazepam was given 15 minutes prior to 325 mg/kg pilocarpine or saline in order to prevent seizures. 2 or 3 days after pilocarpine or saline administration, 200 mg/kg BrdU was given twice, 6 hours apart, in order to label proliferating cells. Mice were perfused the following day. **B.** Quantification of cell proliferation in the dentate gyrus after pilocarpine (2 days, n = 6; 3 days, n = 5) or saline (2 days, n = 5; 3 days, n = 6) administration. The number of positive cells was normalized to the total area of dentate gyrus measured *per* animal.

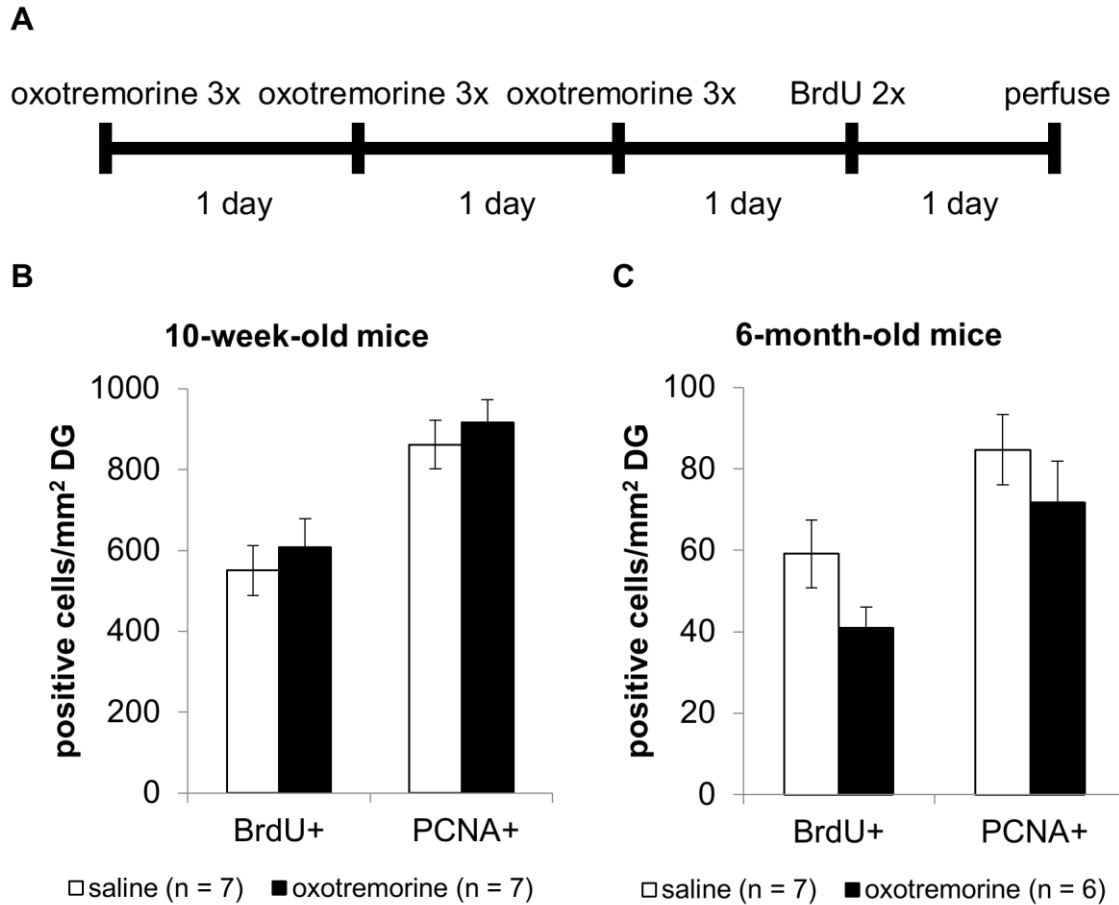


Fig. 2. Multiple injections of oxotremorine do not increase cell proliferation in the dentate gyrus. **A.** Experimental scheme. 1 mg/kg oxotremorine or saline was given 3 times/day, 4 hours apart for 3 consecutive days. 200 mg/kg BrdU was given twice, 6 hours apart, the next day. Mice were perfused 1 day after BrdU labeling. **B.** Quantification of cell proliferation in the dentate gyrus after 3 days of oxotremorine (n = 7) or saline (n = 7) in 10-week-old mice. **C.** Quantification of cell proliferation in the dentate gyrus after 3 days of oxotremorine (n = 6) or saline (n = 7) in 6-month-old mice. The counts of BrdU-positive and PCNA-positive cells were normalized to the total area of dentate gyrus measured *per* animal.

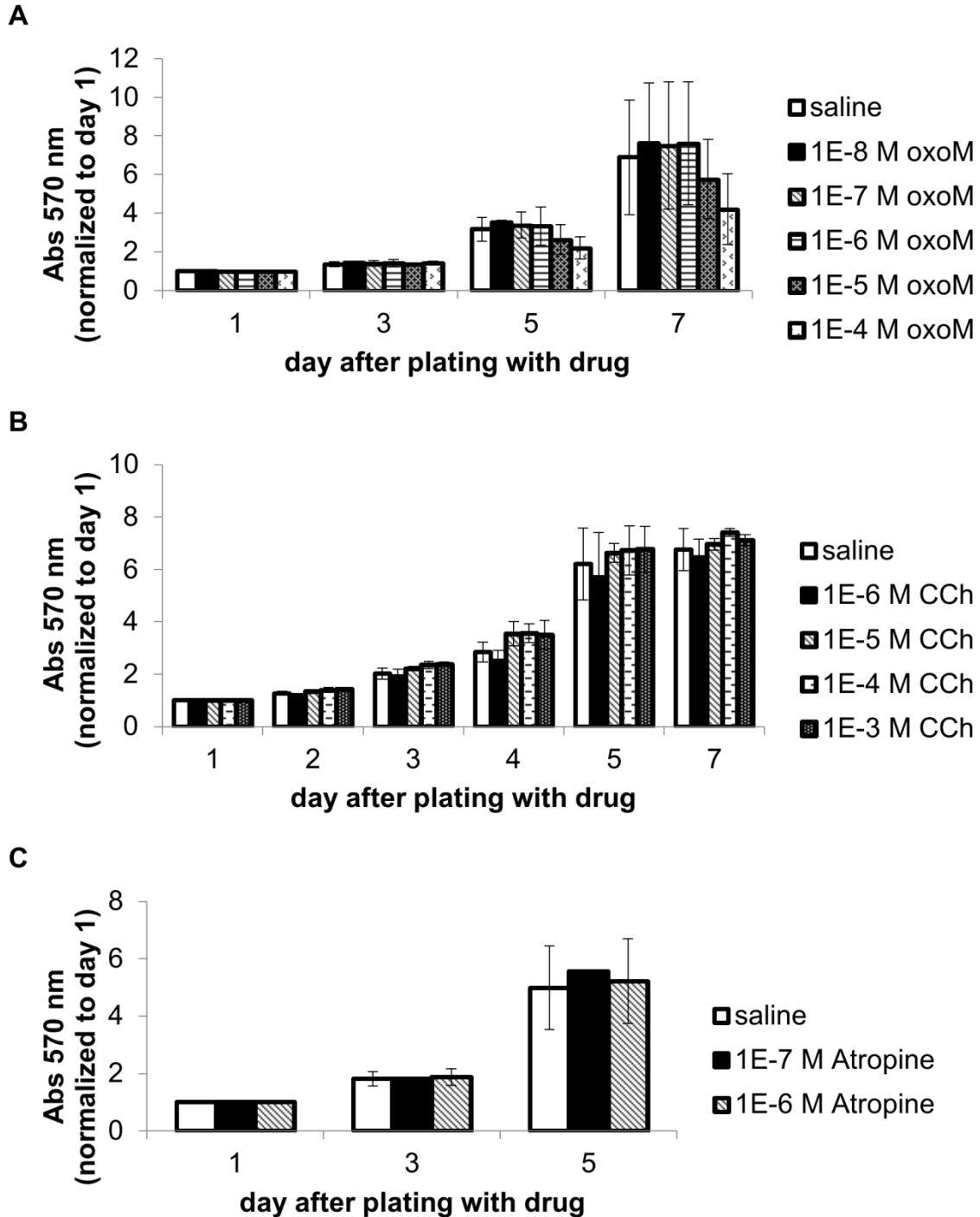


Fig. 3. Muscarinic agonists and antagonists do not alter proliferation of adult neural progenitor cells (aNPCs) *in vitro*. MTT assays were used to measure the number of viable cells in saline-treated and muscarinic agonist or antagonist-treated wells over the course of 5-7 days following plating. MTT assays show proliferation of DG-derived aNPCs treated with different concentrations of oxotremorine M (A), carbachol (B), or atropine (C). Absorbance values were normalized to day 1 values and averaged from at least two replicates.

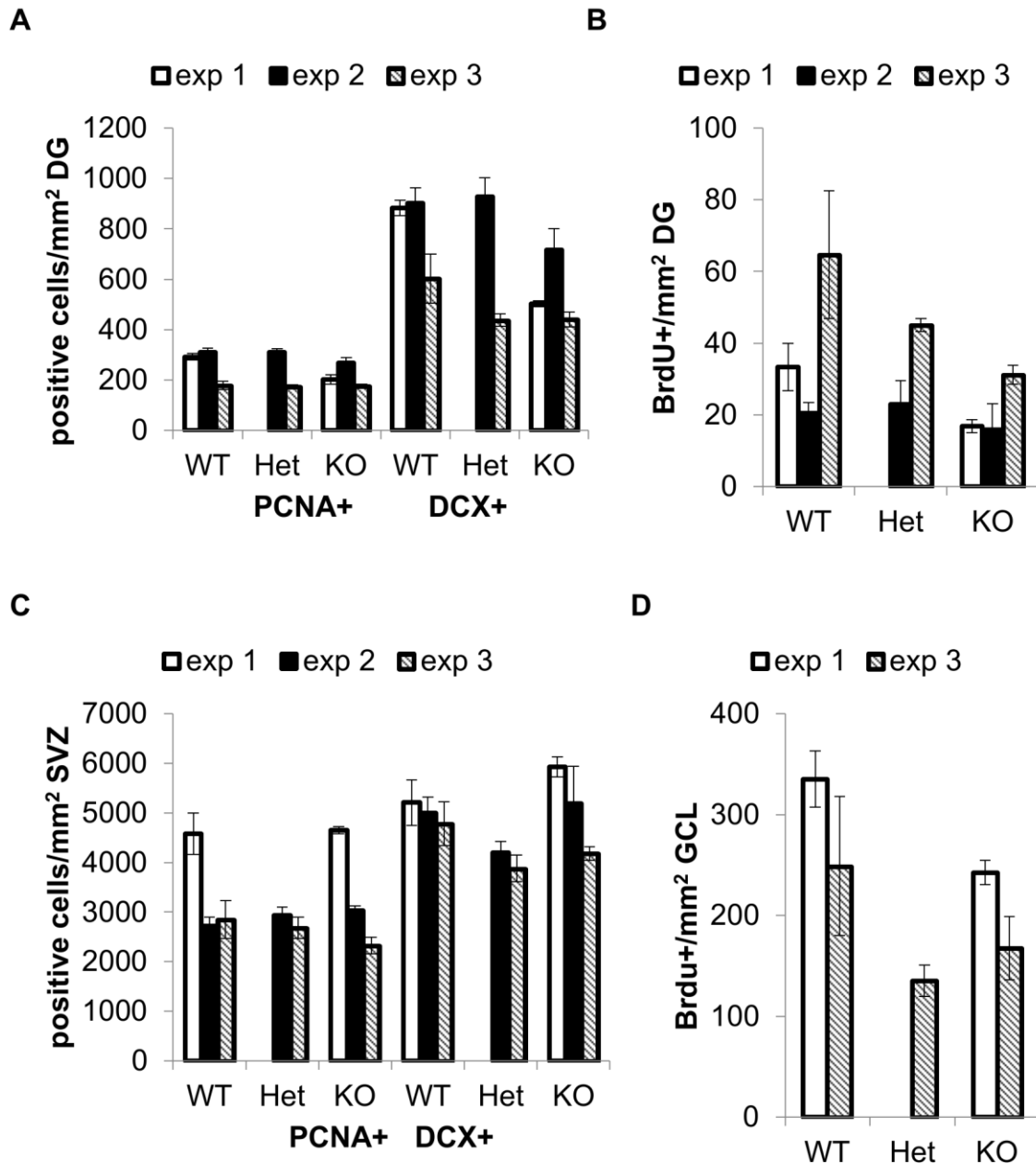


Fig. 4. Loss of M₁ receptor expression does not alter cell proliferation in the dentate gyrus or subventricular zone (SVZ) or cell survival in the dentate gyrus or olfactory bulb. Exp. 1 used WT (n = 5) and M1 KO (n = 4) mice that were age-matched but not littermates. Exp. 2 and 3 used WT (n = 5 for exp. 2; n = 3 for exp. 3), M₁ heterozygotes (Het; n = 4 for exp. 2; n = 11 for exp. 3), and M1 KO (n = 3 for exp. 2; n = 4 for exp. 3) littermates. All mice were approximately 12 weeks old and given BrdU 4 weeks prior. All counts of BrdU or PCNA positive cells were normalized to the area of dentate gyrus, SVZ, or olfactory bulb measured *per* animal. **A.** Quantification of cell proliferation and immature neurons in the dentate gyrus. **B.** Quantification of 4-week-old cells in the dentate gyrus. **C.** Quantification of cell proliferation and immature neurons in the SVZ. **D.** Quantification of 4-week-old cells in the olfactory bulb.

