

Neurochemical Characterization of Huntingtin-Associated Protein 1 and Choline Acetyltransferase in the Adult Mouse Forebrain

成獣マウス前脳におけるハンチンチン関連タンパク質1とコリンアセチルトランス
フェラーゼ の神経化学的特徴



**DEPARTMENT OF BASIC LABORATORY SCIENCES
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MARCH, 2026

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ACADEMIC DISSERTATION

Submitted by

MIRZA MIENUR MEHER

Student ID: 23-9903-004-1

in Partial Fulfilment of the Requirement for the Degree of

DOCTOR OF PHILOSOPHY

(Health Science)



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Submitted by
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Student ID: 23-9903-004-1

**in Partial Fulfilment of the Requirement for the Degree of
DOCTOR OF PHILOSOPHY (PhD)**

Supervisor: Akie Yanai, PhD

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List of Abbreviations

3V	: 3rd ventricle
AA	: Anterior amygdaloid area
aca	: Anterior commissure: anterior part
AcbC	: Accumbens nucleus: core
AcbSh	: Accumbens nucleus: shell
ACh	: Acetylcholine
acp	: Anterior commissure: posterior nerve
AD	: Alzheimer's disease
ALS	: Amyotrophic lateral sclerosis
AOM	: Anterior olfactory area medial part
BFCN	: Basal forebrain cholinergic nuclei
CB	: Calbindin
CBPs	: Calcium binding proteins
CCK	: Cholecystokinin
CGRP	: Calcitonin gene-related peptide
ChAT	: Choline acetyltransferase
CPu	: Caudate putamen nuclei
CPu	: Caudate-putamen
CR	: Calretinin
fmi	: Forceps minor of the corpus callosum
GABA	: Gamma-aminobutyric acid
GFAP	: Glial fibrillary acidic protein
GP	: Globus pallidus
HAP1	: Huntingtin associated protein 1
Hb	: Habenular nucleus
HD	: Huntington's disease
HDB	: Horizontal limb of the diagonal band of Broca
HTT	: Huntingtin
Iba1	: Ionized calcium-binding adaptor molecule 1
ICj	: Islands of Calleja
ir	: Immunoreactive
LAcbSh	: Lateral accumbens shell
LHb	: Lateral Habenula

LHb	: Lateral habenular nucleus
LHbLMc	: Magnocellular part of the lateral division of LHb
LHbLO	: Oval part of the lateral division of LHb
LHbLPc	: Parvocellular part of the lateral division of LHb
LHbMC	: Central part of the medial division of LHb
LHbMMg	: Marginal part of the medial division of LHb
LHbMPc	: Parvocellular part of the medial division of LHb
LHbMS	: Superior part of the medial division of LHb
LO	: Lateral orbital cortex
LS	: Lateral septal nucleus
LSD	: Lateral septal nucleus: dorsal part
LSI	: Lateral septal nucleus: intermediate part
LSV	: Lateral septal nucleus: ventral part
LV	: Lateral ventricle
M1	: Primary motor cortex
MC	: Motor cortex
MCPO	: Magnocellular preoptic nucleus
MHb	: Medial Habenula
MHbC	: Central part of MHb
MHbI	: Inferior part of MHb
MHbL	: Lateral part of MHb
MHbS	: Superior part of MHb
MO	: Medial orbital cortex
MPA	: Medial preoptic area
MS	: Medial septal nucleus
NBM	: Nucleus Basalis of Meynert
NeuN	: Neuronal Nuclei
nNOS	: Neuronal nitric oxide synthase
och	: Optic chiasm
opt	: Optic tract
OVLt	: Vascular organ of the lamina terminalis
PBS	: Phosphate-buffered saline
PD	: Parkinson's disease
PPN	: Pedunculopontine nucleus
PV	: Parvalbumin

PVA	: Paraventricular thalamic nucleus: anterior part
RMTg	: Rostromedial tegmental nucleus
S1	: Primary somatosensory cortex
SCAs	: Spinocerebellar ataxias
SFO	: Subfornical organ
SIB	: Substantia innominata basal part (Meynert nucleus)
SN	: Substantia nigra
SNc	: Substantia nigra pars compacta
SNpc	: Substantia nigra pars compacta
SP	: Substance P
STB	: Stigmoid body
Tu	: Olfactory tubercle
VDB	: Nucleus of the vertical limb of the diagonal band of Broca
VO	: Ventral orbital cortex
VP	: Ventral pallidum
VTT	: Ventral tenia tecta.

Abstract

Huntingtin-associated protein-1 (HAP1) is a neuro-cytoplasmic protein and is involved in both intracellular trafficking and neuroprotection. The neuronal marker Choline acetyltransferase (ChAT) is an enzyme used to recognize the cholinergic neurons; that neuron influences attention, learning, motor control, and emotional responses. Despite their apparent importance to neurodegenerative disorders, little is known about the distribution and the neurochemical heterogeneity throughout the mouse forebrain. This study implemented Western blotting and immunohistochemistry in adult mice to map HAP1 and ChAT immunoreactive (ir) neurons throughout the BFCN, which included the brain area of the medial septum (MS), vertical and horizontal limbs of the diagonal band of Broca (VDB, HDB), and substantia innominata basal part (SIB). Furthermore, research was conducted across the caudate putamen (CPu) and habenula. Neuropeptides (CGRP, CCK), calcium-binding proteins (calbindin, calretinin, parvalbumin), and other markers, including neuronal nitric oxide synthase (nNOS) and Substance P (SP), were employed to assess co-expression as an indicator of neurochemical heterogeneity.

High regionalized HAP1 expression was found in BFCN, with the highest densities found in MS and VDB and medium to high expression in HDB and SIB. In contrast, HAP1 was sparsely distributed over the CPu. There were numerous ChAT-ir neurons in the CPu and across the BFCN, but ChAT neurons in CPu consistently did not co-express with HAP1. The highest co-expression of ChAT and HAP1 was observed in MS and VDB, indicating an enriched association in the forebrain cholinergic populations.

There was intensive co-expression of CGRP and CCK with ChAT neurons in the basal forebrain, particularly in the HDB and CPu. Calretinin had more selective co-expression in the VDB, SIB, and CPu, whereas calbindin was widely distributed and had significant co-localization with ChAT-ir neurons in both the VDB and HDB. However, parvalbumin neurons were not co-localized with ChAT. Furthermore, HAP1 showed selective and region-specific co-expression patterns. HAP1 was found to moderately co-localize with the CGRP and CCK in the MS, VDB, and HDB but didn't co-express with parvalbumin in the studied brain regions.

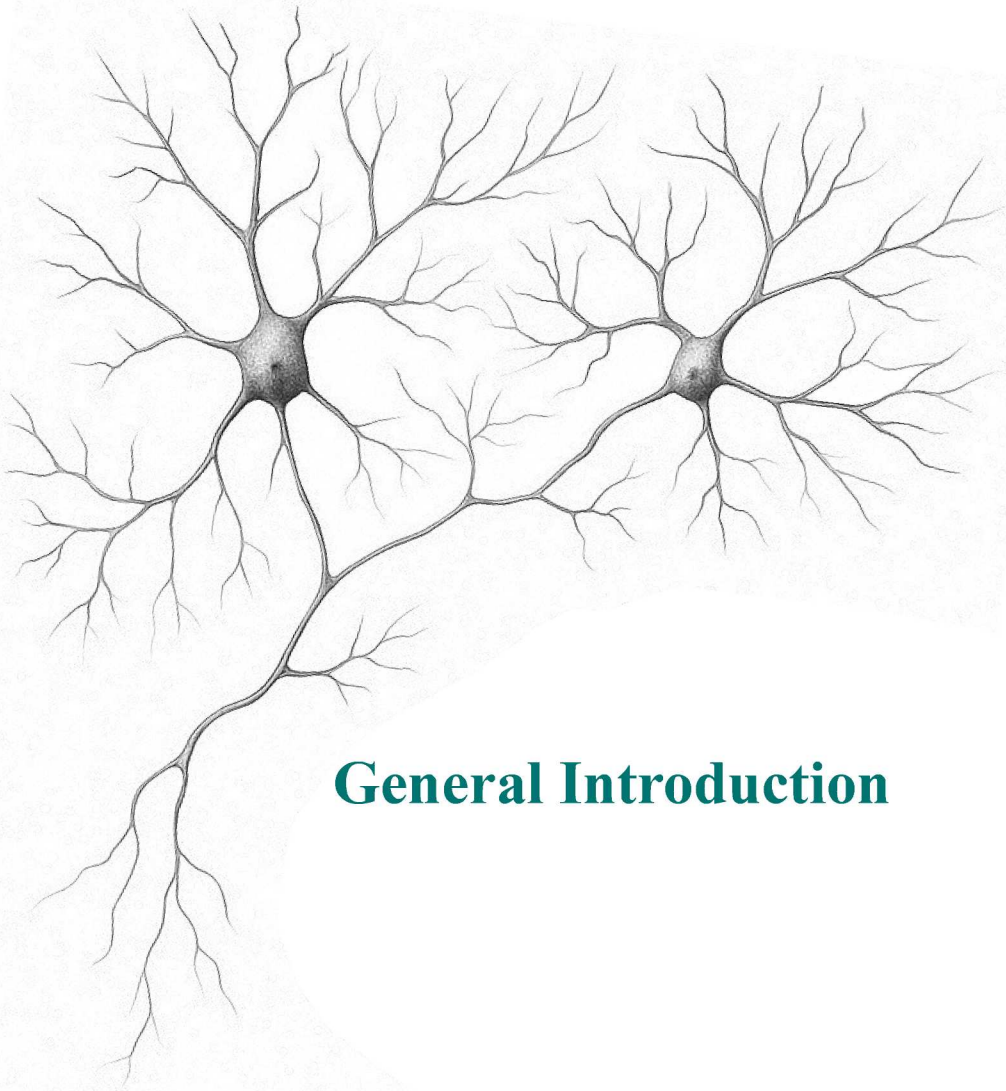
In the habenula, the majority of ChAT-positive neurons were found in the medial habenula (MHb), which lacked expression of HAP1. Conversely, HAP1 was only expressed in the lateral habenula (LHb); specifically, it was enriched in the central and paracentral subnuclei of LHb. In LHb, HAP1 showed the highest co-expression with CGRP and CCK neurons, whereas HAP1 had few

