

# Chapter 3

## A New Aspect of Cholinergic Transmission in the Central Nervous System



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**Abstract** In the central nervous system, acetylcholine (ACh) is an important neurotransmitter related to higher brain functions and some neurodegenerative diseases. It is released from cholinergic nerve terminals and acts on presynaptic and postsynaptic ACh receptors (AChRs). Following release, ACh is rapidly hydrolyzed and the resultant choline is recycled as a substrate for new ACh synthesis. However, this classical concept of cholinergic transmission is currently reevaluated due to new evidence. In the cholinergic synapse, ACh may be itself taken up into postsynaptic neurons by a specific transport system and may act on AChRs at intracellular organelles (Golgi apparatus and mitochondria). Choline for ACh synthesis in cholinergic nerve terminals may be mainly supplied from choline at relevant concentration levels

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present in the extracellular space, rather than recycled from ACh-derived choline. Recent evidence has reopened the issue of classical cholinergic transmission and cognition, and may provide a novel approach to rational drug development for the treatment of neurodegenerative disorders such as Alzheimer's disease.

**Keywords** Cholinergic transmission · Intracellular acetylcholine receptors · Acetylcholine uptake · Acetylcholine esterase · Presynaptic muscarinic receptors

## Abbreviations

ACh	Acetylcholine
AChE	Acetylcholine esterase
AChR	Acetylcholine receptor
AChT	Acetylcholine transporter
Ca <sup>2+</sup>	Calcium
CHT1	High affinity-choline transporter 1
CNS	Central nervous system
DFP	Diisopropylfluorophosphate
ERK	Extracellular regulated kinase
HC-3	Hemicholinium-3
LTP	Long term-potential
mAChR	Muscarinic acetylcholine receptor
MAPK	Mitogen-activated protein kinase
nAChR	Nicotinic acetylcholine receptor
NMDAR	N-methyl-D-aspartate receptor
NMS	N-methyl-scopolamine
PIP <sub>2</sub>	Phosphatidylinositol 4,5-bisphosphate
QNB	Quinuclidinyl benzilate
TEA	Tetraethylammonium

## 3.1 Introduction

In the central nervous system (CNS), acetylcholine (ACh) is one of the major neurotransmitters involved in higher brain functions, including cognitive processes such as learning and memory and extrapyramidal locomotor activity (Everitt and Robbins 1997; Terry and Buccafusco 2003; Mesulam 2004; Wess et al. 2007). In cholinergic transmission, released ACh acts on ACh receptors (AChRs) located on the presynaptic and/or postsynaptic plasma membranes. ACh is also rapidly hydrolyzed by ACh esterase (AChE), leading to the termination of synaptic neurotransmission. Then the resultant choline is transported back into the cholinergic nerve

terminals by the high-affinity choline transporter 1 (CHT1), and is reutilized as a substrate for ACh synthesis (Parsons et al. 1993; Apparsundaram et al. 2000; Okuda et al. 2000; Sarter and Parikh 2005). However, this classical tenet of cholinergic transmission has recently been challenged by several new findings from recent studies. The first finding is with regard to the intracellular distribution and function of AChRs in postsynaptic neurons and neuroblastoma cells (Yamasaki et al. 2010; Uwada et al. 2011, 2014; Anisuzzaman et al. 2013; Muramatsu et al. 2015); the second is the incorporation of ACh itself into postsynaptic neurons (Muramatsu et al. 2016); and the third finding is that ACh-derived choline after hydrolysis may not be largely reused (Muramatsu et al. 2017). In this chapter, these findings were briefly summarized.