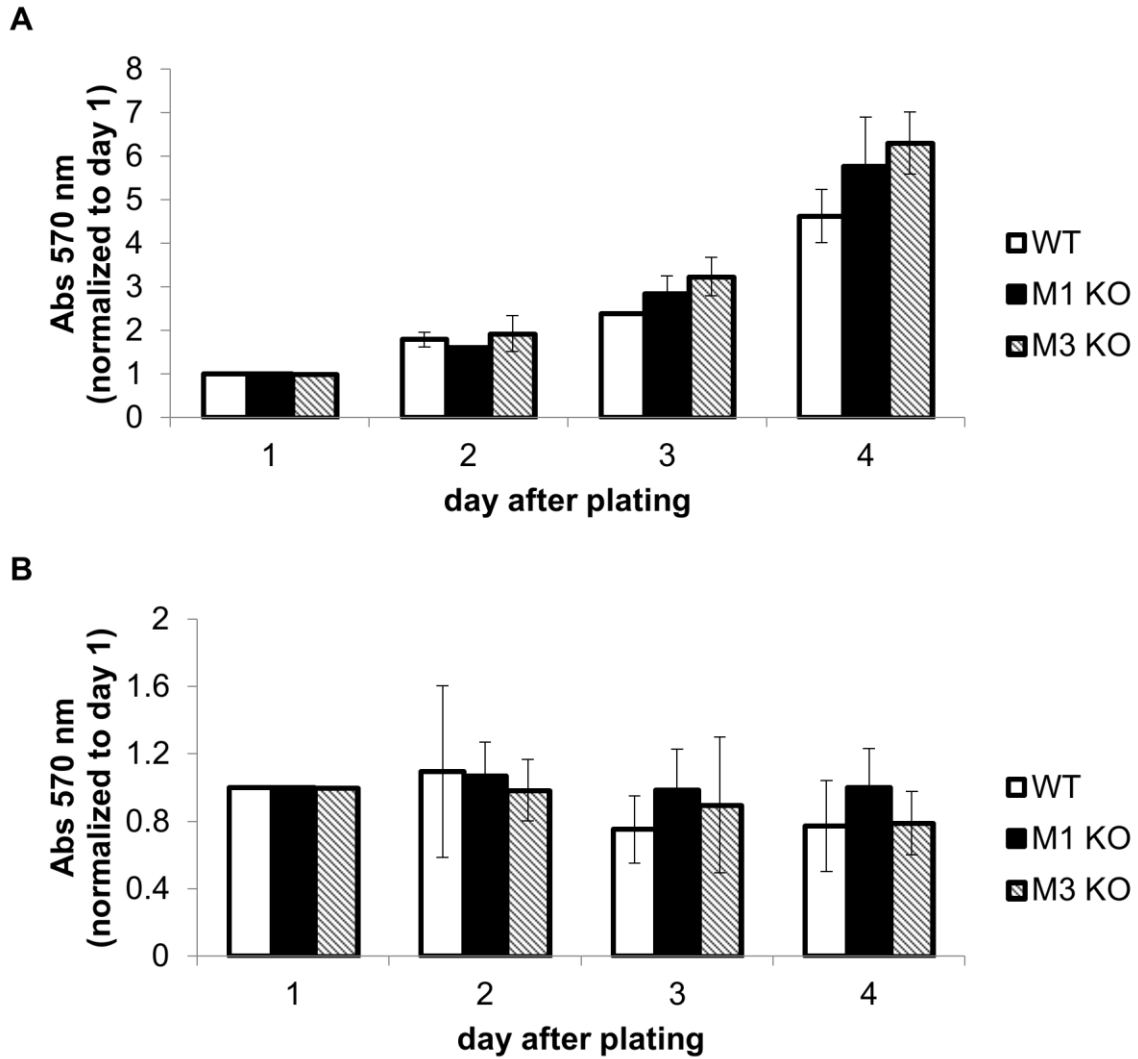


Fig. 5. Loss of the M₁ or the M₃ receptor does not alter proliferation or survival of adult neural progenitor cells *in vitro*. MTT assays were used to measure changes in the number of viable cells over the course of 4 days in normal, growth factor-supplemented media (**A**) or media without growth factors (**B**). Absorbance values were normalized to day 1 values and averaged between the two replicates.

CHAPTER 3

MODULATION OF PILOCARPINE-INDUCED SEIZURES BY CANNABINOID RECEPTOR 1

Introduction

Muscarinic acetylcholine receptors (mAChRs) mediate many of the actions of acetylcholine in the central nervous systems (Eglen, 2006). There are five mAChR subtypes, all of which are G protein-coupled receptors (GPCRs). The M_1 , M_3 , and M_5 subtypes preferentially couple to members of the $G_{q/11}$ family of G-proteins to activate phospholipase C- β (PLC β), while the M_2 and M_4 subtypes preferentially couple to members of the $G_{i/o}$ family to inhibit adenylyl cyclase (Nathanson, 2000). In the brain, muscarinic receptors are involved in processes such as learning, memory, control of movement, nociception, temperature control, as well as in the modulation of signaling by other neurotransmitters (Eglen, 2006; Bubser et al., 2012; Newman et al., 2012). The M_1 subtype is the predominant mAChR in the forebrain with high expression in the hippocampus, cortex, and striatum (Oki et al., 2005), where it has been implicated in learning and memory (Anagnostaras et al., 2003; Eglen, 2006; Digby et al., 2012]. In addition, the M_1 receptor mediates seizure induction due to administration of muscarinic agonists such as pilocarpine (Hamilton et al., 1997).

Pilocarpine is a muscarinic agonist commonly used to induce seizures in rodents because it produces a phenotype that resembles human temporal lobe epilepsy (Turski et al., 1989). After recovery from the initial period of seizure activity, pilocarpine-treated animals develop spontaneous seizures a few weeks later. During this latent period prior to the development of

spontaneous seizures, the brain, especially the hippocampus, undergoes many changes including increased cell proliferation, cell death and mossy fiber sprouting (Mello et al., 1993; Parent et al., 1997). Induction of pilocarpine seizures is blocked by pretreatment with muscarinic antagonists, but subsequent administration of muscarinic antagonists will not terminate seizure activity, indicating that muscarinic receptor activation is required for the induction of seizures but is not required for their maintenance (Turski et al., 1984; Clifford et al., 1987).

Endogenous cannabinoids (endocannabinoids, eCB) and CB₁ receptors agonists have anticonvulsant activity in the electroshock seizure, the spontaneous seizure, and the kainic acid seizure models of epilepsy, while CB₁ antagonists have proconvulsive activity in these models (Wallace et al., 2001; Marsicano et al., 2003; Wallace et al., 2003; Hofmann and Frazier, 2013). CB₁ receptors couple to G_{i/o} proteins and are predominantly located on presynaptic nerve terminals (Howlett et al., 2002). Activation of CB₁ receptors serves as a feedback mechanism to modulate neurotransmitter signaling. The activity-dependent production and release of eCBs from postsynaptic cells leads to the activation of presynaptic CB₁ receptors that inhibit neurotransmitter release (Wilson and Nicoll, 2001). eCB production is increased by either electrical activity or activation of GPCRs such as the M₁ and M₃ receptors that couple to PLCβ, the enzyme involved in the production of 2-arachidonoylglycerol, the most abundant eCB (Ohno-Shosaku et al., 2003; Fukudome et al., 2004; Hashimotodani et al., 2005).

Previous studies on the role of CB₁ receptors in the pilocarpine model of epilepsy have focused on the role of this receptor after the induction of seizures (Wallace et al., 2003; Falenski et al., 2007; Falenski et al., 2009; Karlócai et al., 2011). These studies examined the role of CB₁ receptors during the latent phase following the initial seizures or the chronic phase of spontaneous seizures, which is well past the time when muscarinic receptor activity is necessary

for seizures. Therefore, in order to examine the effect of CB₁ receptor activity on the induction of seizures by activation of muscarinic receptors, we determined the effects of administration of CB₁ receptor agonists and antagonists and of the deletion of the CB₁ receptor gene on the induction of pilocarpine-induced seizures.

Materials and Methods

Animals

All procedures involving animals were approved by the University of Washington Institutional Animal Care and Use Committee under protocols #2239-01 and #3233-05. CB₁ knockout (KO) mice were obtained from Dr. Giovanni Marsicano (Marsicano et al., 2002) and were bred at the University of Washington. C57Bl/6 male mice were obtained from Charles River and used at 13 weeks of age.

Drugs

Pilocarpine hydrochloride was purchased from Sigma Aldrich and dissolved in 0.9% saline. Diazepam (Hospira) and phenobarbital (West-Ward) were purchased as stock solutions dissolved in 0.9% saline from the University of Washington Medical Center Pharmacy. SR141716 was obtained from the NIDA Drug Supply Program and was prepared in pharnasolve/cremophor RH40 (pharnasolve: cremophor RH40: drug, 1:9:40). CP55940 was obtained from the NIDA Drug Supply Program and was prepared in a vehicle solution consisting of cremophor RH40: ethanol: saline (1:1:18).

Behavioral responses to CP55940

Adult male WT or CB₁ KO mice on a C57Bl/6 background were injected intraperitoneally (IP) with 0.3 mg/kg CP55940, and tetrad behaviors were measured as described

by Martin et al. (1991). Core temperature was measured using an anal probe. Locomotion was videotaped over 10 minutes in an Open Field chamber (45 cm x 25 cm x 25 cm), with Noldus Ethovision (Wageningen, the Netherlands). Catalepsy was scored by measuring latency to remove forepaws from a bar placed 3 cm above the bench surface, with a maximum score of 30s. For each animal, three trials were performed, and the trial with the longest latency was recorded. Analgesia was measured by tail flick; tails were immersed up to one cm in 52°C-56°C water bath, and latency to withdraw the tail was measured, up to a maximum time of 15 seconds. Tails were always inspected for tissue damage immediately after the experiment, and monitored the next day.

Seizures

To compare seizure severity after pilocarpine treatment, mice were observed for 1 hour and scored as previously described (Hamilton et al., 1997) by an observer blind to drug treatment or genotype. In this scale, 1: tremor; 2: single myoclonic jerks; 3: clonus; 4: one tonic-clonic seizure; 5: 2 seizures; 6: 3 or more seizures, death, or *status epilepticus* (SE).

To examine cell proliferation and cell death after pilocarpine-induced SE, mice were injected IP with 225-250 mg/kg pilocarpine. After 2 hours of SE or 2.5 hours after pilocarpine administration if no SE occurred, mice were injected IP with 4 mg/kg diazepam to reduce excitability. Forty five minutes later, 30 mg/kg phenobarbital was given IP to maintain the blockade of residual seizure activity

Tissue Processing

Four days after pilocarpine treatment, mice were treated with 140 mg/kg ketamine/10 mg/kg xylazine, perfused, and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight. After brains were soaked in 30% sucrose in PBS, they were frozen on dry ice.

Frozen brains were sectioned at 30 μm with a cryostat and sections were stored at -20°C in a cryoprotectant solution (30% ethylene glycol, 30% glycerol, 0.1 M phosphate buffer, pH 7.4).

Proliferating Cell Nuclear Antigen (PCNA) Immunofluorescence

Thirty μm free-floating sections were washed in PBS and PBST (PBS containing 2% Triton X-100) before antigen retrieval (10 minutes of boiling in 10 mM sodium citrate, pH 6). Sections were blocked in blocking buffer (2% bovine serum albumin, 0.1 M glycine, 0.05% sodium azide in PBST) plus 10% donkey serum for 1 hour at room temperature before overnight incubation at 4°C in 1:1000 mouse anti-PCNA (Santa Cruz) in blocking solution. After multiple PBST washes, sections were incubated in 1:500 donkey anti-mouse IgG Alexa Fluor 488 (Invitrogen) for 3 hours at room temperature. Sections were counterstained with 10 μM Hoechst 33342 in PBS for 10 minutes and mounted with Vectashield (Vector Laboratories).

Fluoro-jade B (FJ) Staining

FJ staining was performed as described by the manufacturer (Millipore). Thirty μm sections were mounted on gelatin-subbed slides and allowed to dry at room temperature overnight. The next day, slides were incubated for 5 minutes in 1% sodium hydroxide in 80% ethanol, 2 minutes in 70% ethanol, and 2 minutes in water. Slides were then incubated for 10 minutes in 0.06% potassium permanganate and 2 minutes in water prior to FJ staining (0.0004% in 0.1% acetic acid for 20 minutes). After 3 washes with water, the slides were allowed to dry at room temperature overnight before coverslipped with DPX mountant for histology (Sigma Aldrich).

Quantification

Images of hippocampal tissue were taken with a 10X objective on a Nikon Eclipse S600 equipped with a QImagine QIClick camera. Positively-stained cells were counted in the hilus

from at least 8 sections from each animal. Counting was done blind to treatment using ImageJ (NIH). The total number of positive cells per animal was normalized to the total hilus area measured per animal.