overlaps with nNOS neurons and a moderate co-expression with calbindin and calretinin. Though the co-localization of HAP1 with parvalbumin and SP neurons was absent. However, the selective expression of CCK and CGRP by ChAT neurons in the MHb revealed unique peptidergic pathways that are mediated by cholinergic nuclei.

These results show neurochemical distinctions between cholinergic and HAP1-expressing neurons. The selective HAP1-ChAT interaction in MS and VDB but not in striatal cholinergic neurons suggests a spatially diverse inherent protective potential of the HAP1 and ChAT. The regionalized and heterogenous immunohistochemical relationships between HAP1, ChAT, neuropeptides, and calcium-binding proteins indicate selective synaptic regulatory and protective effects against neurodegenerative stress. Our results provide a neurochemical foundation for future functional and mechanistic research on HAP1 and ChAT, especially in the context of different neurological diseases.



Chapter 1



General Introduction



1.1. General Introduction

Huntingtin-associated protein 1 (HAP1) is a crucial neuro-cytoplasmic protein that substantially influences the intracellular trafficking and neuroprotection. It was first discovered as a polyglutamine (polyQ)-length-dependent interactor of huntingtin (HTT) (Gutekunst et al., 1998; Li et al., 1995). The HTT is the protein that encoded by the gene responsible for the Huntington's disease (HD) (Gutekunst et al., 1998; Li et al., 1995). Importantly, the HAP1 inhibits the neuro pathogenic protein from inducing apoptosis by engaging with the mutant polyQ-extended HTT (Gutekunst et al., 1998; Li et al., 1995). Furthermore, HAP1 might inhibit cell death caused by the polyQ-expanded androgen receptor in spinal and bulbar muscular atrophy (SBMA) (Takeshita et al., 2006). These statements clearly indicate that HAP1 is a neuroprotective molecule that may deliver defenses against neuronal degeneration. As like the HD and SBMA, HAP1 interacts with additional proteins that linked to a number of neurodegenerative disorders (NDs). These include the TATA-binding protein in spinocerebellar ataxia type 17, the Abelson helper integration site 1 in Joubert syndrome, and the ataxin-3 in Machado-Joseph disease (Prigge and Schmidt, 2007; Sheng et al., 2006; Takeshita et al., 2011). Furthermore, HAP1 contributes to intracellular transportation by regulating vesicle and organelle mobility inside the neurons via connections to transport machinery and motor proteins (Chen et al., 2023). Since trafficking disruptions are common in a number of neurodegenerative disorders, including as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and spinocerebellar ataxias (SCAs). Another significant element of HAP1 is the stigmoid body (STB), a distinct cytoplasmic structure located inside the HAP1 and recognized by the HAP1 antibodies (Chen et al., 2020; Fujinaga et al., 2007, 2004; Gutekunst et al., 1998; Islam et al., 2022, 2017, 2012; Xiang et al., 2014; Yanai et al., 2020). STB are tiny, non-membranous inclusion bodies with a diameter of 0.5 to 3 μm . They have a granular or fuzzy texture and a low-to-moderate electron density (Shinoda et al., 1993, 1992). There are two primary isoforms of HAP1. The HAP1A and HAP1B, that have different C-terminal amino acid sequences (Fujinaga et al., 2007; Li et al., 1998). Experimental research has shown that HAP1A generates STB in the cytoplasm, whereas HAP1B is widely dispersed and may be sequestered into STB when co-expressed with HAP1A (Fujinaga et al., 2007; Li et al., 1998).

STB/HAP1 are significantly expressed in certain mouse brain areas such as the hypothalamus, medial amygdala, and preoptic area. However, some of the brain area such as the cerebellum, thalamus, and cerebral cortex have very little or no STB/HAP1 expression (Gutekunst et al. 1998; Fujinaga et al. 2004, 2007; Islam et al. 2012, 2017, 2020a, 2022, 2025; Chen et al. 2020; Yanai et al. 2020). Interestingly, the brain areas with low or no HAP1 expression are more vulnerable to neurodegeneration, while areas with high HAP1 expression seem to be protective (Fujinaga et al.

2004; Takeshita et al. 2006, 2011; Metzger et al. 2008; Islam et al. 2017, 2020a, 2025). The inverse relationship between HAP1 abundance and neuronal vulnerability led to the advancement of the "STB/HAP1 protection hypothesis," which postulates that HAP1 might protect the neurons against apoptotic and degenerative processes during stress (Fujinaga et al. 2004; Takeshita et al. 2006, 2011; Metzger et al. 2008; Islam et al. 2017, 2020a, 2025).

The physiological relevance of HAP1 in specific neuronal populations is currently unknown, despite the fact that its neuroprotective features are being acknowledged. HAP1 has been associated to gastrointestinal phenomenon, stress reaction, and sensory control, according to prior research (Dragatsis et al., 2004; Islam et al., 2023, 2017; Lin et al., 2010; Niu et al., 2011; Sheng et al., 2008, 2006). Additionally, cholinergic preganglionic autonomic neurons in the brainstem and spinal cord express HAP1, but somatic motor neurons lack this protein (Islam et al., 2022, 2017). According to this distribution pattern, HAP1 could help to protect certain cholinergic neurons from neurodegenerative stress.

Choline acetyltransferase (ChAT) is an enzyme that produces acetylcholine (ACh), the primary neurotransmitter of the cholinergic system (Witzemann, 2007). As a neuronal marker, ChAT provides an accurate confirmation of cholinergic neurons. The cholinergic system is essential for several brain functions, such as memory formation, attention, learning, arousal, and motor regulation (Hampel et al., 2018; Nagori et al., 2024). Cholinergic signaling influences behavioral states, processing of sensory input, and brain plasticity (Hampel et al., 2018; Nagori et al., 2024). Neurodegenerative illnesses such as PD, HD, and AD have been associated with instability or degeneration of neurons involved in cholinergic function (Eickhoff et al., 2022a; Pepeu and Grazia Giovannini, 2017).

In the human brain, basal forebrain cholinergic neurons (BFCNs) comprise the primary cholinergic output channel. The BFCNs include the certain area of brain such as the medial septum (MS), the vertical and horizontal limbs of the diagonal band of Broca (VDB, HDB), and the substantia innominata, basal part (SIB) (Meher et al., 2025b). Neurons of these are involved in regulating the cognitive functions such as memory and attention through a broad projection to the hippocampus and neocortex (Hu et al., 2016; Latina et al., 2018). BFCN degradation is linked to cognitive loss, concentration problems, and sleep disorders. These are the typical feature of AD (Gamage et al., 2020). Similarly, the degeneration of cholinergic neurons in SIB particularly in the nucleus basalis of Meynert (NBM) is associated with an elevated risk of dementia (Bohnen et al., 2022; He et al., 2023).

Another substantial cholinergic population is found in the caudate putamen (CPu, neostriatum). The large, aspiny striatal cholinergic interneurons are critical for reward processing, behavior development, and motor regulation (Ahmed et al., 2019). The ACh may integrate motor and cognitive information by influencing the activity of medium spiny neurons in the neostriatum (Woolf and Butcher, 1981). Moreover, dysfunction of the striatal cholinergic neurons has been associated with schizophrenia, dystonia, PD, and hypertension (Ahmed et al., 2019). Despite their importance, little is known about the neurochemical diversity of these neurons.