## 3.2 Intracellular Distribution of AChRs

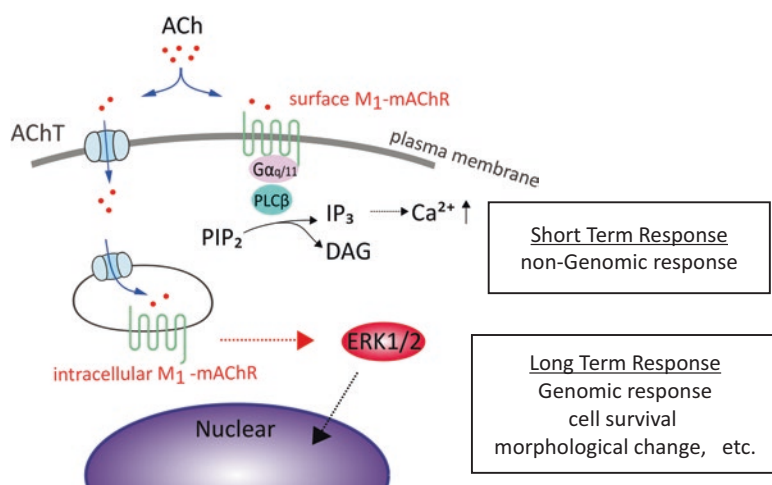
It has been commonly accepted that most neurotransmitter receptors are located on the plasma membrane and transduce extracellular to intracellular signals. However, recent evidence suggests that several G-protein-coupled receptors including AChRs are located, and may also signal from, intracellular sites such as endosomes, Golgi apparatus, endoplasmic reticulum, mitochondria, and nuclear membranes (Boivin et al. 2008; Jong et al. 2009; Benard et al. 2012; den Boon et al. 2012; Uwada et al. 2011, 2014; Anisuzzaman et al. 2013).

### 3.2.1 Muscarinic AChRs

There are five subtypes of muscarinic AChRs (M1–M5 mAChRs), all of which are expressed in the CNS (Caulfield and Birdsall 1998; van Koppen and Kaiser 2003; Nathanson 2008). In general, the M1 subtype is the most abundant within the CNS, M2 and M4 subtypes are moderately expressed, and only low levels of M3 and M5 subtypes have been found (Volpicelli and Lavery 2004). All of the mAChRs are generally located and function at the plasma membrane. However, recent studies have revealed that M1 mAChRs exist not only at the cell surface but also at intracellular membranes in the hippocampus and other telencephalon regions of rodents and humans and in neuroblastoma cells (Uwada et al. 2011; Anisuzzaman et al. 2013). Pharmacologically, the intracellular distribution of mAChRs is evaluated from the different binding densities of cell-permeable (hydrophobic) and cell-impermeable (hydrophilic) radioligands, [<sup>3</sup>H]quinuclidinyl benzilate (QNB) and [<sup>3</sup>H]N-methyl-scopolamine (NMS), respectively, in intact segments of brain tissue or whole neuronal cells to detect total (QNB binding) and cell surface (NMS binding) mAChRs (Muramatsu et al. 2005, 2015), and proportions of intracellular and surface M1 subtypes are estimated from the competing profiles of M1-selective

ligands at the binding sites of both radioligands. Comparable amounts of surface and intracellular M1 mAChRs were identified under conditions where brain tissues or cultured neurons were not stimulated beforehand. Therefore, it is likely that approximately half the amount of M1 mAChR constitutively occurs at intracellular sites in the telencephalon and in neuroblastoma cells. Intracellular M1 mAChRs were immunohistochemically shown to localize at the Golgi apparatus, and a recent molecular biology study revealed that their intracellular localization requires the C-terminal tryptophan-based motif of M1 subtype, which does not exist in other subtypes (Uwada et al. 2014; Anisuzzaman et al. 2013). The abundant distribution of M1 mAChRs in the Golgi apparatus and endoplasmic reticulum of pyramidal neurons but not in astroglia was also demonstrated in immunoelectron microscopic studies (Yamasaki et al. 2010). A previous immunohistochemical study with a specific M1 antibody also reported intracellular detection in the cytoplasm of large and small dendrites and the dendritic spines of cerebral cortex neurons (Mrzljak et al. 1993).