Data analysis

Seizure severity scores are presented as means \pm S.E.M. All imaging data are expressed as means \pm S.E.M. The Mann-Whitney U test was used to test the significance of seizure severity scores. The Fisher's exact test was used for fractions of mice experiencing seizures. The Kruskal Wallis test was used for the quantifications of PCNA- and Fluoro-jade B-positive cells, with post-hoc Bonferroni-corrected Mann-Whitney U tests performed between groups. P values of less than 0.05 were considered statistically significant. Fisher's T-test was used for analysis of cannabinoid tetrad behaviors.

Results

Loss of CB₁ receptor activity increases pilocarpine seizure severity

To investigate the role of the CB₁ receptor in the induction of pilocarpine seizures, we first compared seizure severity after pilocarpine treatment in WT and CB₁ KO mice. Using a submaximal dose of pilocarpine (250 mg/kg), we observed more severe seizure behaviors in CB₁ KO mice than WT mice (Fig. 6). Both the average seizure severity score and the fraction of mice having full seizures (at least one tonic-clonic seizure) were significantly higher in CB₁ KO mice compared to WT mice.

To ensure that the increased sensitivity to pilocarpine-induced seizures in CB₁ KO mice was due to loss of CB₁ receptor activity and not due to a developmental or compensatory difference caused by a lack of CB₁ receptors from birth, we compared pilocarpine-induced

seizures in mice pretreated with the CB₁ receptor antagonist SR141716 (SR1) or vehicle. At submaximal doses of pilocarpine (250 and 275 mg/kg), we saw an increase in the severity of seizure behaviors and in the proportion of mice experiencing full seizures with SR1 pretreatment (Fig. 7). This indicates that activity of the CB₁ receptors, presumably due to the actions of endogenously released eCBs, modulates the sensitivity to seizure induction by pilocarpine.

Pilocarpine seizure-induced increases in cell proliferation and cell death are unchanged by CB₁ antagonist

Pilocarpine-induced seizures, specifically pilocarpine-induced *status epilepticus* (SE), cause significant increases in both cell proliferation and cell death in the brain days following the initial seizures (Mello et al., 1993; Parent et al., 1997). To determine whether blockade of CB₁ receptor activity led to any changes in seizure-induced cell proliferation and cell death, we performed immunohistochemistry focusing on the hilus 4 days after pilocarpine treatment.

We used proliferating cell nuclear antigen (PCNA), a protein commonly used as an endogenous marker of proliferation (Von Bohlen and Halbach, 2011), to measure seizure- and/or drug-induced cell proliferation. PCNA immunoreactivity was greatly increased throughout the hippocampus of mice that experienced SE, as previously reported (Parent et al., 1997; Ledergerber et al., 2005), while it remained mostly limited to the subgranular zone of the dentate gyrus of mice that did not experience SE (Fig. 8A). Since variations in PCNA immunoreactivity in the subgranular zone could also be due to differences in basal neurogenesis, we quantified PCNA immunoreactivity in the hilus, a region that normally does not have significant amounts of cell proliferation in adult mice. Quantification of PCNA-immunopositive cells in the hilus showed a significant increase in cell proliferation in mice that experienced SE compared to those

that did not (Fig. 8B). SR1 pretreatment did not modify the amount of cell proliferation induced by SE, indicating that seizure activity itself caused the increases in cell proliferation.

To measure changes in cell death, we stained sections with Fluoro-jade B, which labels degenerating cells (Schmued et al., 2000). Fluoro-jade B staining showed a significant increase in degenerating cells in the hilus of mice that experienced SE and this response was not altered by SR1-pretreatment (Fig. 8A and 8B).

In line with our result showing that SR1 pretreatment increased the probability and the severity of pilocarpine seizures, only 1 out of the 22 SR1-pretreated mice did not experience SE or death. The amount of cell proliferation and cell death in the hilus of this mouse was similar to vehicle-pretreated mice that did not experience SE (PCNA-positive: 36 cells/mm² versus 27 ± 8 cells/mm²; FJ-positive: 1.6 cells/mm² versus 0.8 ± 0.3 cells/mm²). Together the results indicate that CB₁ receptor antagonism itself does not increase cell proliferation or cell death but rather that the increases in cell proliferation and cell death are due to the increases in pilocarpine seizure severity measured in mice pretreated with the CB₁ receptor antagonist.

Treatment with CB1 agonist does not alter pilocarpine seizure severity

The results above indicate that blockade or genetic deletion of CB₁ receptors increases the severity of pilocarpine-induced seizures, most likely due to elimination of the action of eCB at the CB₁ receptors. In order to test if pretreatment with a CB₁ receptor agonist affected pilocarpine –induced seizures, we first ensured that pretreatment with the CB₁ receptor agonist CP55940 (CP) would elicit CB₁- mediated responses in mice. Administration of 0.3 mg/kg CP (given IP) resulted in characteristic behavioral effects in WT, but not CB₁ KO mice. Locomotor activity in an open field chamber (Fig. 9A) was reduced in WT but not CB₁ KO mice. Similarly, although no mice demonstrated any measureable catalepsy prior to treatment (Fig. 9B), 30

minutes after administration of CP, WT mice developed catalepsy whereas CB₁ KO mice showed no change. CP-induced hypothermia was measured by comparing the core body temperature 5 minutes before, and 30 minutes after drug administration (Fig. 9D). WT mice developed marked hypothermia, whereas CB₁ KO mice were unaffected (WT: $\Delta T = -7.0 \pm 0.3^\circ\text{C}$; CB₁ KO: $\Delta T = 0.4 \pm 0.9^\circ\text{C}$). Finally, analgesia was measured by the tail flick method (Fig. 9C). Prior to CP administration, all animals removed their tails immediately upon immersion, but 30 minutes after drug administration, WT mice took $13.8 \pm 1.0\text{s}$ to remove their tails, whereas CB₁ KO mice continued to remove their tails immediately upon immersion. Based on these results, we confirm previous studies (Wiley et al., 2003; Monory et al., 2007) showing that 0.3 mg/kg CP administered IP results in hypolocomotion, catalepsy, hypothermia, and analgesia in WT but not CB₁ KO mice.

We then tested whether pretreatment with CP reduces pilocarpine-induced seizure severity. We found no difference in seizure severity scores or in the proportion of mice that exhibited clonic-tonic seizures between CP- and vehicle-pretreated mice in response to administration of 325 mg/kg pilocarpine (Fig. 10). Previous studies have shown that stimulation of M₁ and M₃ muscarinic receptors in the hippocampus stimulates eCB production *via* the activation of PLC β (Ohno-Shosaku et al., 2003; Fukudome et al., 2004; Hashimoto et al., 2005). The lack of effect of CP administration on pilocarpine-induced seizures suggests that eCB activity at the CB₁ receptors is already at maximal levels following high dose pilocarpine administration, so administration of exogenous CB₁ receptor agonist cannot further modulate the sensitivity to pilocarpine seizures.

Discussion

Activation of CB₁ receptors has been shown to reduce the severity of seizures in a variety of models of epilepsy, and decreased activation of CB₁ receptors increases the severity of spontaneous, kainic acid-induced, and electroshock-induced seizures (Wallace et al., 2001; Marsicano et al., 2003; Wallace et al., 2003). We show here that the loss of CB₁ receptor activity, due to genetic deletion in knockout animals or by administration of CB₁ receptor antagonists, causes an increased sensitivity to pilocarpine-induced seizures. Previous studies have shown that activation of M₁ and M₃ receptors stimulate the production of endocannabinoids (Ohno-Shosaku et al., 2003; Fukudome et al., 2004). The increased sensitivity to pilocarpine-induced seizures following administration of a CB₁ antagonist and when the CB₁ gene is deleted indicates that eCBs acting at the CB₁ receptor modulate the severity of pilocarpine-induced seizures.

Induction of SE causes many changes in the brain, including increased neurogenesis, gliosis, mossy fiber sprouting, and cell death (Mello et al., 1993; Parent et al., 1997). We confirmed that pilocarpine-induced SE caused the expected increases in cell proliferation and cell death in the hilus in mice that had experienced SE. SE-induced damage was not modified by the loss of CB₁ receptor activity during the seizure period, suggesting that changes in cell proliferation and cell death are not due directly to CB₁ receptor activity but rather reflect the effect of CB₁ receptor activity on seizure severity and ensuing cell proliferation and cell death. Previous studies using kainic acid reported that loss of CB₁ receptor activity caused a decrease in neurogenesis and an increase in cell death and gliosis (Marsicano et al., 2003; Aguado et al., 2007). In the study by Aguado et al. (2007), 10 μ M kainic acid was sufficient to significantly increase neurogenesis in a CB₁-dependent manner *in vitro*. However, when tested *in vivo*, Aguado et al. (2007) did not indicate whether the dose of kainic acid used (15 mg/kg) in WT and

CB1 KO mice evoked seizures or the severity of any seizures evoked. Therefore the decrease in neurogenesis due to loss of CB₁ receptor activity in their study may be mostly or entirely seizure-independent.

Marsicano et al. (2003) also did not indicate whether the quantification of cell death in their study was performed on mice that had experienced SE or not. Because the dose of kainic acid that they used (20 mg/kg) typically caused more severe seizures in CB₁ KO mice compared to WT mice, they may have compared cell death between WT mice that did not have SE to CB₁ KO mice that did have SE. If this was the case, their results are consistent with the fact that CB₁ KO mice are more sensitive to kainic acid-induced seizures but do not indicate whether or not CB₁ activity modified kainic acid-induced seizure damage.

While we and others found that loss of CB₁ receptor activity increased seizure incidence and severity, we saw no effect of CB₁ receptor agonist pretreatment on the sensitivity or severity of pilocarpine-induced seizures. This resistance to exogenous CB₁ agonist administration suggests that CB₁ receptor activity was already maximally modulating pilocarpine seizures *via* the actions of eCBs. In contrast, increasing CB₁ receptor activity reduced the severity of electroshock, spontaneous, and kainic acid-induced seizures (Wallace et al., 2001; Marsicano et al., 2003; Wallace et al., 2003). CB₁ receptor agonists reduced or abolished seizures in the electroshock and spontaneous seizure model (Wallace et al., 2001; Wallace et al., 2003), while the eCB reuptake inhibitor UCM707 reduced the severity of kainic acid seizures (Marsicano et al., 2003). This suggests that eCB production may differ between models of seizure induction.

Kainic acid, pilocarpine, and seizure activity itself have been shown to increase eCB levels, but the identity and the quantity of the eCB increased have yet to be fully determined. While anandamide but not 2-AG was increased after kainic acid seizures (Marsicano et al., 2003),

only changes in 2-AG were measured after pilocarpine seizures (Wallace et al., 2003). Future comparisons of changes in eCB levels in response to kainic acid or pilocarpine administration may allow determination of whether differences in drug- or seizure-induced eCB levels account for differences in the anticonvulsive activity of exogenously applied CB₁ receptor agonists.

The extent of change in the expression level of CB₁ receptors over the course of epilepsy development is remarkably different depending on the models that are studied. While there are some discrepancies between studies looking at CB₁ receptor expression following pilocarpine seizures, CB₁ receptor expression decreases shortly after seizures and increases in epileptic mice (Falenski et al., 2007; Falenski et al., 2009; Maglóczy et al., 2010; Karlócai et al., 2011). Specifically, CB₁ receptor expression on presynaptic GABAergic terminals increased (Maglóczy et al., 2010). A short-term decrease in CB₁ receptor expression could be due to receptor internalization, while long-term changes could be due to a compensatory mechanism (Karlócai et al., 2011). Interestingly, a decrease in CB₁ receptor expression is seen in human temporal lobe epilepsy patients (Ludányi et al., 2008). A comparable analysis of CB₁ receptor expression levels following kainic acid seizures could indicate whether differences in CB₁ receptor expression levels correlates with differences in the anticonvulsive activity of CB₁ activators in different epilepsy models.

In conclusion, we have demonstrated a role for CB₁ receptors in the induction phase of pilocarpine-induced seizures. While loss of CB₁ receptor activity changed the outcome of subthreshold pilocarpine-induced seizures, administration of a CB₁ receptor agonist did not alter the severity of pilocarpine-induced seizures, suggesting that the ability of CB₁ receptor to reduce pilocarpine-induced seizure severity is already maximized by muscarinic receptor-induced eCB. Further investigation of the effects of the CB₁ receptor in this and other seizure models will

expand our understanding on the actions and limitations of CB₁ receptors in the regulation of seizures.