The habenula, a little epithalamic structure with two subnuclei, medial (MHb) and lateral (LHb), may also include cholinergic neurons. It is critical for motivation, reward processing, aversion, and mood modulation (Browne et al., 2018; Yang et al., 2018). In particular, the LHb connects limbic and midbrain regions as well as it influences monoaminergic systems implicated in stress and emotional behavior (Groos and Helmchen, 2024; Lecca et al., 2020; Mondoloni et al., 2022). A variety of neurotransmitters, neuropeptides, and calcium-binding proteins (CBPs) are characterized by the neurons in habenula which demonstrating the neurochemical diversity in the habenula. However, the neurochemical characteristics and connections of HAP1 and ChAT inside the habenula are yet unknown.

Neuropeptidergic and calcium-binding proteins are crucial indicators for defining neuronal heterogeneity. Cholecystokinin (CCK) and calcitonin gene-related peptide (CGRP) are two peptides that regulate eating habits, pain, and anxiety (Arzani et al., 2020; Iyengar et al., 2019). Calbindin, calretinin, and parvalbumin are the examples of calcium-binding proteins (CBP) that define interneuron subtypes with unique firing characteristics and calcium-buffering abilities (Pace et al., 2020; Parent et al., 1996; Qi et al., 2022a; Udakis et al., 2020; Wagatsuma et al., 2023). Calbindin offers calcium buffering and neuroprotection against excitotoxicity (Bezprozvanny and Mattson, 2008), while calretinin controls firing accuracy (Qi et al., 2022b). In GABAergic interneurons, parvalbumin permits fast-spiking activity while preserving neuronal oscillations and the equilibrium between excitation and inhibition (Udakis et al., 2020; Wagatsuma et al., 2023). Although HAP1 has been widely explored in the brainstem and hypothalamus, little is known about its distribution and neurochemical interactions in the caudate putamen, habenula, and basal forebrain. It is crucial to understand HAP1 distribution, because the HAP1 expressing brain areas may be more stable to neurodegenerative stress than the areas without HAP1. Co-expression of HAP1 with ChAT and other neurochemical markers may give essential information on neuronal protection and susceptibility in neurodegenerative diseases.

Based on the background, firstly we hypothesized that HAP1 is expressed in forebrain cholinergic nuclei and has particular immunohistochemical interactions with ChAT neurons. This hypothesis indicates that HAP1 has a neuroprotective function in the forebrain cholinergic system. Furthermore, it is believed that the neurochemical relationship between HAP1 and ChAT in the BFCN and CPu may contribute to the functional diversity of these regions. These relationships may further possess the factors associated with neurodegenerative and neurobehavioral disorders. Assuming the first hypothesis is correct, we further hypothesized that ChAT-immunoreactive (ChAT-ir) and HAP1-immunoreactive (HAP1-ir) neurons in the BFCN and CPu exhibit distinct neurochemical heterogeneity, which may be identified by their distinctive co-expression with neuropeptides and CBPs. HAP1 is also thought to have a neuroprotective and modulatory role since its co-expression with various neuropeptidergic and CBP markers. Lastly, according to previous investigations, HAP1 has been hypothesized to be predominantly expressed in certain habenula neuronal populations, where it may aid in maintaining complex modulatory actions and protecting neurons. It is anticipated that the distinct distribution of HAP1 in the habenula would be associated with specific neurochemical variations, suggesting its role in cellular specialization. Similar to this, it is believed that cholinergic (ChAT-expressing) neurons in the habenula exhibit neurochemical heterogeneity and may interact or co-localize with HAP1. Moreover, unique patterns that support the molecular structure and functional diversity of habenular circuits may be shown by the expression of HAP1 and ChAT with other neuronal markers such as nNOS, SP, CCK, CGRP, calbindin, calretinin, and parvalbumin. Therefore, in light of the above hypothesis the present study was formulated the following aims:

- **Aim 1:** to determine the expression, comparative distribution, and immunohistochemical relationships between HAP1 and ChAT in the adult mouse forebrain cholinergic nuclei.
- **Aim 2:** to investigate the neurochemical heterogeneity of ChAT-ir and HAP1-ir neurons in the adult mouse forebrain by determining the co-expression patterns of neuropeptides and CBPs in BFCN and CPu.
- **Aim 3:** to ascertain the expression, comparative distribution, and neurochemical properties of HAP1 and ChAT with different neuronal marker such as nNOS, SP, CCK, CGRP, calbindin, calretinin, and parvalbumin in the mouse habenula.

1.2. Rationale of this Study

As we are well known about the protective role of HAP1, especially its existence to certain brain areas leads to be protective that area against neurodegenerative disorders. However, the exact distribution and neurochemical properties of HAP1 in some of the brain regions are still unknown. Previous studies have focused on the brainstem and hypothalamus with little attention given to the key areas of the forebrain such as the caudate putamen, habenula and basal forebrain. Such regions are believed to be involved in memory formation, movement control, motivation and emotional output. However, all these brain functions are altered in conditions such as Parkinson's, Alzheimer's and Huntington's diseases.

ChAT is an important enzyme in the synthesis of acetylcholine, a neurotransmitter that is necessary for learning, attention, and mental function. Many neurodegenerative diseases are characterized by cholinergic neuronal loss or dysfunction that is detected by the ChAT as cholinergic neuronal marker. Therefore, examining the connection between HAP1 and ChAT may aid in understanding why some cholinergic neurons are less vulnerable to neurodegeneration than other neurons.

The molecular spectrum of neurons in various brain areas will be revealed through investigation of the interactions between HAP1 and ChAT as well as their relationship to other neurochemical markers (neuropeptides and calcium-binding proteins). Understanding this difference has a great impact on defining mechanisms that support neuronal survival and resilience. This study provides novel insights on the protective role of HAP1 in the brain, which could enhance our understanding of the adaptive response of the brain and its protection in certain diseases.

Understanding the selective vulnerability of neurons is of paramount importance for the development of the neuroprotective strategy. HAP1 may have the role in intracellular trafficking and thus reduce apoptotic signaling. however, the role of HAP1 in the cholinergic projections of the BFCN, CPu, and habenula remains unclear. Understanding these relationships may yield insight into the reasons why some neurons are stable and continue to function in spite of losing the neurons that normally cause neurodegeneration.

Moreover, the habenula and basal forebrain are central modulators of motivational control, reward processing and emotional behavior. Dysregulation in these brain regions is associated with psychiatric and cognitive disorders. The study on HAP1 and ChAT expression and co-localization with other neurochemical markers will help to provide a cellular level explanation for the functional resilience or susceptibility to neurodegeneration.

Finally, this research may serve as the basis for translational applications. The knowledge of the HAP1 and ChAT interactions will have implications for therapeutic strategies to enhance neuronal survival. It will also help to identify the populations who are vulnerable to neurodegenerative or any

psychiatric diseases. It can also be used to find potential biomarkers for early disease progression and treatment response.

1.3. Socioeconomic Importance of this Study

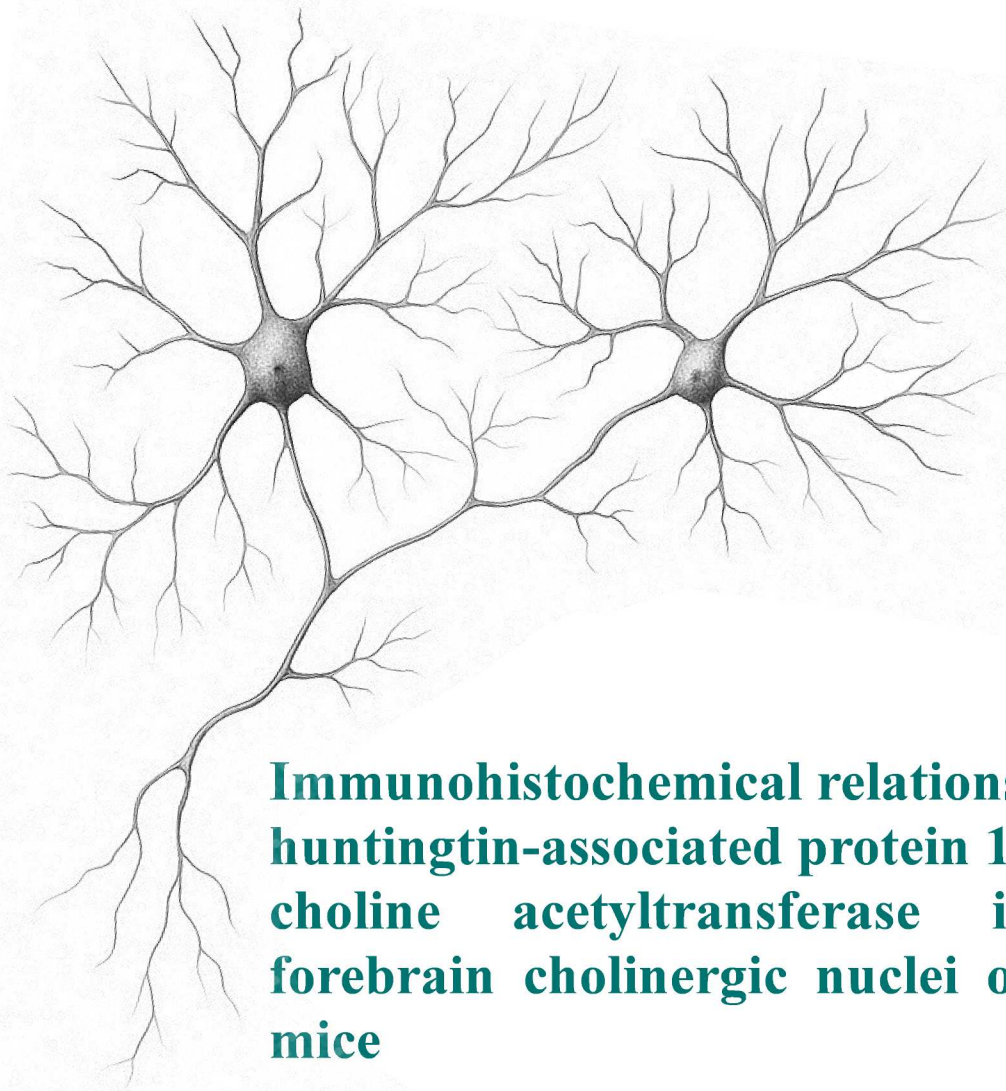
Now a days the neurodegenerative disorders including Alzheimer's, Parkinson's, and Huntington's are becoming more prevalent around the world. These diseases result in long-term incapacity, loss of independence, emotional breakdown, and economic challenges for patients, families as well as healthcare systems. The number of individuals who are affected by several ND have been increasing dramatically. Moreover, the medical treatment and social support systems are gradually under tremendous stress because of the higher proportion of aged population.