M1 mAChRs cause phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) hydrolysis leading to calcium (Ca<sup>2+</sup>) upregulation and activation of the mitogen-activated protein kinase (MAPK) pathway through the G $\alpha_{q/11}$  protein (van Koppen and Kaiser 2003; Morishima et al. 2013). In rat hippocampal and cortical neurons and neuroblastoma cells, the PIP<sub>2</sub>-Ca<sup>2+</sup> response is exclusively mediated by surface M1 mAChRs on a time scale of seconds. On the other hand, the extracellular signal-regulated kinase1/2 (ERK1/2) pathway is activated by intracellular M1 mAChRs on a slow time scale of minutes (Uwada et al. 2011; Anisuzzaman et al. 2013). These results indicate that M1 mAChRs at each site may be specifically activated and involved in distinct neuronal functions with different temporal courses (Fig. 3.1).



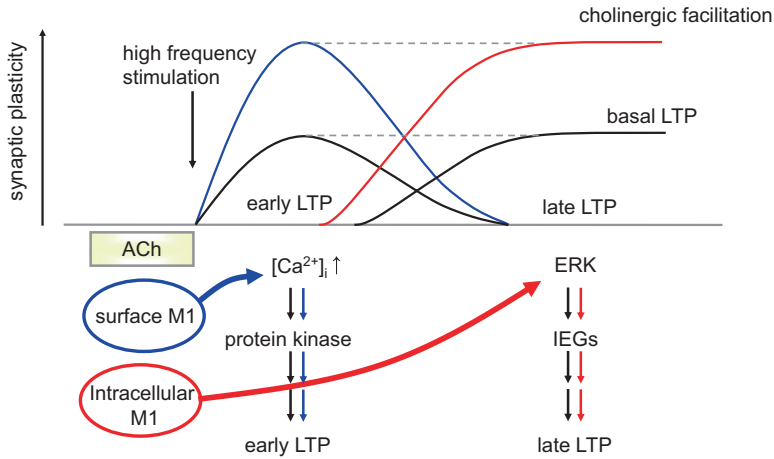
**Fig. 3.1** Schematic representation of surface and intracellular M1 mAChRs in postsynaptic neurons, and their possible signal transduction pathways and physiological responses

As aforementioned, M1 mAChRs predominantly exist in the CNS and are involved in cognitive enhancement (Everitt and Robbins 1997; Kruse et al. 2014; Terry and Buccafusco 2003; Mesulam 2004; Wess et al. 2007). In the hippocampus, cholinergic activation induces a theta rhythm of neuronal activity and enhances or induces long-term potentiation (LTP) (Huerta and Lisman 1995; Williams and Kauer 1997; Fernandez de Sevilla et al. 2008), the primary experimental model for investigating the synaptic basis of learning and memory (Bliss and Collingridge 1993; Seol et al. 2007). M1 mAChR-knockout mice have severe deficits in working memory and memory consolidation, as well as impaired hippocampal LTP (Anagnostaras et al. 2003; Shinoe et al. 2005; Wess et al. 2007). M1-specific agonists have been shown to facilitate the induction of LTP and improve cognitive function in several animal models of amnesia (Caccamo et al. 2006; Langmead et al. 2008; Ma et al. 2009). Thus, the specific distribution of intracellular M1 mAChRs in the telencephalon may correlate with synaptic plasticity.