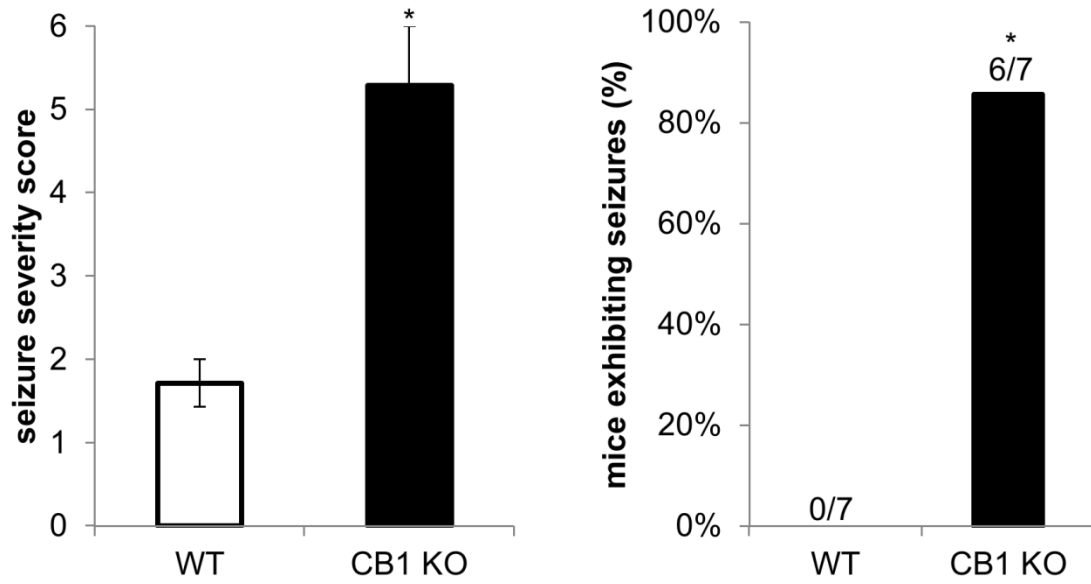


Fig. 6. CB₁ KO mice are more sensitive to pilocarpine. Seizure severity scores and the proportion of mice having at least one clonic-tonic seizure after injection with 250 mg/kg pilocarpine were compared in male CB₁ KO (n = 7) and WT (n = 7) littermates. * p < 0.05; ** p < 0.005.

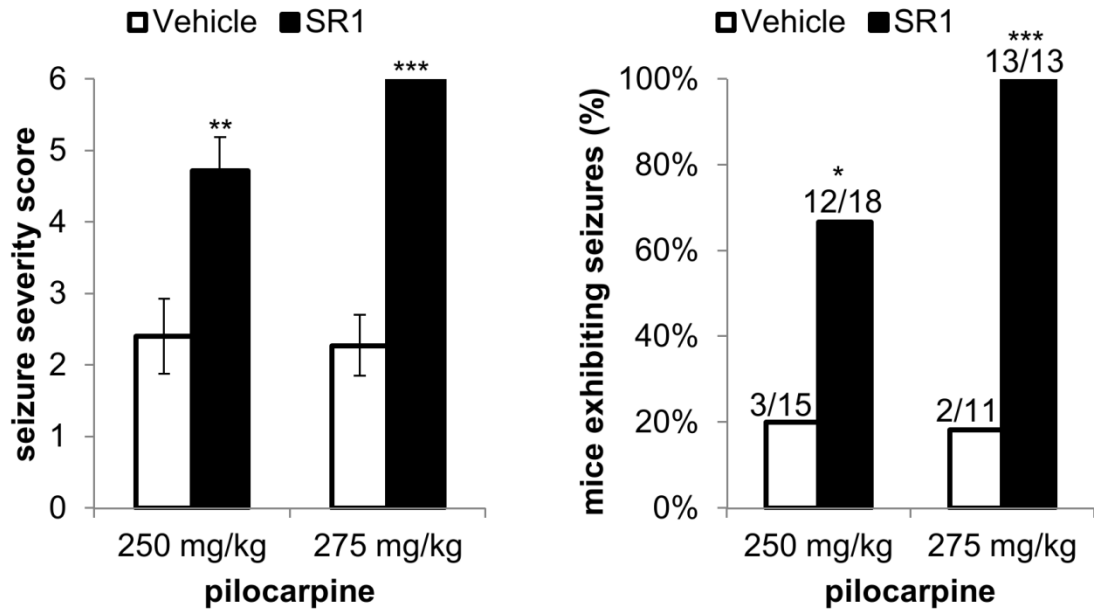


Fig. 7. CB₁ receptor antagonist pretreatment increases pilocarpine seizure sensitivity. SR141716 (SR1, 10 mg/kg) or the corresponding vehicle were given 2 hours prior to injection of 250 or 275 mg/kg pilocarpine. Seizure severity scores and the proportion of mice exhibiting at least one tonic-clonic seizure were compared between SR1 (250 mg/kg, n = 18; 275 mg/kg, n = 13) and vehicle-treated mice (250 mg/kg, n = 15; 275 mg/kg, n = 11). * p < 0.05; ** p < 0.005; *** p < 0.0001

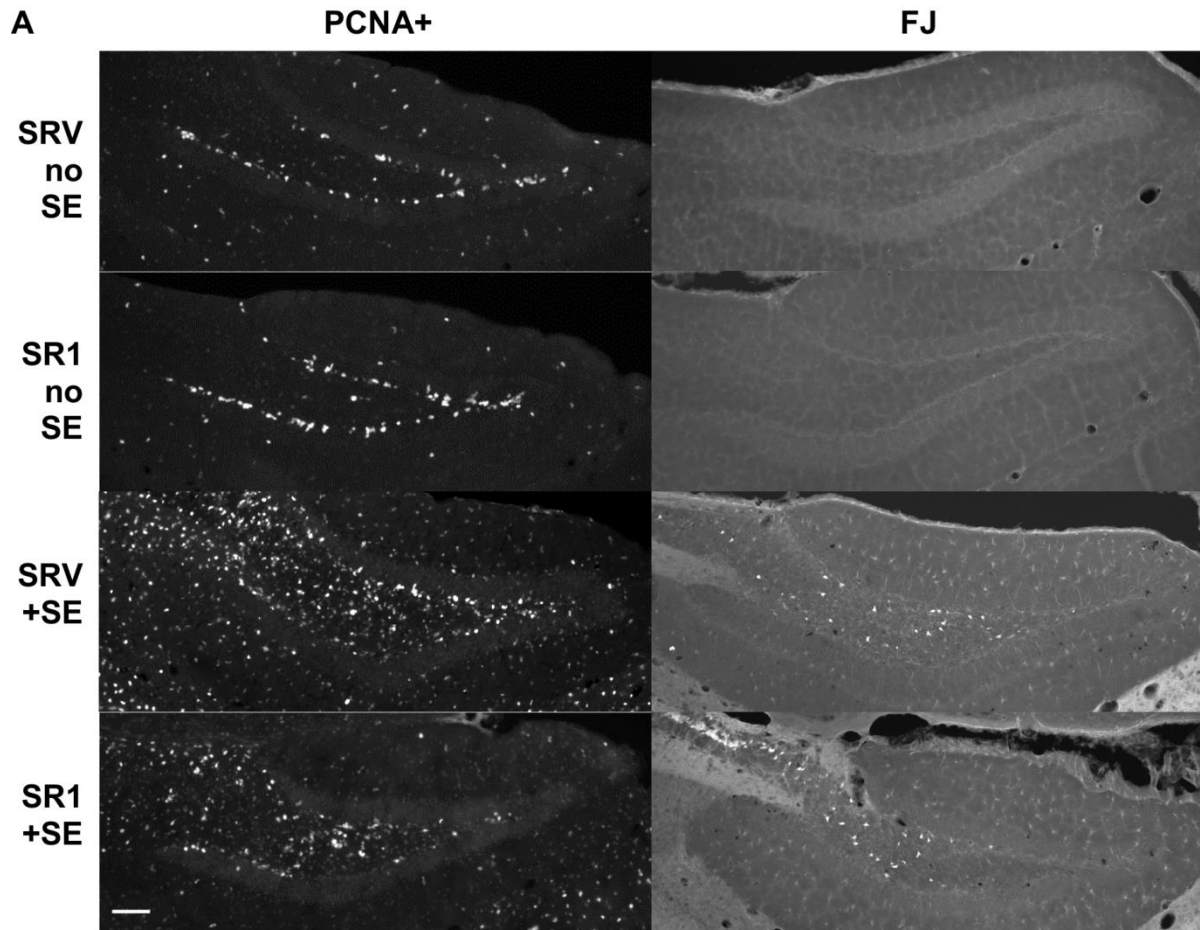
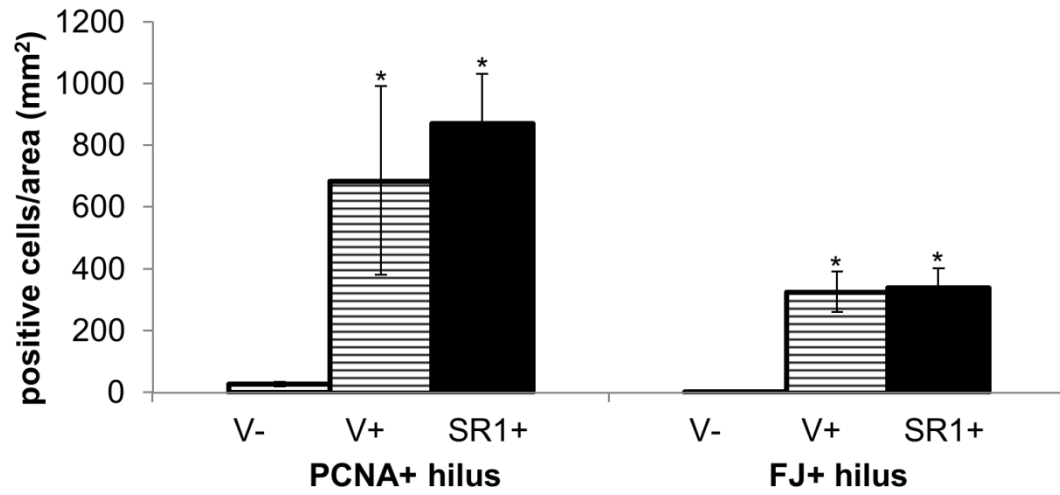


Fig. 8. Cell death and cell proliferation after pilocarpine-induced SE are unchanged by SR1 pretreatment. Mice were pretreated for 2 hours with SR141716 (10 mg/kg) or vehicle before injection with 225-250 mg/kg pilocarpine. Brains were harvested for immunohistochemistry analysis 4 days after pilocarpine treatment. **A.** Representative images of proliferating cell nuclear antigen (PCNA) immunofluorescence and Fluoro-jade B (FJ) staining from (top to bottom) vehicle-treated mice without SE (V-), SR1-treated mice without SE (SR1-), vehicle-treated mice with SE (V+), and SR1-treated mice with SE (SR1+). Scale bar = 100 μ m.

B



B. PCNA and FJ quantifications in the hilus of vehicle-treated mice without SE (V-, n = 6), vehicle-treated mice with SE (V+, n = 3), and SR1-treated mice with SE (SR1+, n = 5). The number of positive cells was normalized to the area of hilus measured *per* animal. * p < 0.05 when compared to vehicle-treated mice without SE (V-).

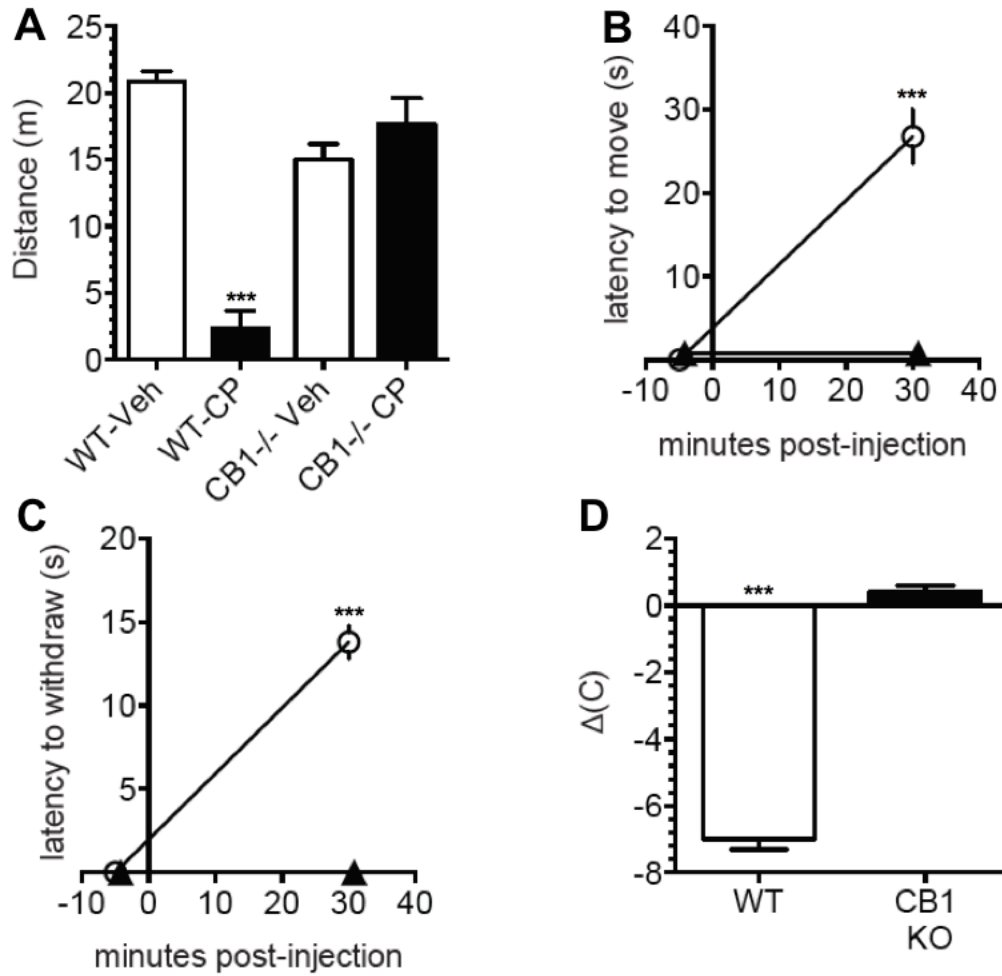


Fig. 9. Administration of CP55940 results in CB₁-dependent cannabinoid responses. Cannabinoid tetrad behaviors (hypolocomotion, catalepsy, analgesia, and hypothermia) were measured after i.p. administration with 0.3 mg/kg CP in WT (n = 5) and CB₁-KO mice (n = 5). **A.** Mice were treated with either vehicle or CP, and placed in an open-field chamber 30 minutes after treatment. **B.** Catalepsy was measured by the bar test either 5 minutes before, or 30 minutes after CP treatment in WT and CB₁ KO mice. **C.** Analgesia was measured by tail flick both before and after CP treatment. **D.** Hypothermia was measured by comparing core body temperature before and 30 minutes after treatment with CP. ***p < 0.001