Comprehending the neuronal mechanisms of significant molecules such as ChAT or HAP1 might lead to novel approaches for the early detection, avoidance, and management of ND. Addressing similar pathways may lead to novel neuroprotective treatments if HAP1 aids in the resistance of certain neurons to degeneration. In addition to enhancing patients' quality of life, this would lower the long-term medical expenses related to chronic neurodegenerative diseases.

In a larger sense, this work builds upon the basis of neuroscience research by uncovering cellular and molecular patterns that promote brain health. This study may be the basic for the advanced strategies to mitigate or halt the development of ND.



Chapter 2



**Immunohistochemical relationships of
huntingtin-associated protein 1 with
choline acetyltransferase in the
forebrain cholinergic nuclei of adult
mice**



Abstract

Huntingtin-associated protein 1 (HAP1) is a core component of the stigmoid body (STB) and a neuroprotective interactor with the causative agents of several neurodegenerative diseases (NDs). The cholinergic system is often affected by NDs. Our previous studies suggest that cholinergic brainstem/spinal cord motoneurons are more vulnerable to neurodegeneration due to a lack of STB/HAP1 protectivity. The forebrain cholinergic nuclei are also major neurodegenerative or psychotic targets; however, the relationships of HAP1 with choline acetyltransferase (ChAT) have yet to be determined there. This study used western blotting and immunohistochemistry to evaluate the comparative distribution of HAP1 with ChAT and their immunohistochemical relationships in the adult mouse forebrain cholinergic nuclei. The results showed that HAP1-immunoreactive (ir) neurons were highly distributed in the basal forebrain cholinergic nuclei, including medial septal nucleus (MS), nucleus of vertical limb of the diagonal band of Broca (VDB), nucleus of horizontal limb of the diagonal band of Broca (HDB), and substantia innominata basal part (SIB). HAP1-ir neurons were sporadically scattered in the striatum. The substantial highest co-expression ratio of HAP1 with ChAT was observed in MS and VDB. In contrast, the ChAT-ir neurons never contained HAP1 in the caudate putamen of the striatum. These suggest that due to having putative HAP1 protectivity, the cholinergic neurons in MS and VDB that are mainly projected to the hippocampal/parahippocampal regions might be protected to regulate social memory formation, emotion, and other psychological functions. Consequently, the lack of HAP1 protectivity might make cholinergic neurons in the striatum more prone to neurodegeneration in certain NDs.

Keywords: *Cholinergic neurons; Forebrain; HAP1; Neurodegeneration; Psychotic stress; Striatum.*

2.1. Introduction

Huntingtin-associated protein-1 (HAP1) has recently emerged as a neuroprotective binding partner with causal agents of several neurodegenerative diseases (NDs). The stigmoid body (STB), a dot-like structure in the cytoplasm of neurons, is generally identified by HAP1 immunohistochemistry (Gutekunst et al. 1998; Fujinaga et al. 2004, 2007; Islam et al. 2012, 2017, 2022, 2025; Xiang et al. 2014; Chen et al. 2020; Yanai et al. 2020). STB was initially detected in our previous study by an antibody produced by the human placental antigen complex X-P2 and later defined as a spherical to ovoid shape (diameter of 0.5 - 3 μ m) of granulated to fuzzy, non-membranous inclusion bodies with a moderate to low electron density (Shinoda et al., 1993, 1992). HAP1 has two spliced isoforms, HAP1A and HAP1B, each featuring a distinct C-terminal sequence (Fujinaga et al., 2007; Li et al., 1998). Transfection of HAP1B cDNA can lead to diffuse cytoplasmic expression, while transfection of HAP1A cDNA can result in the formation of cytoplasmic STB in cultured cells. Interestingly, when co-expressed with HAP1A, HAP1B associates with HAP1A and is sequestered to STB from the early formation of small STB particles (Fujinaga et al., 2007).

HAP1 was first identified as a polyglutamine (polyQ) length-dependent interactor of huntingtin (HTT), the protein synthesized by the gene responsible for Huntington's disease (HD) (Gutekunst et al., 1998; Li et al., 1995). HTT can cause apoptosis and cell death when the polyQ structure gets longer, and HAP1 can stop the process of apoptosis by binding with polyQ HTT (Fujinaga et al., 2004; Li et al., 2003; Metzger et al., 2008). In addition, it is known that the polyQ androgen receptor-induced apoptosis in spinal and bulbar muscle atrophy (SBMA) can also be inhibited by HAP1 (Takeshita et al., 2006). Growing evidence shows that HAP1 participates in intracellular trafficking and binds with different proteins as part of cargo-motor molecules (Chen et al., 2023). The failure of intracellular trafficking is known to have a substantial impact on the development of NDs, including HD, SBMA, Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and spinocerebellar ataxia (SCA) (Chen et al., 2023). Likely, the dysfunction of HAP1 may account for certain NDs.

STB/HAP1 expression is highly present in the medial amygdala, preoptic area, and hypothalamic regions. However, in rodent brains, there is either no or minimal expression of STB/HAP1 in the cerebellum, thalamus, and cerebral neocortex (Gutekunst et al. 1998; Fujinaga et al. 2004, 2007; Islam et al. 2012, 2017, 2020a, 2022, 2025; Chen et al. 2020; Yanai et al. 2020). Interestingly, brain regions with higher STB/HAP1 expression seem resistant to neuronal cell death, whereas areas with low STB/HAP1 expression consistently become targets for neurodegeneration in

NDs. These findings lead to the idea that STB/HAP1 shields neurons against degeneration (Fujinaga et al. 2004; Takeshita et al. 2006, 2011; Metzger et al. 2008; Islam et al. 2017, 2020a, 2025).

Nevertheless, the precise physiological functions of HAP1 remain unclear. HAP1 has been reported to influence dietary patterns, stress responses, brain development, and even alterations in specific sensory processes (Dragatsis et al., 2004; Islam et al., 2023, 2017; Lin et al., 2010; Niu et al., 2011; Sheng et al., 2008, 2006). The results of our previous research indicate that STB/HAP1 might be involved in changes to specific cholinergic neuronal activity in the brainstem, as HAP1 is highly expressed in the cholinergic preganglionic autonomic neurons, laterodorsal tegmental nucleus (LDTg) and pedunculopontine tegmental nucleus (PPTg). However, the cholinergic motoneuron in the brainstem and spinal cord do not have HAP1 immunoreactivity (Islam et al., 2022, 2017). These suggest that because of lacking HAP1 protectivity, cholinergic motoneurons are more susceptible to neuronal degeneration than HAP1-expressing cholinergic neurons in preganglionic autonomic nuclei, PPTg and LDTg (Islam et al., 2022, 2017).