Our electrophysiological study with rat hippocampal slices revealed that cholinergic induction of a theta rhythm and cholinergic facilitation in the early part of *N*-methyl-d-aspartate receptor (NMDAR)-dependent LTP were mainly mediated by surface M1 mAChRs, whereas cholinergic facilitation in the late part of NMDAR-dependent LTP and the majority of non-NMDAR-dependent LTP were evoked by activation of intracellular M1 mAChRs (Anisuzzaman et al. 2013). The induction of LTP requires elevation of postsynaptic  $\text{Ca}^{2+}$  (Lynch et al. 1983) and activation of protein kinases (Soderling and Derkack 2000), whereas the maintenance of late-phase LTP depends on the MAPK/ERK cascade, gene transcription, protein synthesis, and posttranslational modification (Davi et al. 2000; Giovannini 2006; Nguyen et al. 1994; Frey et al. 1996; Bliss and Collingridge 1993; Routtenberg and Rekart 2005; Gold 2008). Taking these mechanisms into consideration, it is likely that cholinergic stimulation may primarily cause the selective enhancement of respective signaling processes in the early and late phases of LTP through M1 mAChRs located at two distinct sites, leading cholinergic facilitation (Fig. 3.2).

### 3.2.2 Nicotinic AChRs

The nicotinic AChRs (nAChRs) are widely expressed in the brain, where they maintain various neuronal functions including learning and memory (Terry and Buccafusco 2003; Dineley et al. 2015; Wu et al. 2016). They also control survival/death, proliferation/neurite outgrowth, and neurotransmitter release in neuronal cells (Akaike et al. 1994; Kihara et al. 2001; Shimohama et al. 1996; 2009; Rosa et al. 2006). The major nAChR subtypes in the CNS are heteromeric  $\alpha 4\beta 2$ ,  $\alpha 3\beta 2$ ,  $\alpha 7\beta 2$  and homomeric  $\alpha 7$  assemblies, all of which have high permeability for  $\text{Ca}^{2+}$  as ionic channels and also transduce signals through the phosphatidylinositol 3-kinase (PI3K) signaling pathway (Kihara et al. 2001; Dajas-Bailador and Wonnacott 2004; also see other chapters in this book). To date, these responses have been mainly



**Fig. 3.2** Involvement of surface and intracellular M1 mAChRs in cholinergic facilitation of LTP in rat hippocampus. Note that early and late stages of basal LTP are represented to be primarily mediated by two distinct signal pathways, which are independently enhanced by surface and intracellular M1 mAChRs. Blue: surface M1 mAChR-mediated. Red: intracellular M1 mAChR-mediated. IEGs immediately early genes

discussed with special reference to nAChRs located on the plasma membrane; however, the intracellular distribution of nAChRs, especially in the mitochondria, was recently reported in immunochemical studies (Lykhmus et al. 2014; Gergalova et al. 2014). In brain mitochondria,  $\alpha 7\beta 2$  nAChRs mainly stimulate the PI3K/AKT pathway, and  $\alpha 3\beta 2$  and  $\alpha 4\beta 2$  nAChRs inhibit Src- and  $\text{Ca}^{2+}$ /calmodulin-dependent protein-kinase II pathways. Mitochondrial nAChRs and associated signaling pathways are associated with the induction of mitochondrial apoptosis (Lykhmus et al. 2014). However, more detailed analysis from different approaches is required in this issue.

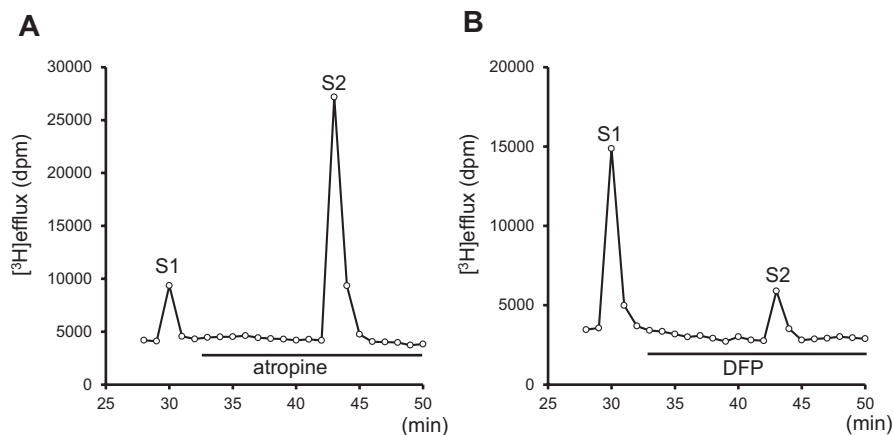
### 3.3 Incorporation of ACh into Postsynaptic Neurons