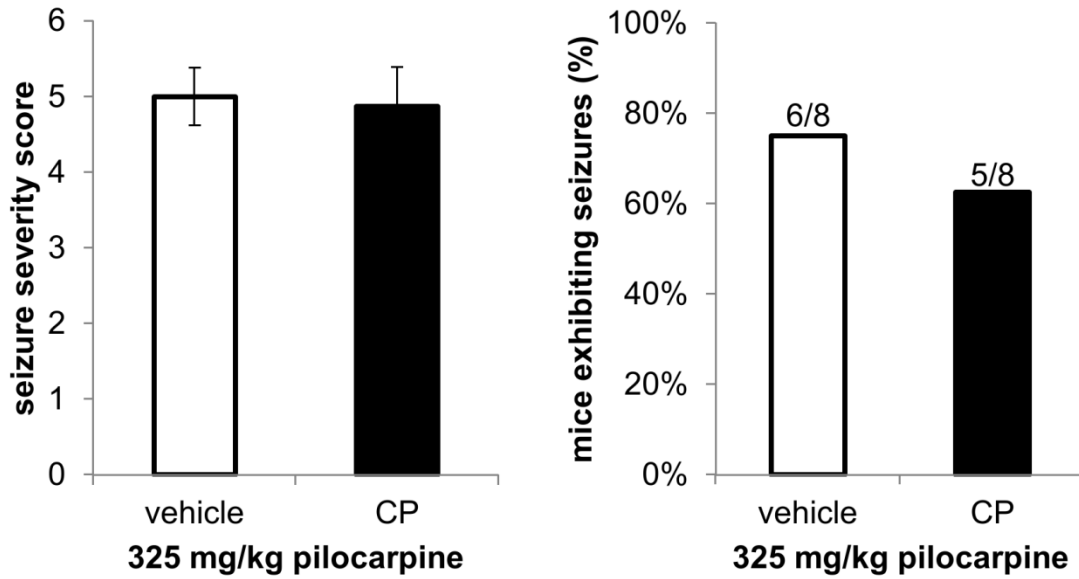


Fig. 10. CB₁ receptor agonist pretreatment does not reduce pilocarpine seizure severity. Mice were pretreated with CP 55940 (CP, 0.3 mg/kg) or vehicle 30 minutes prior to pilocarpine (325 mg/kg). Seizure severity scores and the proportion of mice having at least one clonic-tonic seizure were compared between CP-treated (n = 8) and vehicle-treated mice (n = 8).

CHAPTER 4

DIFFERENTIAL REGULATION OF PILOCARPINE AND PARAOXON SEIZURES BY M_1 AND CB_1 RECEPTORS

Introduction

The toxic properties of organophosphates make them useful as insecticides but also as weapons for chemical warfare (Newmark, 2004; Rusyniak and Nañagas, 2004).

Organophosphate poisoning is caused by the inhibition of acetylcholinesterase (AChE) due to formation of a covalent enzyme-inhibitor complex, leading to an increase in acetylcholine (ACh) levels and excessive activation of nicotinic and muscarinic acetylcholine receptors. These receptors mediate the actions of ACh at the neuromuscular junction, target organs of the autonomic nervous system, and neurons of the peripheral and central nervous systems.

Symptoms of organophosphate poisoning include involuntary movements, respiratory depression, cardiac arrhythmia, seizures, and excessive salivation. Current treatment for organophosphate poisoning typically includes administration of a muscarinic receptor antagonist such as atropine, which treats the most serious symptoms of organophosphate poisoning, and an oxime such as 2-pralidoxime (2-PAM), which reacts with the inactivated AChE to restore enzyme activity.

While the respiratory depression caused by of organophosphate poisoning is the most immediate life-threatening event, organophosphate-induced seizures can cause massive brain damage that results in long-term neurological impairments (see review by Chen, 2012).

Organophosphate seizures are blocked by pretreatment with centrally-acting muscarinic antagonists but not peripheral-selective muscarinic antagonists or nicotinic receptor antagonists,

indicating that centrally-expressed muscarinic receptors mediate the initiation of organophosphate seizures (Capacio and Shih, 1991; Shih et al., 1991). There are 5 muscarinic receptor subtypes, all of which are expressed in the brain (Caulfield and Birdsall, 1998). Muscarinic receptors in the brain regulate many functions including learning and memory, locomotion, body temperature, body weight, and nociception (see reviews by Wess, 2004; Eglén, 2006; Wess et al., 2007). Muscarinic agonist-induced seizures require M₁ receptor activity, as the muscarinic agonist pilocarpine cannot induce seizures in M₁ knockout (KO) mice while it is still able to induce seizures in mice with deletion of the genes encoding any of the other four muscarinic receptor subtypes (Hamilton et al., 1997, Bymaster et al., 2003).

Muscarinic receptor regulation of organophosphate-induced seizures shares some similarities with muscarinic agonist pilocarpine-induced seizures. Muscarinic antagonists can only inhibit pilocarpine-induced seizures if administered before or shortly after pilocarpine administration (Turski et al., 1984; Clifford et al., 1987). Similarly, muscarinic antagonists only block organophosphate-induced seizures if administered within 20-40 minutes of organophosphate exposure (McDonough and Shih, 1993). Moreover, M₁-preferring muscarinic antagonists were more effective than atropine in blocking the seizure activity of the organophosphate soman (Capacio and Shih, 1991). While these studies suggest that the mechanism for initiation of organophosphate seizures is the same as for pilocarpine seizures, a requirement for the M₁ receptor in organophosphate seizure initiation has yet to be confirmed.

We have recently observed that pilocarpine-induced seizures are modulated by cannabinoid CB₁ receptors (CB₁ receptors); deletion of the CB₁ receptor gene or administration of CB₁ receptor antagonists resulted in an increased susceptibility of mice to pilocarpine-induced seizures (see Chapter 3). However, while pilocarpine selectively acts at muscarinic receptors,

organophosphates not only inhibit AChE, thus increasing ACh availability at both muscarinic and nicotinic receptors, but also inhibit multiple serine hydrolases with varying potencies, including fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), the enzymes that degrade the endogenous cannabinoids anandamide and 2-AG respectively (Casida and Quistad, 2005). Organophosphates have also been shown to decrease ligand binding at CB₁ receptors (Casida and Quistad, 2005; Nallapaneni et al., 2006). Organophosphates do not appear to directly bind to CB₁ receptors; the reduction in available CB₁ receptor binding sites appeared to be due to stabilization and increased binding of endocannabinoids (eCBs) to CB₁ receptors (Casida et al., 2008; Nomura et al., 2008). Not only could organophosphates increase eCB levels by inhibiting FAAH and MAGL, organophosphates could also increase eCB levels by increasing ACh-mediated activation of M₁ and M₃ receptors, which increase eCB release via activation of phospholipase C β (Ohno-Shosaku et al., 2003; Fukudome et al., 2004; Hashimoto et al., 2005).

In this paper we investigated whether the requirement for M₁ receptor activation and the regulation by CB₁ receptors for seizures induced by pilocarpine were also true for seizures induced by the organophosphate paraoxon. Here we report that, in contrast to pilocarpine-induced seizures, paraoxon-induced seizures do not require M₁ receptor activity and are not modulated by CB₁ receptor activity. We also observed that pilocarpine but not paraoxon caused a seizure-independent increase in ERK activation in the hippocampus and that this increase was dependent on M₁ receptor activity. Since M₁-dependent activation of ERK is not observed after paraoxon administration, this suggests that paraoxon does not evoke a high level of M₁ receptor activation in the hippocampus.

Materials and Methods

Animals

M₁ KO mice were generated and bred at the University of Washington as described previously (Hamilton et al., 1997). CB₁ KO mice were obtained from Dr. Giovanni Marsicano (Marsicano et al., 2002) and were bred at the University of Washington. For CB₁ agonist and antagonist seizure studies, C57Bl/6 male mice were purchased from Charles River and used at 12-13 weeks of age. For studies looking at ERK activation in the hippocampus, WT and M₁ KO mice were used at 10 weeks of age. All procedures involving animals were approved by the University of Washington Institutional Animal Care and Use Committee.

Drugs

Paraoxon was purchased from Chem Service. Pilocarpine hydrochloride and pyridine-2-aldoxime methochloride (2-PAM) were purchased from Sigma Aldrich. Diazepam (Hospira) was purchased as a stock solution dissolved in 0.9% saline from the University of Washington Medical Center Pharmacy. SR141716 was obtained from the NIDA Drug Supply Program and was prepared in pharماسolve/cremophor RH40 (pharماسolve: cremophor RH40: drug, 1:9:40). CP55940 was obtained from the NIDA Drug Supply Program and was prepared in a vehicle solution consisting of cremophor RH40: ethanol: saline (1:1:18). All drugs except for SR141716 and CP55940 were made as stock solutions in 0.9% saline.

Drug Treatments

For seizure studies, mice were given 90 mg/kg 2-PAM by intraperitoneal (IP) injection 5 minutes prior to IP injection with paraoxon. Seizure activity was observed for 1 hour and scored on a 8-point scale as follows: 0 – no visible response; 1 – sedation, loss of locomotion; 2 – Straub tail, shortened gait; 3 – circling, head bobbing, and/or mouth gaping; 4 – tremors, wild

running, and/or cornering; 5 – single myoclonic jerks; 6 – clonic convulsions; 7 – clonic/tonic seizures; 8 – clonic hind limb extension or death. Scoring was done blind to drug treatment and genotype.

For studies in which seizures were prevented, mice were given 4 mg/kg diazepam by IP injection 15 minutes prior to IP injection of either 350 mg/kg pilocarpine or 6 mg/kg paraoxon, or equivalent volumes of 0.9% saline for controls. In paraoxon experiments, 2-PAM was also given to mice by IP injection 5 minutes prior to paraoxon to minimize peripheral toxicity. 15 minutes after pilocarpine or paraoxon injection, mice were euthanized by cervical dislocation.

Tissue Processing

Euthanized mice were perfused with 4% paraformaldehyde and their brains removed. Brains were fixed overnight at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were then soaked in 30% sucrose in PBS before frozen on dry ice. Frozen brains were sectioned at 40 µm, and sections were stored at -20°C in a cryoprotectant solution (30% ethylene glycol, 30% glycerol, 0.1 M phosphate buffer, pH 7.4).

Phospho-ERK TSA/NeuN Immunofluorescence

Phospho-ERK was detected using tyramide signal amplification (TSA) prior to NeuN immunofluorescence. TSA for phospho-ERK was performed as described by Sindreu et al. (2007) using the TSA Cyanine 3 kit (Perkin Elmer) with some modifications. To block phosphatase activity, 50 mM NaF was added to every solution up through the phospho-ERK antibody incubation. Free-floating sections were washed multiple times with PBS before they were incubated for 15 minutes in 1% NaBH₄ in PBS and 20 minutes in 0.1 M phosphate buffer, pH 7.4, containing 1.5% H₂O₂ and 10% ethanol. Sections were then washed in PBST (PBS + 0.2% Triton X-100) before blocking with TNB blocking buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl,

0.5% blocking reagent). Sections were incubated at 4°C overnight in 1:5000 rabbit anti-phospho-ERK (Cell Signaling) in TNB blocking buffer. After primary antibody incubation, sections were washed with PBST before incubation for 1 hour at room temperature in 1:100 anti-rabbit IgG HRP (GE Healthcare) in TNB blocking buffer. Sections were washed again with PBST before incubated in Cyanine 3 Tyramide working solution (Cyanine 3 Tyramide stock solution diluted 1:66 in amplification reagent) for 10 minutes at room temperature.

NeuN immunofluorescence was then performed after residual Cyanine 3 Tyramide solution was removed with multiple PBST washes. Sections were blocked for 1 hour at room temperature in blocking solution (0.1 M glycine, 2% bovine serum albumin, 0.05% sodium azide, 10% donkey serum in PBST) before overnight incubation at 4°C in 1:1000 mouse anti-NeuN (Millipore) in blocking solution. After multiple washes with PBST, sections were incubated for 3 hours at room temperature in 1:500 donkey anti-mouse IgG Alexa Fluor 488 (Invitrogen) in blocking solution. Sections were counterstained with 10 µM Hoechst 33342 in PBS before mounted with Vectashield (Vector Laboratories).