It is well known that in addition to the brainstem and spinal cord, acetylcholine (ACh) is also derived from the basal forebrain nuclei (Eickhoff et al., 2022b) that express choline acetyltransferase (ChAT). In the brain or spinal cord, cholinergic neurons are identified by the ChAT enzyme, which catalyzes the biosynthesis of the ACh (Witzemann, 2007). The degeneration or alteration of ChAT-immunoreactive (ir) neurons in various brain regions may be linked to several NDs, such as HD and ALS (Witzemann, 2007). Specifically, cognitive deficits related to AD and PD are caused by the deletion or alteration of ChAT-ir neurons in the forebrain nuclei (Pepeu and Grazia Giovannini, 2017). Moreover, reduced neurite arborization and decreased ChAT immunoreactivity have been associated with autism spectrum disorders, schizophrenia, and sudden infant death syndrome (Witzemann, 2007). ChAT is believed to play a pivotal role in fundamental brain processes such as learning, memory, awareness, and sleep (Witzemann, 2007). Given that ChAT in the forebrain regulates various brain functions and that the degeneration of ChAT-ir neurons can result in neurodegenerative and psychotic diseases, it is essential to clarify the role of HAP1 in forebrain cholinergic nuclei. To date, only an *in situ* hybridization study has sparingly determined the neuroanatomical distribution of HAP1 mRNA in the mouse forebrain (Fujinaga et al., 2004). However, due to the lack of comprehensive immunohistochemical analysis, the protective role of HAP1 in the forebrain cholinergic system remains uncertain. To address this, the present work utilized immunohistochemistry and Western blotting to determine the expression, comparative distribution, and immunohistochemical relationships between HAP1 and ChAT in the adult mouse forebrain cholinergic nuclei.

2.2. Materials and methods

2.2.1. Animals

Eight-week-old adult male mice (C57BL/6J) were obtained from Japan SLC Inc. in Shizuoka, Japan. While the onset of adulthood can be debated, 8 weeks is a standard threshold used to categorize mice as adults. Mice can reach sexual maturity and the ability to reproduce around 6-8 weeks of age (Jackson et al., 2017). The mice were kept in cages with a controlled temperature range of 22°C to 24°C. The mice were provided a standard pellet diet and water throughout the 12 h light-dark cycle (from 8:00 AM to 8:00 PM). The experimental methods used in this research were approved by the Committee on the Ethics of Animal Experimentation at Yamaguchi University School of Medicine and conducted in accordance with Japan's regulations for animal research (Law No. 105, Notification No. 6). For this study, a total of thirty mice were sacrificed. Six mice were utilized for Western blotting, while twenty-four were used for immunohistochemistry (fourteen mice for immunoperoxidase staining and ten for immunofluorescence staining). Every effort was made to minimize the suffering and the number of mice sacrificed. Mice were deeply anesthetized using a mixture of medetomidine chloride (Domitor, Orion Pharma, Espoo, Finland; 0.3 mg/kg), midazolam chloride (Midazolam, Sandoz, Basel, Switzerland; 4.0 mg/kg), and butorphanol tartrate (Vetorphale, Meiji Seika Pharma, Tokyo, Japan; 5.0 mg/kg). Each mouse received an injection of 0.25-0.30 ml of the anesthetic mixture intraperitoneally. After achieving a deep level of surgical anesthesia, indicated by the loss of pedal withdrawal and corneal reflexes, the mice were sacrificed via transcardial perfusion with either normal saline for Western blotting or 4% paraformaldehyde in 0.1 M phosphate buffer. (PB; pH 7.4) for immunohistochemistry. The perfusion was conducted using a peristaltic pump (AP-3300, AdVANTEC, Tokyo, Japan). The mice were then decapitated, and the whole brain was removed.

2.2.2. Sample preparation for Western blotting

Segments of the forebrain and brainstem were homogenized with T-PER tissue protein extraction reagent (78510; Thermo Scientific, Rockford, IL, USA) with 5 µl/ml of protease inhibitor (P8340; Sigma-Aldrich). The homogenization process was conducted on ice using a Portable Power Homogenizer (S-203, AS ONE Corporation, Osaka, Japan). The homogenates were subjected to centrifugation at 17,000 rpm for 30 minutes at a temperature of 4 °C. The supernatant was collected, and the BCA Protein Assay Kit (Pierce™, Thermo Scientific, Waltham, MA, USA) was employed to determine the protein content of each lysate.

2.2.3. Tissue preparation for immunohistochemistry

After collection, the brains were immersed in the same fixative agent overnight and kept in a 30% sucrose solution in 0.1 M PB for 5-7 d. Subsequently, the brain was frozen in powdered dry ice and placed in a cryostat (Leica CM1900, Germany) for the coronal sections at a thickness of 30 μ m.

2.2.4. Antibodies

The specifications and working dilutions of the primary antibodies are presented in Table 1, and the details of the secondary antibodies are described in Table 2.

Table 2.1. Detailed information on primary antibodies used in the present study

Antibody	Immunogen	Host/ Clonality	Code	Source	Dilution	Reference
Anti-HAP1 (R19)	Rat HAP1 C-terminus	Goat polyclonal	Cat# sc-8770, RRID: AB_647322	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:20,000	Tarif et al., 2021
Anti-HAP1 (EB07787)	Mouse HAP1 C-terminus	Goat polyclonal	Cat# EB07787, RRID: AB_2116122	Everest Biotech Ltd, Oxfordshire, UK	1:10,000	Islam et al., 2022
Anti-ChAT	Synthetic peptide within Pig Choline Acetyltransferase aa 150–250	Rabbit monoclonal	Cat# ab178850, RRID: AB_2721842	Abcam, Cambridge, UK	1: 5,000	Islam et al., 2022
Anti-ChAT	Human placental ChAT	Goat polyclonal	Cat# AB144P, RRID: AB_11214092	Millipore, Billerica, MA, USA	1:5,000	Islam et al., 2017
Anti-Iba1	Synthetic peptide corresponding to the C terminus of Iba1	Rabbit polyclonal	Cat# 019-19741, RRID: AB_839504	Wako, Osaka, Japan	1:1,000	Islam et al., 2022
Anti-GFAP	Purified human brain GFAP	Rabbit polyclonal	Cat# G9269, RRID: AB_477035	Sigma-Aldrich, St. Louis, MO, USA	1:1,000	Islam et al., 2022
Anti-NeuN	Synthetic peptide of Human NeuN aa 1–100	Rabbit Polyclonal	Cat# ab177487, RRID: AB_2532109	Abcam, Cambridge, UK	1: 5,000	Islam et al., 2022
Anti- α tubulin	Microtubule derived from chicken embryonic brain	Mouse monoclonal	Cat# T6199, RRID: AB_477583	Sigma-Aldrich, St. Louis, MO, USA	1:200,000	Islam et al., 2022

Table 2.2. Detailed information on secondary antibodies used in the present study

Antibody	Immunogen	Host	Code/Source	Application	Dilution	References
HRP-labelled anti-goat	Goat IgG, F(ab) 2	Donkey	Cat# 3851, RRID: AB_641200 Santa Cruz Biotechnology, Santa Cruz, CA, USA	Western blotting	1:10,000	Islam et al., 2022
HRP-labelled anti-rabbit	Rabbit whole antibody	Donkey	Cat# NA934V, Healthcare Life Sciences, Buckinghamshire, UK	Western blotting	1:5000	Islam et al., 2022
HRP-labelled anti-mouse	Mouse IgG whole molecule	Sheep	Cat# NA931, RRID: AB_772210GE Healthcare, Buckinghamshire, UK	Western blotting	1:20,000	Islam et al., 2022
Biotinylated donkey anti-goat	IgG, isolated from goat serum	Donkey	Cat# AP180B, RRID: AB_92569 Millipore, Darmstadt, Germany	Immunoperoxidase staining	1:1,000	Islam et al., 2022
Biotinylated donkey anti-rabbit	IgG, isolated from rabbit serum	Donkey	Cat# E0432, RRID: AB_2313609, Dako, Denmark	Immunoperoxidase staining	1:1,000	Jahan et al., 2015
Alexa Fluor 594 donkey anti-goat	IgG, isolated from goat serum	Donkey	Cat# A-11058, RRID: AB_2534105 Invitrogen, Eugene, OR, USA	Immunofluorescence staining	1:1,000	Yanai et al., 2020
Alexa Fluor 488 donkey anti-rabbit	IgG, isolated from goat serum	Donkey	Cat# A32790, RRID: AB_2762833 Invitrogen, Eugene, OR, USA	Immunofluorescence staining	1:1,000	Yanai et al., 2020
HRP-conjugated streptavidin	N/A	N/A	Cat# P0397, Agilent Dako, Glostrup, Denmark	Immunoperoxidase staining	1:1,000	Islam et al., 2012

2.2.5. Western blotting

The Western blotting was performed following our previous studies (Islam et al. 2017, 2022). In summary, 80 µg of each protein was loaded and separated using 7.5% SDS-polyacrylamide gel electrophoresis. After that, it was transferred onto a polyvinylidene difluoride membrane. Then, 5% skimmed milk was used to block the membrane for 1 h at 20°C after being washed in Tris-buffered saline containing 0.1% Tween-20 (TBST). The samples were incubated overnight at 4°C in the goat polyclonal anti-HAP1 R19 (1:20,000), rabbit monoclonal anti-ChAT (1:5,000), or mouse monoclonal anti- α tubulin (1:200,000) primary antibody (Table 1). A specific blocking peptide was added to the diluted primary antibody (HAP1 R19), and it was incubated overnight at 4°C for the HAP1 pre-adsorption test. After washing with TBST, the membrane was incubated for 2 h at 20°C with the horseradish peroxidase-linked anti-goat (1:10,000), anti-rabbit (1:5,000) or anti-mouse IgG (1:20,000) secondary antibody (Table 2). Following three rounds of washing with TBST, protein bands were examined using enhanced chemiluminescence reagents (ECL select, GE Healthcare), and images were captured using an Amersham Imager 600 (GE Healthcare). ImageJ software (NIH, Bethesda, MD, USA) was used to determine the relative optical density ratio of the HAP1-ir/ α -tubulin and ChAT-ir/ α -tubulin bands.