To activate intracellular AChRs, the endogenous agonist ACh must cross the postsynaptic plasma and endosomal membranes. Because ACh is a hydrophilic molecule, the presence of a specific ACh transport system such as the ACh transporter (AChT) has been postulated (Muramatsu et al. 2016) (Fig. 3.1). Although detailed information on the AChT is lacking, incorporation of ACh into brain slices was reported more than four decades ago (Polak and Meeuws 1996; Liang and Quastel 1969; Katz et al. 1973; Kuhar and Simon 1974). More recently, previous evidence showing that  $[^3\text{H}]\text{ACh}$  is actively taken up into brain segments in time- and

temperature-dependent manners was confirmed (Muramatsu et al. 2016, 2017). The uptake was clearly observed in the presence of irreversible but not reversible AChE inhibitors. [ $^3\text{H}$ ]ACh uptake was high in the CNS but was negligible or minor in the peripheral tissues. The uptake was comparable among brain regions but was not related to the density of cholinergic innervation. Hemicholinium-3 (HC-3) and tetraethylammonium (TEA) inhibited [ $^3\text{H}$ ]ACh uptake in a concentration-dependent manner. However, the uptake was little affected by the excitatory or inhibitory amino acid neurotransmitters glutamate and gamma-aminobutyric acid, respectively, biogenic amines, tetrodotoxin, and atropine. Therefore, it is likely that ACh uptake is facilitated by an intrinsic transport system (AChT), rather than a change in neuronal excitability and the involvement of amino acid neurotransmitters. Interestingly, [ $^3\text{H}$ ]ACh uptake was potently suppressed by AChE inhibitors including drugs clinically used to treat the cognitive symptoms of Alzheimer's disease such as donepezil, galantamine and rivastigmine (Muramatsu et al. 2016). These results raise the interesting possibility that ACh concentrations released in the synapse may be regulated by both AChE and the postsynaptic uptake of ACh, which may be relevant to cholinergic therapy in Alzheimer's disease. As mentioned above, intracellular M1 mAChRs selectively activate ERK in hippocampal neurons and participate in the late stage of cholinergic facilitation of LTP. The intracellular M1-mediated responses both were inhibited by TEA and HC-3 at concentrations that inhibit ACh uptake. However,  $\text{PIP}_2$  hydrolysis and the early phase of cholinergic facilitation of LTP, which were selectively caused by plasma membrane M1 AChRs, were not affected by TEA and HC-3 (Uwada and Masuoka unpublished observations). These pharmacological results further support the fact that cholinergic facilitation of LTP is caused independently by surface and intracellular M1 mAChRs through distinct signaling pathways (Figs. 3.1 and 3.2). These results also indicate that the ACh transport system serves as an intrinsic route for released ACh to access intracellular AChRs in postsynaptic neurons.

### 3.4 Regulation of Synaptic ACh Concentrations and the Choline-ACh Cycle

In the CNS, release of ACh from the cholinergic nerve terminals is negatively regulated through presynaptic mAChRs (Raiteri et al. 1989; Starke et al. 1989; Zhang et al. 2002; Alquicer et al. 2016). In vitro, this release is monitored with the superfusion technique, where ACh in synaptosomes (Raiteri and Raiteri 2000; Pittaluga 2016) and brain slices (Richardson and Szerb 1974; Zhang et al. 2002; Alquicer et al. 2016) has been synthesized/prelabeled in advance with [ $^3\text{H}$ ]choline followed by superfusion. Figure 3.3a shows a representative result obtained from a superfusion experiment with rat striatal slices. Atropine dramatically increased [ $^3\text{H}$ ]efflux evoked by electrical stimulation, indicating that blockade of presynaptic mAChRs suppressed autoinhibition of ACh release. Although the presynaptic mAChR