Quantification of Phospho-ERK Fluorescence

Images of hippocampal tissue were taken with a 10X objective on a Nikon Eclipse S600 equipped with a QImagine QIClick camera at 8-bit resolution. Hoechst staining and NeuN immunofluorescence were used in order to determine the location and size of the stratum lucidum of each tissue section. Phospho-ERK fluorescence was determined by measuring the mean grey value of the stratum lucidum using ImageJ (NIH). Background fluorescence was subtracted and the corrected fluorescence values for each tissue section were averaged per animal. Imaging of the tissue and the measurement of phospho-ERK fluorescence were done blind to treatment.

Data analysis

All data are presented as means \pm S.E.M. The Mann-Whitney U test was used to test the significance of seizure severity scores. The Fisher's exact test was used for fractions of mice experiencing seizures. Two-way ANOVA was used to determine if there was a genotype effect on the observed phospho-ERK signal following drug or vehicle treatment. Bonferroni-corrected Student t-tests were performed between vehicle- and drug-treated mice of the same genotype. P values of less than 0.05 were considered statistically significant.

Results

M₁ receptor activity is not necessary for paraoxon-induced seizures

Previous work with the muscarinic agonist pilocarpine showed that pilocarpine-induced seizure behaviors were reduced and clonic-tonic seizures were absent only from M₁ KO mice and not from mice lacking any of the four other muscarinic subtypes (Hamilton et al., 1997; Bymaster et al., 2003). Because organophosphates also require muscarinic receptor activity in order to initiate seizures (Capacio and Shih, 1991; Shih et al., 1991), organophosphates and muscarinic agonists could share a similar muscarinic receptor requirement for seizure induction. To determine if M₁ receptor activity was also necessary for paraoxon-induced seizures, we compared seizures induced by 5 mg/kg paraoxon in WT and M₁ KO mice. We observed no differences in seizure severity scores or the proportion of mice exhibiting clonic-tonic seizures after 5 mg/kg paraoxon administration in WT and M₁ KO mice (Fig. 11). Thus, in contrast to pilocarpine-induced seizures, the M₁ receptor is not only unnecessary for paraoxon-induced seizures but it also does not significantly modulate sensitivity to paraoxon.

Pilocarpine, but not paraoxon, activates ERK in a seizure-independent manner in the hippocampus

To further investigate the activity of the M₁ receptor following pilocarpine or paraoxon administration, we examined the ability of pilocarpine and paraoxon to activate ERK in the hippocampus. The M₁ receptor is the predominant muscarinic receptor subtype in the hippocampus and cortex (Oki et al., 2005). Previous studies indicated that M₁ receptors mediated muscarinic agonist-induced ERK activation in these regions *in vitro* (Berkeley et al., 2001; Hamilton and Nathanson, 2001). We chose to look at the hippocampus because administration of pilocarpine or the organophosphate soman increased hippocampal ERK activation within 15 minutes *in vivo* (Berkeley et al., 2002; RamaRao et al., 2011).

In order to distinguish muscarinic receptor activity-dependent ERK activation following pilocarpine or paraoxon from seizure-induced ERK activation, we pretreated mice with diazepam in order to block seizure activity. Previous work by Berkeley et al. (2002) demonstrated that pilocarpine could induce ERK activation in the hippocampus even with diazepam pre-treatment, indicating that pilocarpine could increase ERK activation in the absence of seizures. In order to confirm that seizure-independent activation of ERK by pilocarpine was M₁-dependent *in vivo*, we compared the magnitude and location of seizure-independent ERK activation in the hippocampus of WT and M₁ KO mice following saline or 350 mg/kg pilocarpine treatment. We observed basal phospho-ERK immunoreactivity in the stratum lucidum that was enhanced by pilocarpine administration in WT mice (Fig. 12). However, we did not see an increase in phospho-ERK immunoreactivity in the dentate gyrus or in the CA1 which was seen after pilocarpine administration in mice not pretreated with diazepam (Berkeley et al., 2002). This suggests that the activation of ERK in the dentate gyrus and CA1 of those mice was due to

pilocarpine-induced seizure activity. Pilocarpine-induced increases in phospho-ERK immunoreactivity in the stratum lucidum was absent in M₁ KO mice, indicating that ERK activation following pilocarpine administration was mediated by the M₁ receptor.

We then compared seizure-independent phospho-ERK levels in the hippocampus of WT and M₁ KO mice following saline or 6 mg/kg paraoxon treatment. In order to eliminate death due to peripheral organophosphate-induced toxicity, we also administered 2-PAM prior to saline or 6 mg/kg paraoxon. The pattern of phospho-ERK immunoreactivity was unaffected by the addition of 2-PAM, but, in contrast to pilocarpine, paraoxon did not increase phospho-ERK immunoreactivity in the hippocampus of WT or M₁ KO mice (Fig. 13). This indicates that paraoxon is unable to increase ERK activation in a seizure-independent manner and suggests that it may be due to a lack of sufficient activation of M₁ receptors in the hippocampus.

CB₁ receptor activity does not affect paraoxon-induced seizures

Recently we observed that pilocarpine-induced seizures were sensitive to CB₁ receptor activity (see Chapter 3). Loss of CB₁ receptor activity either through pharmacological antagonism or genetic deletion increased the seizure severity scores seen with submaximal doses of pilocarpine, suggesting that endogenously released eCBs were modulating the sensitivity to seizure induction by pilocarpine. Loss of CB₁ receptor activity also increased the severity of kainic acid and spontaneous seizures, indicating that eCB activity at CB₁ receptors was generally anticonvulsive (Marsicano et al., 2003; Wallace et al., 2003). Organophosphates can inhibit FAAH and MAGL, causing significant increases in eCB levels (Casida et al., 2008; Nomura et al., 2008). 0.4 mg/kg paraoxon significantly inhibited FAAH activity *in vivo* and caused greater toxicity in mice with reduced CB₁ receptor expression, suggesting that eCBs can modulate paraoxon toxicity via CB₁ activity (Nallapaneni et al., 2006).

To determine whether loss of CB₁ receptor activity affected paraoxon-induced seizures, we compared seizure activity induced by 4 mg/kg paraoxon in WT and CB₁ KO mice. We saw no differences in seizure severity scores or proportion of mice experiencing clonic-tonic seizures between WT and CB₁ KO mice (Fig. 14A). Pre-treatment with CB₁ receptor antagonist SR141716 (SR1) also did not significantly alter seizure severity scores or the proportion of mice experiencing clonic-tonic seizures after 3 or 4 mg/kg paraoxon treatment (Fig. 14B), confirming that paraoxon-induced seizures, in contrast to pilocarpine-induced seizures, are unaffected by the lack of CB₁ receptor activity.

Administration of 0.4 mg/kg paraoxon was reported to cause significant inhibition of FAAH, which should result in increased levels of eCBs; nevertheless, CB₁ agonists were still able to reduce paraoxon-induced increases in involuntary movements and parasympathomimetic toxicity (Nallapaneni et al., 2006). To determine whether CB₁ agonist treatment could similarly reduce the severity of paraoxon-induced seizures, we compared seizures induced by paraoxon in vehicle- and CB₁ agonist CP55940 (CP) pretreated mice. CP pretreatment did not alter seizure scores or the proportion of mice that experienced clonic-tonic seizures (Fig. 14C). Altogether these results suggest that paraoxon-induced seizures are unaffected by CB₁ receptor activity.

Discussion

Since the identification of the M₁ receptor as the muscarinic receptor subtype necessary for pilocarpine-induced seizures (Hamilton et al., 1997), some studies have suggested that centrally-acting M₁-selective antagonists would be sufficient in preventing muscarinic receptor-induced seizures (Capacio and Shih, 1991; Sheffler et al., 2009). However, even though organophosphates initiate seizures in a muscarinic receptor-dependent manner (Capacio and Shih,

1991; Shih 1991), we observed that paraoxon-induced seizures do not require M₁ receptor activity.

Paraoxon-induced seizures were not only present but were similar in severity in WT and M₁ KO mice. While this finding does not exclude the M₁ receptor from possibly contributing to the initiation of paraoxon-induced seizures in WT mice, it does show that other muscarinic receptor subtypes can fully initiate paraoxon seizures in the absence of the M₁ receptor. A difference in the brain regions involved in seizure initiation could explain the differential M₁ receptor requirement for pilocarpine- and paraoxon-induced seizures. Pilocarpine caused an increase in high-voltage spiking in the hippocampus followed by the cortex, suggesting that pilocarpine-induced seizures begin in the hippocampus (Turski et al., 1984). In contrast, the organophosphate soman did not display a consistent sequence of activation; sometimes it increased hyperexcitability in the cortex before the hippocampus and sometimes after the hippocampus (McDonough and Shih, 1993). In addition, ACh could evoke seizure activity when injected focally in the amygdala, hippocampus, or cortex, though the most sensitive region was the “area tempestus” (Gale, 1988). A comparative analysis of excitability in the brain following paraoxon administration could determine which regions are most sensitive to paraoxon-induced excitability.

The difference in seizure-independent ERK activation following pilocarpine and paraoxon is consistent with the observed difference in muscarinic receptor requirement for seizure induction. Using doses of pilocarpine and paraoxon that would normally induce seizures in a majority of mice, we observed that only pilocarpine administration caused a seizure-independent increase in ERK activation. The fact that the pilocarpine-induced increase in ERK activation was absent from M₁ KO mice confirmed that the M₁ receptor mediated muscarinic

agonist-induced ERK activation in the hippocampus as seen previously with carbachol (Berkeley et al., 2001). If we use ERK activation as a measure of M₁ receptor activity in the hippocampus, then the lack of ERK activation following paraoxon administration in WT mice suggests that paraoxon administration does not increase M₁ receptor activity to a sufficient extent in the hippocampus to lead to ERK activation.

Even though ERK activation is not necessary for seizure induction, spontaneous seizures, pilocarpine-induced seizures, and soman-induced seizures have all been shown to increase ERK phosphorylation in the hippocampus (Berkeley et al., 2002; Houser et al., 2008; RamaRao et al., 2011). If paraoxon is not sufficiently activating M₁ receptors in the hippocampus in order to observe ERK activation, then paraoxon seizure-induced ERK activation could be mediated by other neurotransmitter receptors in the hippocampus. Many other receptors, including metabotropic and ionotropic glutamate receptors, β -adrenergic receptors, and serotonin receptors, have been shown to activate ERK activity in the hippocampus (Sala et al., 2000; Errico et al., 2001; Berkeley and Levey, 2003).

In addition, we observed a difference in CB₁ receptor regulation of pilocarpine and paraoxon-induced seizures. While CB₁ receptor antagonism increased the severity of pilocarpine-induced seizures, indicating that eCB activity at CB₁ receptors normally decreased the sensitivity towards pilocarpine seizures, treatment with CB₁ receptor antagonists, CB₁ receptor agonists or deletion of the gene encoding the CB₁ receptor had no effect on the severity of paraoxon-induced seizures, indicating that CB₁ receptor activity did not affect paraoxon-induced seizures.

The lack of CB₁ receptor modulation of paraoxon-induced seizures is unexpected, considering that behaviors characteristic of increased cholinergic activity caused by 0.4 and 0.6

mg/kg paraoxon was affected by CB₁ agonist treatment or decreases in CB₁ receptor expression (Nallapneni et al., 2006). Nallapaneni et al. (2006) also observed significant block of FAAH activity and CB₁ receptor binding sites by paraoxon *in vivo*, indicating that paraoxon interacts with components of the cannabinoid system. However, if CB₁ receptor activity modulated paraoxon-induced seizures, then at least one of the following should have been observed. If the anticonvulsive activity of CB₁ receptors was already maximal due to eCB activity, then reduction of CB₁ receptor activity by CB₁ antagonist pretreatment should have increased paraoxon-induced seizures severity. On the other hand, if eCB activity at CB₁ receptors was too low to significantly affect seizure severity, then increasing CB₁ receptor activity with addition of CB₁ agonists should have reduced paraoxon-induced seizure severity. The inability of CB₁ agonists and CB₁ antagonists to alter the severity or the proportion of clonic-tonic seizures induced by paraoxon indicates that CB₁ receptor activity does not regulate paraoxon-induced seizure activity.

While paraoxon-induced seizures were not regulated by CB₁ receptors, we cannot exclude the possibility that seizures induced by other organophosphates might be sensitive to CB₁ receptor activity. The ability of CB₁ receptors to regulate non-seizure toxicity of organophosphates has been reported to be different depending on the organophosphate examined. While CB₁ agonists decreased both involuntary movements and salivation caused by paraoxon administration, they only decreased involuntary movements and not salivation following diisopropylfluorophosphate (DFP) administration (Nallapaneni et al., 2006; Nallapaneni et al., 2008). Unexpectedly, loss of CB₁ receptor expression did not increase involuntary movement or salivation caused by chlorpyrifos administration (Baireddy et al., 2011). A multitude of factors, including pharmacokinetic properties and ability to inhibit FAAH and/or MAGL, may influence the ability of CB₁ receptor activity to regulate different aspects of organophosphate toxicity.