2.2.6. Immunoperoxidase staining

Single immunoperoxidase immunohistochemical protocol was adapted from our previous studies (Islam et al. 2020b, 2023; Jahan et al. 2015; Wroblewski et al. 2018). In summary, free-floating forebrain sections were blocked with 0.3% Triton X-100-containing 10% normal donkey serum or 10% normal goat serum. After that, the sections were pretreated with 1.5% hydrogen peroxide and 50% methanol. Next, goat anti-HAP1 R19 (1:20,000) or rabbit anti-ChAT (1:5000) primary antibody was applied (Table 1) for five days at 20°C. A specific blocking peptide (sc-8770P, Santa Cruz Biotechnology, Santa Cruz, CA, USA) against the HAP1 R19 antibody was added to the diluted primary antibody overnight at 4°C for the HAP1 pre-adsorption test. Then, the sections were incubated for 3 h at 20°C with either biotinylated donkey anti-goat or goat anti-rabbit secondary antibody (Table 2). Then, the sections were incubated for 2 h at 20°C with peroxidase-conjugated streptavidin (P0397, Dako, Glostrup, Denmark). A nickel-enhanced diaminobenzidine (DAB; Wako Pure Chemical Industries, Ltd., Osaka, Japan) reaction was conducted to stain the sections for 10 to 20 min at 4°C. Subsequently, the sections were mounted on glass slides, allowed to air dry for half an hour, dehydrated using ascending grades of alcohol and xylene solutions, and then embedded with Entellan Neu. (Merck KGaA, Darmstadt, Germany).

2.2.7. Double-label immunofluorescence staining

The immunofluorescence immunohistochemistry approach was adapted from previously published works (Jahan et al. 2015; Islam et al. 2017, 2025; Yanai et al. 2020). In brief, 10% NDS containing 0.3% Triton X-100 was added to the free-floating brain sections for 2 to 3 h at 20°C for blocking. Next, the sections were incubated with either a combination of goat anti-HAP1 R19 (1:20,000) and rabbit anti-ChAT (1: 5,000) primary antibodies or goat anti-HAP1 R19 antibody (1:20,000) in combination with rabbit anti-GFAP (1:1,000), rabbit anti-Iba1 (1:1,000) or rabbit anti-neuronal nuclei (NeuN; 1:5,000) primary antibodies for 5 days at 20°C diluted with 1% NDS in PBS with 0.3% Triton X-100 (Table 1). Following washing, brain sections were then incubated for 3 h at 20°C with appropriate species-specific secondary antibodies [donkey anti-goat conjugated to Alexa 594 (A11058) or donkey anti-rabbit conjugated to Alexa 488 (A32790)] (Table 2). PBS with 1% NDS was used to dilute the secondary antibodies. After washing with PBS, the sections were placed on glass slides, permitted to air dry for half an hour, and then coverslipped with Fluoromount/Plus (K048, Diagnostic Biosystems, Pleasanton, CA, USA).

2.2.8. Photomicrographs

Photomicrographs for immunoperoxidase staining images were taken using a color digital USB 2.0 camera (Lumenera Corporation, Ottawa, Canada) equipped with an Eclipse E80i photomicroscope (Nikon). Immunofluorescence photomicrographs were obtained using a 20x and 40x objective on a confocal microscope (Leica Stellaris-8 CRS, Wetzlar, Germany). Subsequently, the images were imported into Adobe Photoshop Elements 2021 (Adobe Systems, Inc., San Jose, CA, USA).

2.2.9. Tissue analyses and mapping of STB/HAP1 or ChAT-ir cells

The distribution of HAP1 or ChAT-ir neurons in the mouse forebrain was ascertained using coronal sections with 30 µm thicknesses typically ranging from bregma 3.92 mm to -1.58 mm. Using Nissl stain on adjacent sections, the specific locations of HAP1 or ChAT-ir cells in the forebrain cholinergic nuclei were identified. The data were presented in tabular form, where –, no HAP1 or ChAT immunoreactivity; +/-, weak HAP1 or ChAT immunoreactivity; +, weak to moderate HAP1 or ChAT immunoreactivity; ++, moderate to strong HAP1 or ChAT immunoreactivity; +++, strong HAP1 or ChAT immunoreactivity (Table 3).

2.2.10. Cell counting

Immunofluorescence images were captured using a 20x objective and subsequently imported into the ImageJ program (NIH, Bethesda, MD, USA) to count HAP1 or ChAT-ir neurons. The precise count of HAP1 or ChAT-ir neurons, along with those neurons double-labeled for HAP1 with ChAT, was used to ascertain the co-expression ratios of HAP1 in ChAT or ChAT in HAP1-ir neurons, employing the counting methodology established in our prior research (Jahan et al. 2015; Islam et al. 2017, 2020a, 2025). The HAP1 or ChAT-ir cells were counted in the caudate putamen nuclei (CPu; approximately bregma 1.54 mm to -1.58 mm), nucleus accumbens core (AcbC; approximately bregma 1.34 mm to 0.62 mm), olfactory tubercle (Tu; approximately bregma 2.10 mm to 0.74 mm), medial septal nucleus (MS; between bregma 1.18 mm and 0.74 mm), substantia innominata basal part (Meynert nucleus) (SIB; around bregma 0.62 mm to -0.46 mm), ventral pallidum (VP; around bregma 1.78 mm to 0.26 mm), magnocellular preoptic nucleus (MCPO; around bregma 0.50 mm to -0.34 mm), the nucleus of the horizontal limb of the diagonal band of Broca (HDB; around bregma 0.86 mm to -0.34 mm), the nucleus of the vertical limb of the diagonal band of Broca (VDB; approximately bregma 1.34 mm to 0.74 mm), the lateral septal nucleus (LS; approximately bregma 1.18 mm and 0.14 mm), and the habenular nucleus (Hb; around bregma -0.94 mm to -1.58 mm). Four brain sections from a single mouse were used for each location, maintaining a gap of at least 60 μ m between sections. A total of six mice were employed for cell counting.

2.2.11. Statistical analysis

The Statistical Package for Social Sciences (SPSS), version 25.0, was used to examine all of the data. For the purpose of computation (mean, standard error of mean) and graphical representation, the relative band optical density ratio data for the HAP1-ir/ α -tubulin or ChAT-ir/ α -tubulin band were imported into MS Excel (Microsoft Office Excel 2019, USA). After that, the data were moved to SPSS v. 25, and one-way Analysis of Variance (ANOVA) was carried out, then the post-hoc test (Tukey's test) was used to compare the means of HAP1/ChAT ratio and ChAT/HAP1 ratio in different regions of the mouse forebrain. The student's t-test was used to compare the HAP1/ChAT ratio and ChAT/HAP1 ratio in the same brain areas. Conversely, ANOVA followed by post-hoc test (Tukey's test) was used to determine the significance of HAP1/ α -tubulin and ChAT/ α -tubulin in different segments of the mouse forebrain. For every test, p-values of less than 0.05 were deemed significant.

2.2.12. Terminology

The present work used terminology outlined in a mouse brain atlas ([Franklin and Paxinos, 2008](#)).

2.3. Results

2.3.1. The general expression of HAP1 and ChAT in the mouse forebrain

The expression of HAP1 and ChAT immunoreactivity in the forebrain was determined by immunohistochemistry and Western blotting. The two isoforms of HAP1 (HAP1A and HAP1B) were detected as around 80 kDa and 90 kDa, respectively, in different segments of the forebrain, including basal forebrain nuclei, CPu, and cortex (**Figure 2.1A**). The expressions of HAP1 were considerably lower in CPu and cortex than other forebrain areas and control (brainstem). The HAP1 antibody with the blocking peptide led to a substantial decrease of HAP1-ir bands in the pre-adsorption test (**Figure 2.1A**). In the basal forebrain nuclei, CPu, and cortex, ChAT-ir bands were observed as about 60 kDa where the expression was a little bit higher in basal forebrain nuclei than other areas (**Figure 2.1A**). The quantitative assessment of the relative strength of HAP1 and ChAT (ratio of HAP1 and ChAT to the α -tubulin) using Western blots established that there were significant ($p < 0.01$) differences in HAP1 immunoreactivity in different segments of the mouse forebrain, with the highest in BFCN and the lowest in CPu. However, there were no significant ($p > 0.05$) differences in ChAT immunoreactivity in different segments of the mouse forebrain (**Figure 2.1B**).