**Fig. 3.3** Effects of atropine and diisopropyl phosphorofluoridate in  $[^3\text{H}]$ efflux in superfusion experiments. Rat striatal segments were incubated with  $0.1 \mu\text{M}$   $[^3\text{H}]$ choline for 30 min and then superfused. Electrical stimulation (3 Hz, for 30 s) was applied two times (S1 and S2). (a)  $0.1 \mu\text{M}$  atropine. (b)  $300 \mu\text{M}$  diisopropyl phosphorofluoridate (DFP). Ordinate:  $[^3\text{H}]$  count (dpm) in superfusate collected every minute. Abscissa: time after superfusion

subtypes that participate in autoregulation have been the subject of debate, it was recently concluded that the M2 subtype has dominant involvement and there is only minor participation of the M4 subtype (Dolezal and Tucek 1998; Fadel 2011; Zhang et al. 2002; Alquicer et al. 2016).

Released ACh is rapidly hydrolyzed by AChE. Compared with the peripheral tissues, AChE activity in the brain (except cerebellum) is extremely high, and esterase activity in the CNS is closely related to the density of cholinergic innervation (Muramatsu et al. 2016). The esterase activity in the rat striatum is more than five times higher than that in the cerebral cortex and hippocampus, whereas the lowest activity is in the cerebellum, on par with that of the heart and colon. Thus, concentrations of released ACh appear to be effectively regulated by AChE in the CNS.

Inhibition of AChE would be thought to elicit a dramatic increase in synaptic ACh concentration; however, the scenario is not so simple. As mentioned above, ACh release is negatively regulated by presynaptic autoreceptors. Thus, it is likely that AChE inhibitors suppress hydrolysis of released ACh, which in turn, enhances stimulation of presynaptic mAChRs. Figure 3.3b shows a representative result in rat striatal slices, where  $[^3\text{H}]$ efflux evoked by electrical stimulation (3 Hz, 30 s) was reduced after treatment with an irreversible AChE inhibitor (diisopropyl fluorophosphate [DFP]). This inhibitory effect of DFP was abolished by atropine (Muramatsu unpublished observations). These results strongly suggest that ACh release and synaptic concentrations of ACh are precisely controlled by the subtle balance between AChE activity and presynaptic autoinhibition.

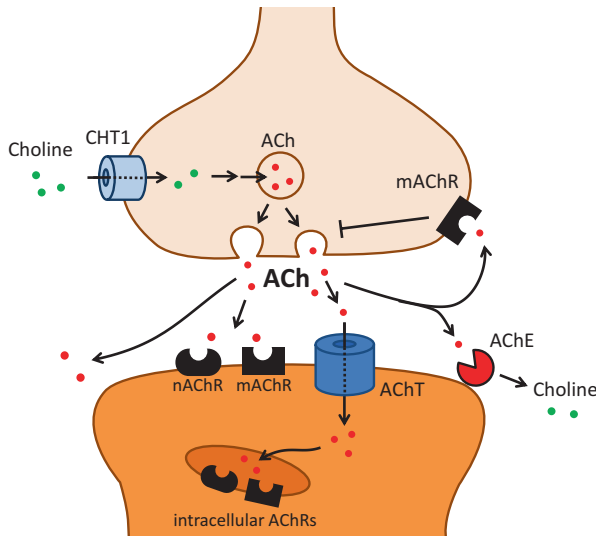


In addition to presynaptic AChRs and AChE, the postsynaptic uptake of ACh itself also regulates synaptic ACh concentrations (Muramatsu et al. 2017). In Sect. 3.3, it was noted that ACh uptake is inhibited by TEA and HC-3. The concentration of both drugs that suppressed ACh uptake significantly increased the evoked [ $^3\text{H}$ ] efflux by electrical stimulation in superfusion experiments. The effects of TEA or HC-3 were not related to inhibitory actions on presynaptic mAChRs or AChE activity, because the effects of both drugs were observed under conditions which mAChRs and AChE were completely inhibited. Therefore, it is possible that a portion of released ACh may be incorporated into postsynaptic neurons by AChT, participating in the regulation of synaptic ACh concentrations.