Further study is necessary to determine whether modulation of the cannabinoid system, especially the activity of CB₁ receptors, is a reasonable strategy to treat any of the various organophosphate-induced toxicities including organophosphate-induced seizures.

In summary, we have observed significant differences in the regulation by the M₁ and CB₁ receptors of seizures induced by pilocarpine and paraoxon. These results suggest that therapies appropriate for the prevention or treatment of pilocarpine-induced seizures may not be effective in the prevention or treatment of organophosphate-induced seizures. In addition, they suggest that drugs targeting the cannabinoid system would not work as an alternative to current therapies for managing organophosphate seizures. Further studies on the regulation of cholinergic agent-induced seizures by using other muscarinic agonists and organophosphates will help determine the shared and the cholinergic agent-specific mechanisms of cholinergic seizure induction.

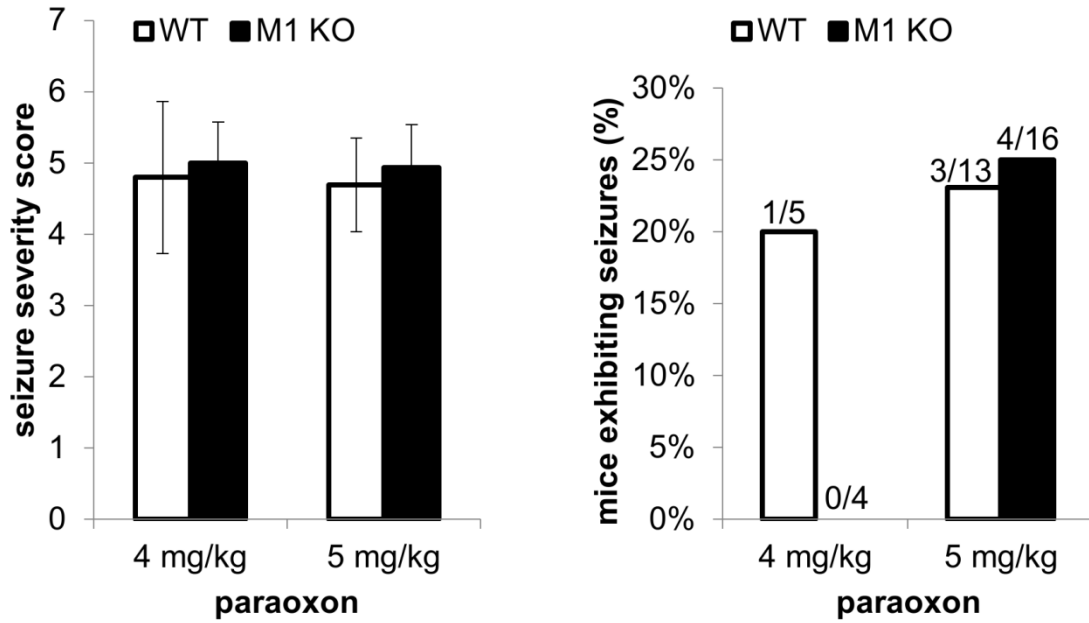


Fig. 11. Paraoxon induces seizures to a similar degree in WT and M₁ KO mice. Seizure severity scores and the proportion of mice having at least one clonic-tonic seizure after 5 mg/kg paraoxon administration were compared in male WT (n = 13) and M₁ KO mice (n = 16).

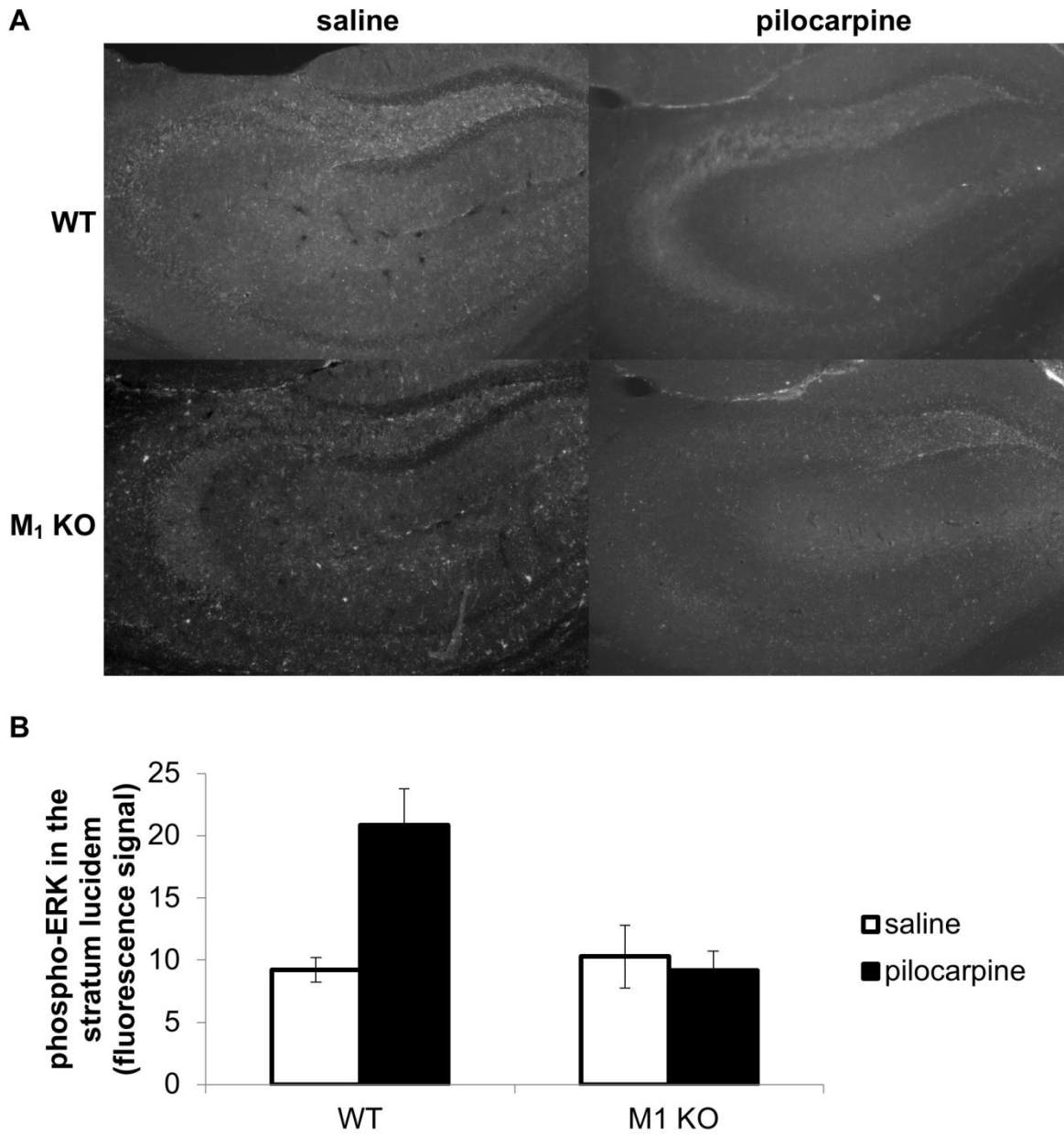


Fig. 12. Seizure-independent ERK activation by pilocarpine is absent from M₁ KO mice. **A.** Representative images of phospho-ERK immunofluorescence in the CA3 region of seizure-blocked male WT and M₁ KO mice 15 minutes after saline or 350 mg/kg pilocarpine administration. 4 mg/kg diazepam was given 15 minutes prior to pilocarpine to prevent seizure activity. **B.** Quantification of phospho-ERK fluorescence in the stratum lucidum of seizure-blocked male WT and M₁ KO mice 15 minutes after saline (n = 7 for WT; n = 6 for M₁ KO) or 350 mg/kg pilocarpine (n = 8 for WT; n = 7 for M₁ KO) administration. * p < 0.05.

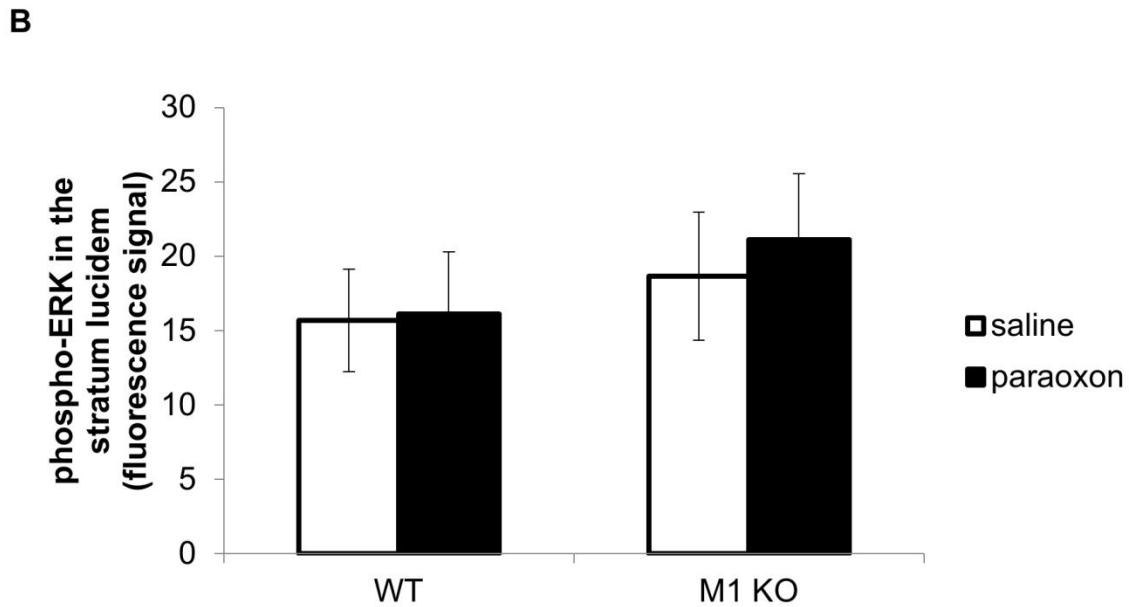
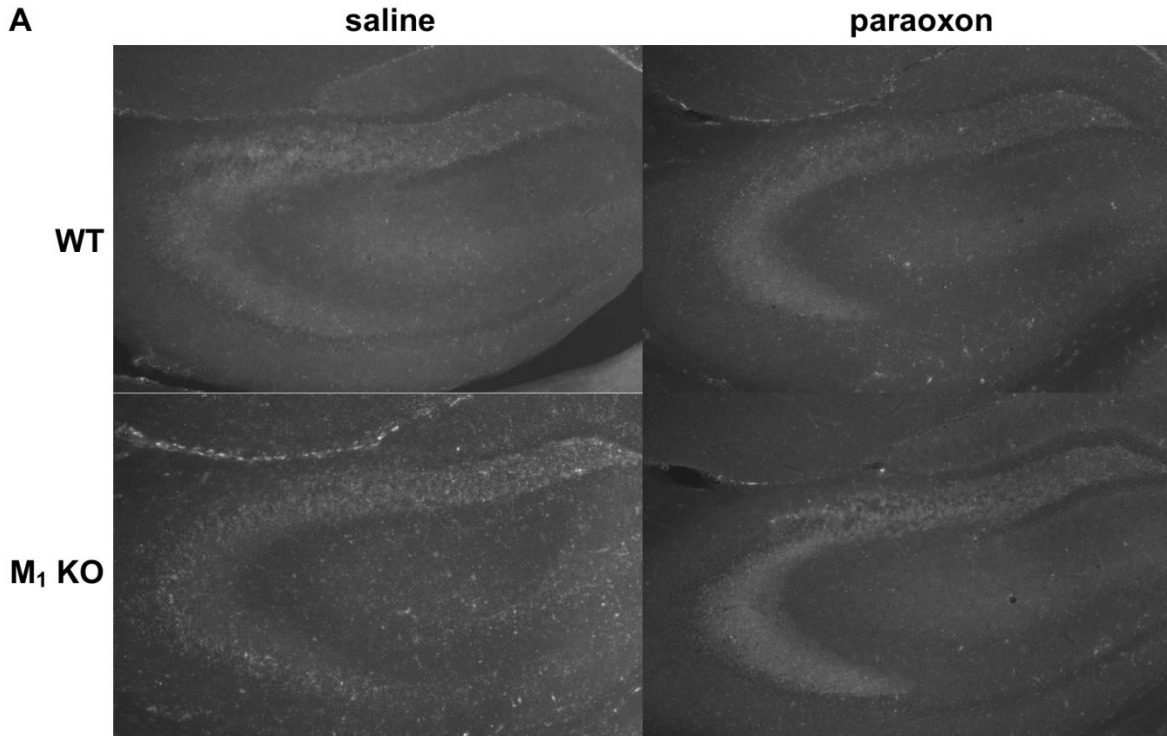


Fig. 13. ERK is not activated by paraoxon in seizure-blocked mice. **A.** Representative images of phospho-ERK immunofluorescence in the CA3 region of seizure-blocked male WT and M₁ KO mice 15 minutes after saline or 6 mg/kg paraoxon administration. 4 mg/kg diazepam was given 15 minutes prior to paraoxon to prevent seizure activity and 90 mg/kg 2-PAM was given 5 minutes prior to prevent peripheral toxicities. **B.** Quantification of phospho-ERK fluorescence in the stratum lucidum of seizure-blocked male WT and M₁ KO mice 15 minutes after saline (n = 7 for WT; n = 6 for M₁ KO) or 6 mg/kg paraoxon (n = 6 for WT; n = 7 for M₁ KO) administration.