By employing immunohistochemistry, HAP1 immunoreactivity was also evident in the septal area of the forebrain (**Figure 2.1C**). In this study, cells exhibiting HAP1 immunoreactivity typically displayed distinct HAP1-ir STB in their diffusely immunopositive cytoplasm (**Figure 2.1D**). However, certain HAP1-ir cells only revealed a diffuse HAP1 immunoreactivity in the cytoplasm, lacking any clearly discernible STB (**Figure 2.1D**). The pre-adsorption test significantly reduced HAP1 immunoreactivity by using a specific blocking peptide against the anti-HAP1 primary antibody (**Figure 2.1E**).

Double-label immunofluorescence immunohistochemistry for HAP1 and GFAP (a marker for astrocytes), Iba1 (a marker for microglial cells), or NeuN (a marker for nuclear neurons) was carried out to recognize the type of HAP1-ir cells in the cholinergic nuclei of the mouse forebrain. Though the HAP1-ir cells did not colocalize with GFAP (**Figure 2.1F-H**) or Iba1 (**Figure 2.1I-K**), a large number of HAP1-ir cells had the NeuN immunoreactivity (**Figure 2.1L-N**). These results indicated that the HAP1-ir cells in the forebrain exhibited neuronal properties but were devoid of any glial cell characteristics.

2.3.2. Comparative distribution of HAP1 and ChAT-ir neurons in the mouse forebrain

The immunohistochemical distribution of HAP1 and ChAT-ir neurons was examined throughout the forebrain regions. Adjacent coronal sections were analyzed by a series of mappings to compare the regional distribution of HAP1 and ChAT-ir neurons throughout the rostrocaudal extent

of the forebrain cholinergic nuclei of adult mice (**Figure 2.2A-F, 2.3A-H, 2.4A-H, 2.5A-H**) and summarized in Table 3.

The basal ganglia are situated at the base of the forebrain and consist of a group of sub-cortical nuclei ([Lanciego et al., 2012](#)), which include the striatal and pallidal components. The striatum is made up of a dorsal or neostriatum section, known as the CPu, and a ventral section formed by the AcbC along with several associated cell groups, such as the Tu and the islands of Calleja (ICj). The pallidum comprises the dorsal pallidum or globus pallidus (GP) and the VP ([Medina and Abellán, 2012; Voorn et al., 2004](#)). HAP1-ir neurons were sporadically scattered in the CPu (**Figure 2.6A**) and AcbC (**Figure 2.6B**) of the dorsal and ventral striatum, respectively. Conversely, elongated and fusiform multipolar intensely stained ChAT-ir neurons were present throughout the CPu (**Figure 2.6D**) and AcbC (**Figure 2.6E**). In the Tu, weakly stained HAP1-ir neurons were located in the ventral digitations of the pyramidal cell layer (**Figure 2.6C**). In contrast, ChAT-ir neurons were moderately expressed throughout the pyramidal and polymorph cell layers of the Tu (**Figure 2.6F**). ICj were primarily restricted to the Tu, which exhibited no ChAT immunoreactivity (**Figure 2.2F, 3E-H; 4E**), although scattered HAP1-ir neurons were present there (**Figure 2.2D, 3A-D; 4A**).

The basal forebrain cholinergic nuclei (BFCN) have been classified into four distinguished cell categories such as (i) MS, (ii) VDB, (iii) HDB, and (iv) basal magnocellular complex that consists of the SIB ([Ma and Geyer, 2018](#)), the MCPO, and the VP ([Ballinger et al., 2016](#)). In the BFCN, strongly stained HAP1-ir neurons were observed in the MS (**Figure 2.7A**) and VDB (**Figure 2.7B**). Moderate to strongly stained HAP1-ir neurons were detected in the HDB (**Figure 2.7C**), and SIB (**Figure 2.7D**). Elongated and larger-sized strongly stained ChAT-ir neurons were present in the MS (**Figure 2.7E**), VDB (**Figure 2.7F**), HDB (**Figure 2.7G**), and SIB (**Figure 2.7H**). In the nucleus of the diagonal band of Broca (VDB, HDB), both HAP1 and ChAT-ir neurons were found to lie along the fibers of the diagonal band and were continuous with those in the MS.

The forebrain pallidum comprises the GP and VP. The VP exhibited moderate HAP1-ir neurons (**Figure 2.8A**), while strongly stained multipolar ChAT-ir neurons (**Figure 2.8C**) were present in the VP. In contrast, the GP lacked STB/HAP1 and ChAT-ir neurons (**Figure 2.4D, 2.4H; 2.5A-H**). Additionally, a group of magnocellular neurons in the MCPO displayed moderate staining for HAP1 (**Figure 2.8B**), but demonstrated strong immunoreactivity for ChAT (**Figure 2.8D**).

The LS is situated in the rostradorsal septal region, just medial to the lateral ventricles. The LS is divided into three main parts: lateral septum dorsal part (LSD), lateral septum ventral part (LSV),

and lateral septum intermediate part (LSI). Interestingly, the LSD had no ChAT-ir neurons (**Figure 2.3F-H; 2.4E-H**) but contained very few HAP1-ir neurons (**Figure 2.3B-D; 2.4A-D**). Numerous highly stained HAP1-ir neurons were found throughout the LSV (**Figure 2.9A**) and LSI (**Figure 2.9B**). In comparison, the LSV (**Figure 2.9D**) and LSI (**Figure 2.9E**) displayed a moderate number of ChAT-ir cells.

The Hb subdivision is typically described as the habenular complex, which consists of two primary components: the lateral habenular nucleus (LHb) and the medial habenular nucleus (MHb). Within the Hb, HAP1-ir neurons were prominently expressed in the LHb, particularly at the edge between the LHb and MHb (**Figure 2.9C**). The HAP1-ir neurons were utterly absent in the MHb (**Figure 2.9C**). In contrast, the ChAT immunoreactivity of the Hb differs from that of HAP1. Strongly stained ChAT-ir neurons were found in the MHb while lacking in the LHb (**Figure 2.9F**).

Next, to clarify the immunohistochemical relationships of HAP1 with ChAT-ir neurons, we conducted double-label immunofluorescence staining for HAP1 and ChAT in the cholinergic nuclei of forebrain regions where both HAP1 and ChAT-ir neurons were evident, including the basal ganglia, BFCN, LS, and Hb.

Table 2.3. Expression of STB/HAP1 and ChAT in the different location in mouse forebrain

Category	Area	Intensity of expression	
		HAP1	ChAT
Basal ganglia	Caudate Putamen (CPu)	+/-	+++
	Nucleus accumbens core (AcbC)	+	++
	Olfactory tubercle (Tu)	+	++
	Medial septal nucleus (MS)	+++	+++
Forebrain cholinergic nuclei	Vertical limb of the diagonal band of Broca (VDB)	+++	+++
	Horizontal limb of the diagonal band of Broca (HDB)	++	+++
	Substantia innominata basal part (Meynert nucleus; SIB)	+++	+++
	Ventral pallidum (VP)	++	+++
Lateral septal nucleus	Magnocellular preoptic nucleus (MCPO)	++	+++
	Lateral septal nucleus dorsa part (LSD)	+/-	-
	Lateral septal nucleus ventral part (LSV)	+++	++
	Lateral septal nucleus intermediate part (LSI)	+++	++
Habenular nucleus	Medial Habenular nucleus (MHb)	-	+++
	Lateral Habenular nucleus (LHb)	+++	-

(- indicates no expression, +/- indicates weak, + indicates weak to moderate, ++ indicates moderate to strong, and +++ indicates strong expression).