After hydrolysis by AChE, it has been classically proposed that ACh-derived choline is transported back into cholinergic nerve terminals and recycled for new ACh synthesis. In this process, choline uptake is mediated through CHT1, which shows high affinity for HC-3 ( $K_i = 0.001\text{--}0.01\ \mu\text{M}$ ) and choline ( $1\text{--}5\ \mu\text{M}$ ) (Guyenet et al. 1973; Haga and Noda 1973; Okuda et al. 2012). In most previous superfusion experiments,  $10\ \mu\text{M}$  HC-3 had been added into perfusion medium to suppress CHT1 activity. HC-3 at this concentration might act on presynaptic mAChRs in addition to inhibiting choline and ACh uptake. On the other hand, lower concentrations ( $0.1\text{--}1\ \mu\text{M}$ ) of HC-3, which selectively inhibit CHT1 but do not affect ACh uptake, failed to increase [ $^3\text{H}$ ]efflux evoked by electrical stimulation in superfusion experiments (Muramatsu et al. 2017). These recent results suggest that the increase in [ $^3\text{H}$ ] efflux by HC-3 is caused by an inhibition of ACh uptake but not choline uptake, implying that ACh-derived choline may not be significantly transported back into cholinergic terminals. Physiological concentrations of choline are relatively high ( $10\text{--}50\ \mu\text{M}$  in plasma and  $5\text{--}7\ \mu\text{M}$  in cerebrospinal fluid) (Lockman and Allen 2002; Sweet et al. 2001), so that endogenous choline can be continuously supplied from extracellular spaces as a substrate for ACh synthesis. It is interesting to note that perturbations in brain choline homeostasis produce central cholinergic dysfunction (Koppen et al. 1993; Jenden et al. 1990; Sarter and Parikh 2005). These recent results were summarized in this review, and the modified cholinergic transmission mechanisms are proposed in Fig. 3.4.

### 3.5 Perspectives

Cholinergic transmission as well as adrenergic transmission is a prototype of neurotransmission, in which the transmitter is synthesized, stored and released from nerve terminals, and then acts on postsynaptic membrane receptors. Following release, the transmitter undergoes degradation and/or presynaptic reuptake, leading to termination of synaptic transmission. However, recent studies have revealed that the neurotransmission mechanisms may be more complex. In cholinergic transmission in the



**Fig. 3.4** A proposed model of cholinergic transmission in CNS. After release from cholinergic nerve terminals, ACh acts on plasma membrane and intracellular AChRs (mAChRs and/or nAChRs) in postsynaptic pyramidal neurons, in addition to presynaptic mAChRs. Thus, synaptic ACh concentrations are regulated by AChE hydrolysis, AChT-mediated postsynaptic uptake and presynaptic modulation of release itself. Choline for ACh synthesis may be largely supplied from endogenous choline present at relevant concentrations ( $>5 \mu\text{M}$ ) in the extracellular space, rather than from ACh-degraded choline. Red circles: ACh. Green circles: choline

CNS, released ACh may act both the plasma membrane and intracellular receptors in postsynaptic neurons. ACh itself may be taken up into postsynaptic neurons and act on intracellular receptors. The proposed model (Fig. 3.4) has reopened the issue of classical cholinergic and other neurotransmission mechanisms, and may provide a significant impetus for the pharmacological therapy of neurodegenerative disorders such as Alzheimer's disease.

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**Conflict of Interest** The authors have no conflicts of interest to declare.

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