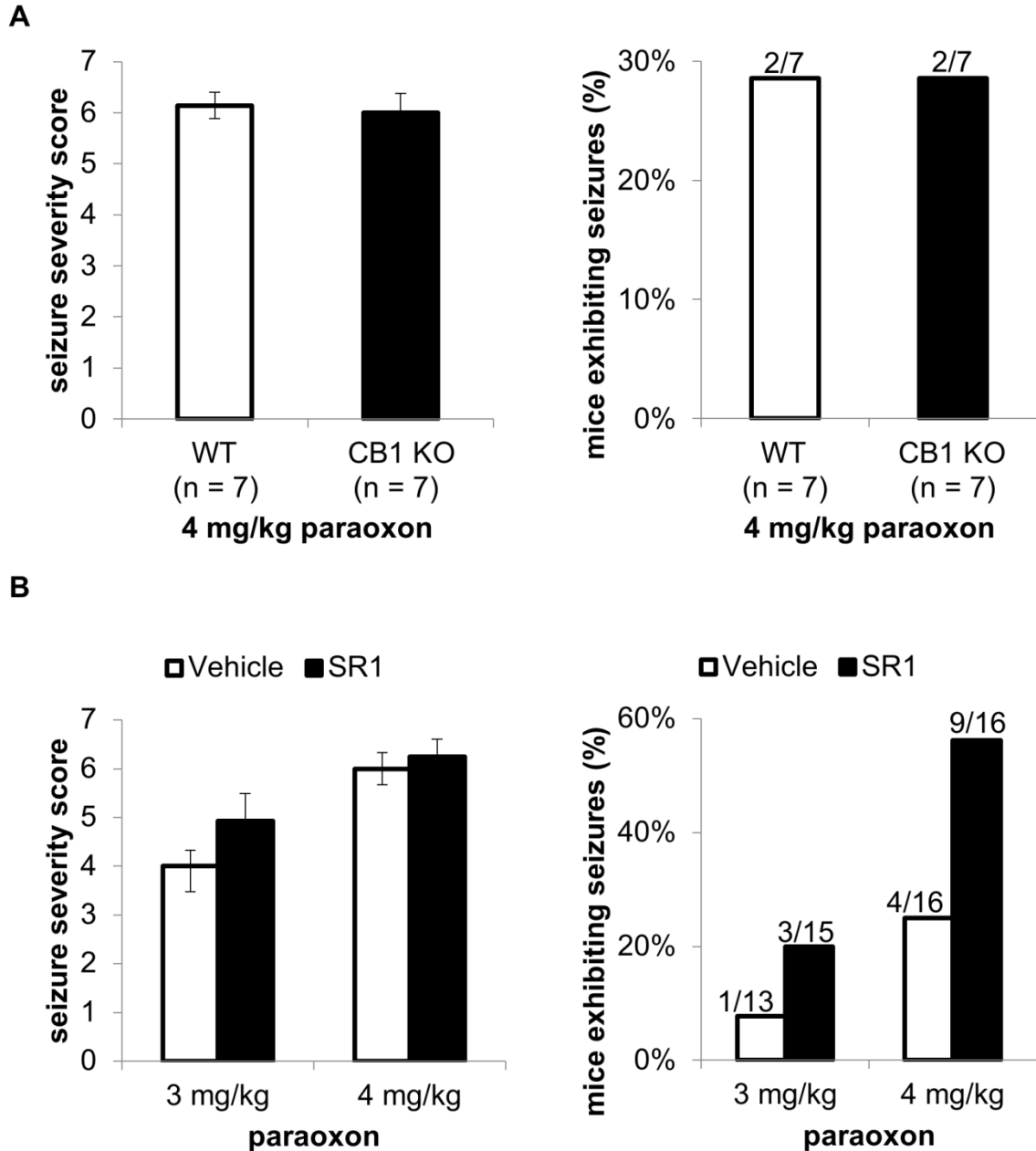
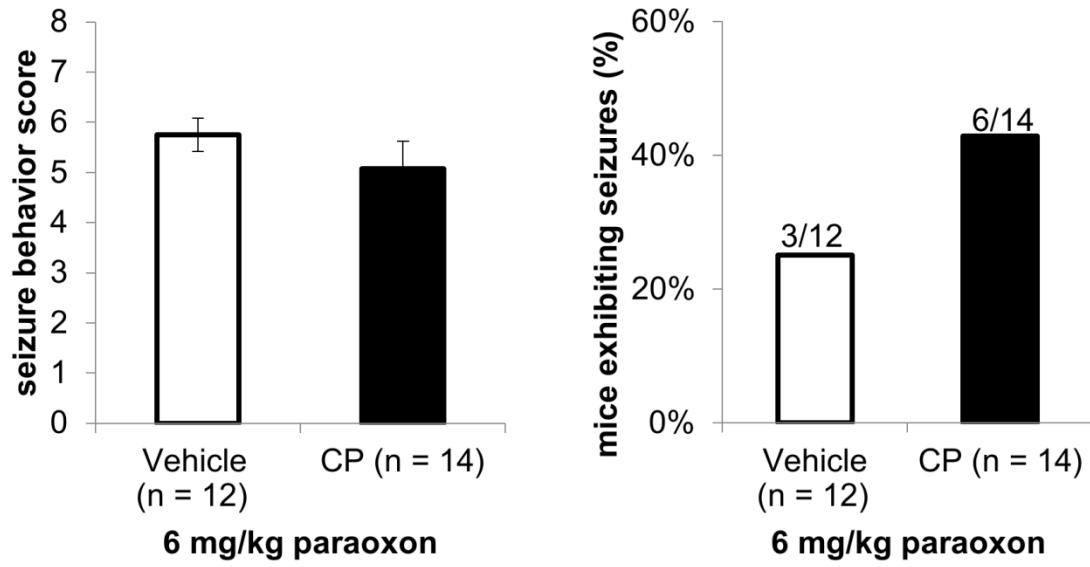


Fig. 14. CB₁ activity does not alter severity of paraoxon seizures. Seizure severity scores and the proportion of mice having at least one clonic-tonic seizure were compared between the following groups of mice. **A.** Male CB₁ KO (n = 7) and WT (n = 7) littermates. **B.** CB₁ receptor antagonist and vehicle-pretreated mice. SR141716 (SR1, 10 mg/kg) or the corresponding vehicle was given 2 hours prior to 3 mg/kg paraoxon (n = 13 for SR1; n = 16 for vehicle) or 4 mg/kg paraoxon (n = 15 for SR1; n = 16 for vehicle).

C



C. CB₁ receptor agonist or vehicle. CP 55940 (CP, 0.3 mg/kg) or the corresponding vehicle was given 30 minutes prior to 6 mg/kg paraoxon (n = 12 for CP; n = 14 for vehicle).

CONCLUSIONS

In the previous chapters we have further investigated the actions of muscarinic acetylcholine receptors (mAChRs) in the brain. All 5 mAChR subtypes are expressed in the brain; most brain regions express multiple subtypes. Using muscarinic agonists, muscarinic antagonists, and mAChR subtype-specific KO mice, many roles for mAChRs have been identified including the regulation of learning and memory, cholinergic seizures, locomotion, body temperature, body weight, and nociception.

In Chapter 2, we investigated a possible role for mAChRs in regulation of adult neurogenesis. Neurogenesis persists in two areas in the adult brain, the subventricular zone (SVZ) and the subgranular zone (SGZ), and is modified by many factors including age, genetic background, environmental factors, hormones, and neurotransmitters. Increased neurogenesis after muscarinic agonist administration was previously reported with pilocarpine treatment of rats; however, it appeared that seizure damage upregulated adult neurogenesis in those rats. Recent studies in which rats were given the muscarinic agonist oxotremorine and/or the muscarinic antagonists pirenzepine and scopolamine implied that mAChR activation could positively regulate adult neurogenesis independently of seizures. Therefore, we first tried to confirm that muscarinic agonists could increase cell proliferation in the dentate gyrus of mice. We found that a single high-dose of the muscarinic agonist pilocarpine did not increase cell proliferation in the dentate gyrus when its seizure activity was blocked. In addition, multiple doses of oxotremorine over a 3-day period did not increase cell proliferation, even when given to older mice that had reduced basal levels of adult neurogenesis. Similarly, the proliferation rate

and the viability of *in vitro* cultures of adult neural progenitor cells (aNPCs) were unaffected by addition of muscarinic agonists oxotremorine M or carbachol.

Because we did not detect an increase in adult neurogenesis following administration of mAChR agonists, we determined whether loss of mAChR activity could decrease adult neurogenesis. Since the M₁ receptor is the predominant mAChR subtype expressed in the forebrain, we compared basal neurogenesis in 12-week-old WT and M₁ KO mice. We found no difference in cell proliferation or new cell survival between WT and M₁ KO mice, indicating that M₁ receptors do not mediate the actions of endogenous acetylcholine on adult neurogenesis. Similarly, aNPCs derived from M₁ KO mice had similar rates of proliferation and survival as WT aNPCs. The muscarinic antagonist atropine, which blocks activity at all mAChR subtypes, also did not alter proliferation or survival of aNPCs *in vitro*. Altogether these results suggest that mAChRs do not regulate adult neurogenesis. However, to fully eliminate a role for mAChRs in the regulation of adult neurogenesis, studies need to be done with other mAChR subtype-specific KO mice or double KO mice.

In Chapters 3 and 4, we investigated the regulation of cholinergic seizures using the muscarinic receptor-specific agonist pilocarpine and the organophosphate paraoxon. Both muscarinic receptor-specific agonists and organophosphates, which indirectly increase mAChR activity by inhibiting the breakdown of acetylcholine by acetylcholinesterases, induce seizures in a mAChR-dependent manner. Because the muscarinic agonist pilocarpine is unable to induce seizures in M₁ KO mice, the M₁ receptor has been considered the mAChR subtype that mediates seizures resulting from administration of all cholinergic agents. Activation of M₁ receptors leads to activation of typical G_q signaling pathways including increased IP₃ production, increased intracellular calcium, and activation of ERK. Activation of M₁ receptors also increases the

release of endocannabinoids, which act at CB₁ receptors to reduce excitatory transmission. Because CB₁ receptors have previously been shown to modulate the sensitivity to seizure induction by electroshock or kainic acid, we examined the role of CB₁ receptors in pilocarpine-induced seizures. In Chapter 3, we found that loss of CB₁ receptor activity, either by genetic deletion or antagonist pretreatment, increased the proportion and the severity of seizure behaviors following administration of submaximal doses of pilocarpine, indicating that endocannabinoids were acting at CB₁ receptors to reduce some of the excitotoxicity of pilocarpine. On the other hand, CB₁ agonist pretreatment did not affect the induction of pilocarpine seizures or their severity. This suggested that endocannabinoids levels during pilocarpine administration were already maximizing the ability of CB₁ receptors to reduce pilocarpine toxicity.

In Chapter 4, we compared the regulation of pilocarpine- and paraoxon-induced seizures by M₁ and CB₁ receptors. Paraoxon seizures were unaffected by the loss of M₁ or CB₁ receptors. CB₁ agonists also did not affect paraoxon seizure severity, which indicates that paraoxon seizures are insensitive to CB₁ receptor modulation. We then investigated the activity of M₁ receptors following pilocarpine or paraoxon administration. We pretreated mice with diazepam in order to look at seizure-independent ERK activation in the hippocampus. Pilocarpine was able to activate ERK in the stratum lucidum of WT mice but not M₁ KO mice, indicating the dependence of muscarinic agonist-induced ERK activation on M₁ receptor expression. Interestingly, paraoxon was unable to activate ERK in a seizure independent manner in both WT and M₁ KO mice. This suggests that paraoxon does not activate enough M₁ receptors to activate ERK in the hippocampus.

Interestingly, the results of these studies suggest a diminished importance of M₁ receptor activation in adult neurogenesis and cholinergic seizures. Despite being the most abundant mAChR subtype in the forebrain, particularly in areas associated with adult neurogenesis and epilepsy in humans, M₁ receptors are not necessary for increases in cell proliferation and survival in neurogenic regions by endogenous ACh and not necessary for all cholinergic agent-induced seizures. Nevertheless, forebrain regions still express a significant number of M₂, M₃, and M₄ receptors, which all have the capability to increase proliferation and/or survival. M₂ and M₃ receptors could also mediate paraoxon-induced excitotoxicity in the forebrain. M₂ receptors expressed on GABAergic neurons can reduce their inhibitory transmission, while M₃ receptors have been shown to act similarly to M₁ receptors in the cortex, albeit producing much smaller effects. In addition, there is the possibility that paraoxon-induced seizures do not need to initiate in the hippocampus, cortex, or other regions where M₁ receptors are the most abundant mAChR subtype, as focal application of acetylcholine indicated that other regions like the amygdala or the “area tempestus” could initiate seizures. Further understanding of the regulation of adult neurogenesis and cholinergic agent-induced seizures by mAChRs will come from analysis of these processes in other mAChR subtype KO mice or double KO mice, as well as more detailed studies on the signaling pathways activated following administration of cholinergic agents and their regional specificities.

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