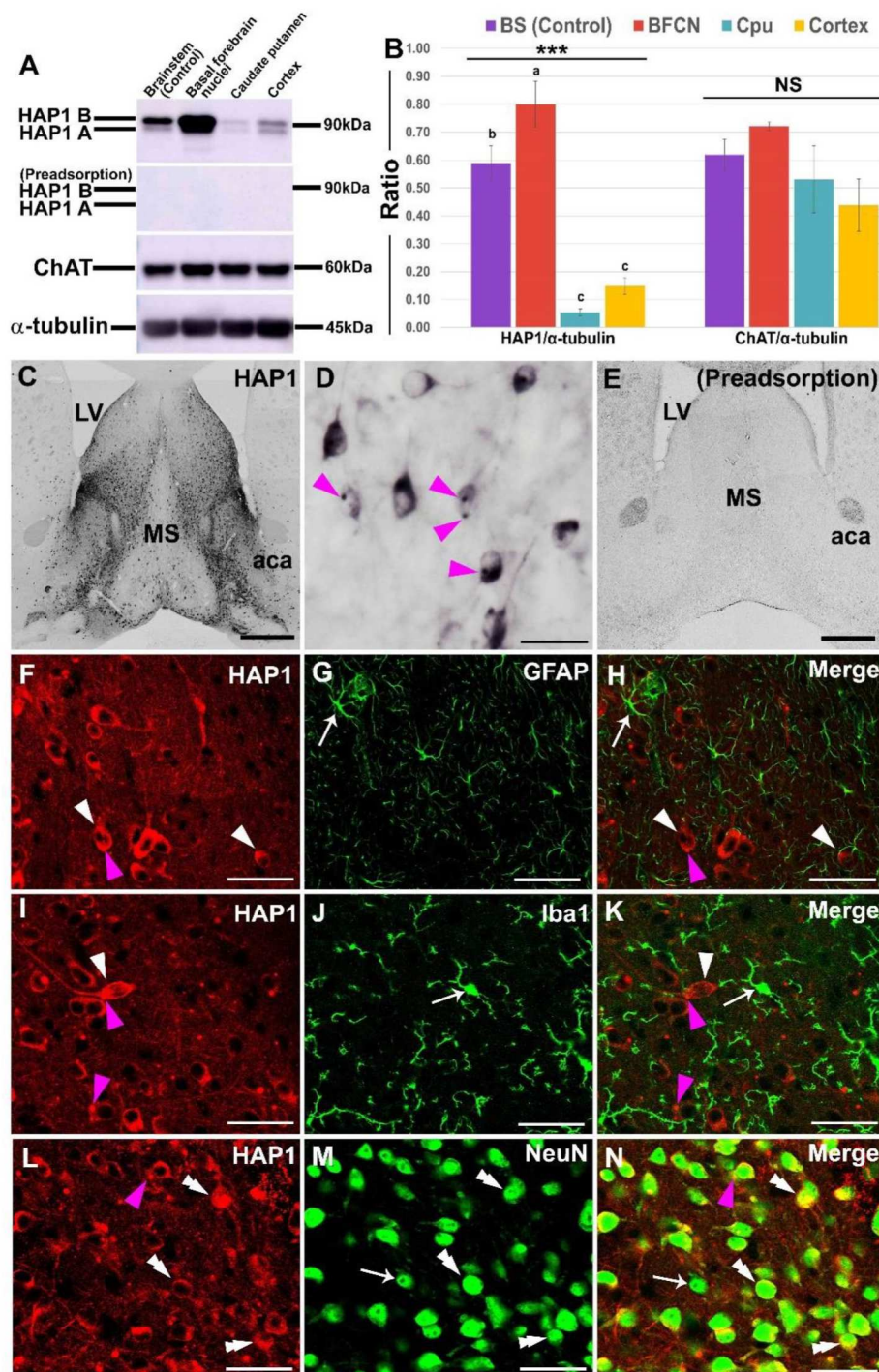


Figure 2.1. Western blotting, immunoperoxidase and double-label immunofluorescence immunohistochemistry for HAP1 with Glial or Neuronal markers. (A) Western blot (WB) analysis showing the HAP1 and Chat immunoreactive (ir) bands; Pre-adsorption of the anti-HAP1 antibody resulted in the disappearance of HAP1-ir bands. α -tubulin was used for loading control. (B) The ratios (mean) of HAP1 and ChAT-ir bands to α -tubulin on the Western blots ($n=3$); ^{a,b,c} denotes different superscript letters differ significantly; **, $p<0.05$. (C) Immunoperoxidase staining showing the presence of HAP1 immunoreactivity in the forebrain. Figure "D" is the enlargement of * in Figure "C", indicating the HAP1-ir stigmoid body (STB). (E) Preincubation with a blocking peptide against the anti-HAP1 antibody eliminated the HAP1 immunoreactivity. Photomicrograph showing double-label immunofluorescence staining of HAP1 and GFAP (f-h), Iba1 (i-k), or NeuN (l-n) in the forebrain. Double arrowheads indicate the cells positive for both HAP1 and NeuN. Single white arrowheads indicate cells single-positive for HAP1. White arrows indicate cells single-positive for GFAP, Iba1, and NeuN. Pink arrowheads indicate HAP1-immunoreactive (ir) STB. Scale bar = 50 μ m in "C" and "E-N"; scale bar = 20 μ m in "D". BS, brainstem; BFCN, basal forebrain cholinergic nuclei; CPu, Caudate Putamen (striatum); GFAP, Glial fibrillary acidic protein; HAP1, Huntingtin-associated protein 1; Iba1, Ionized calcium-binding adaptor molecule 1; NeuN, Neuronal Nuclei; STB, stigmoid body.

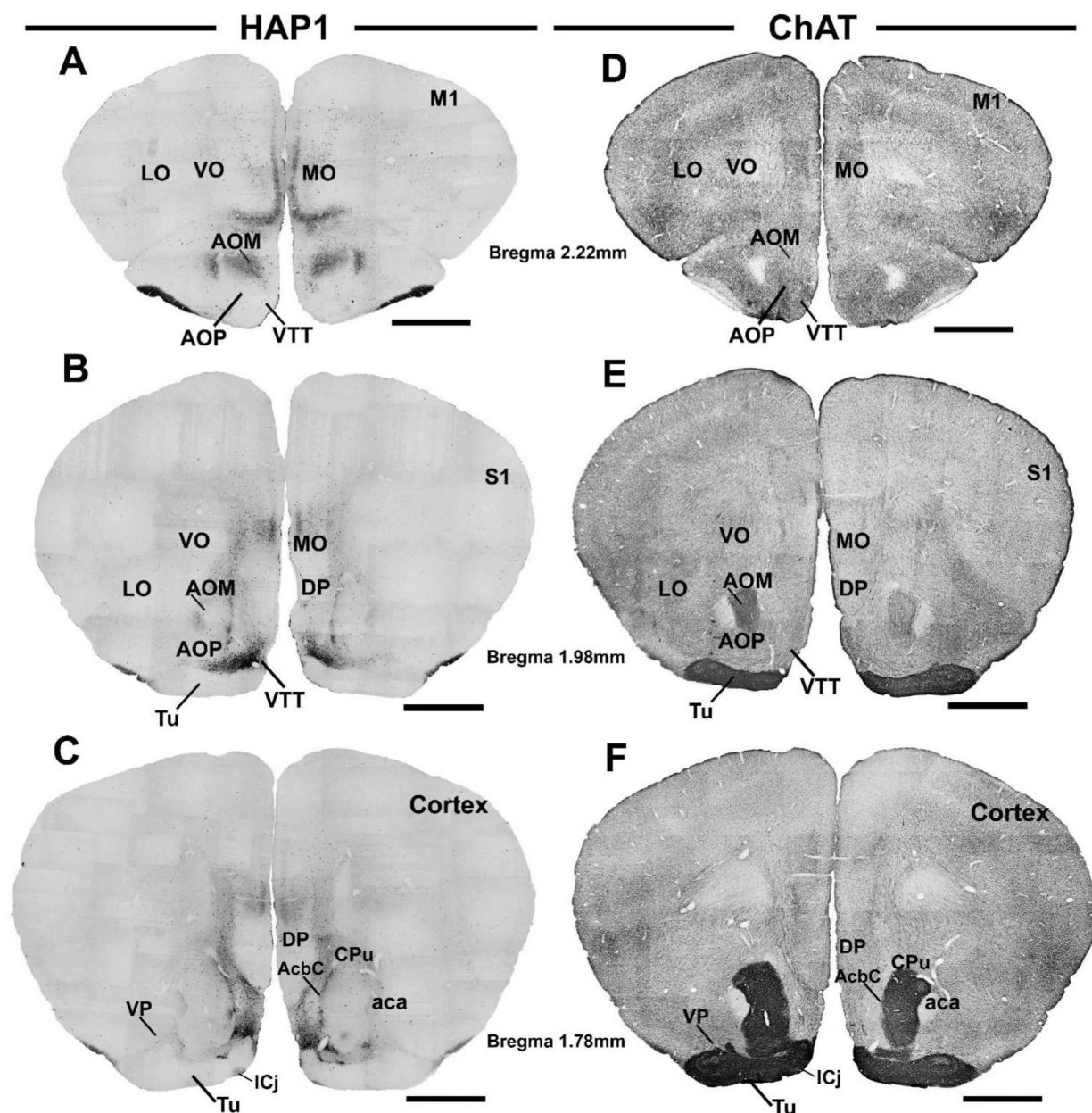


Figure 2.2. HAP1 and ChAT immunoreactivity in the forebrain of the mouse (Bregma 2.22mm to 1.78mm). Photomicrographs showing the HAP1-ir cells (A-C) and ChAT-ir cells (D-F) are distributed throughout the forebrain. Scale bar = 500µm. For details on abbreviations, see Abbreviations.

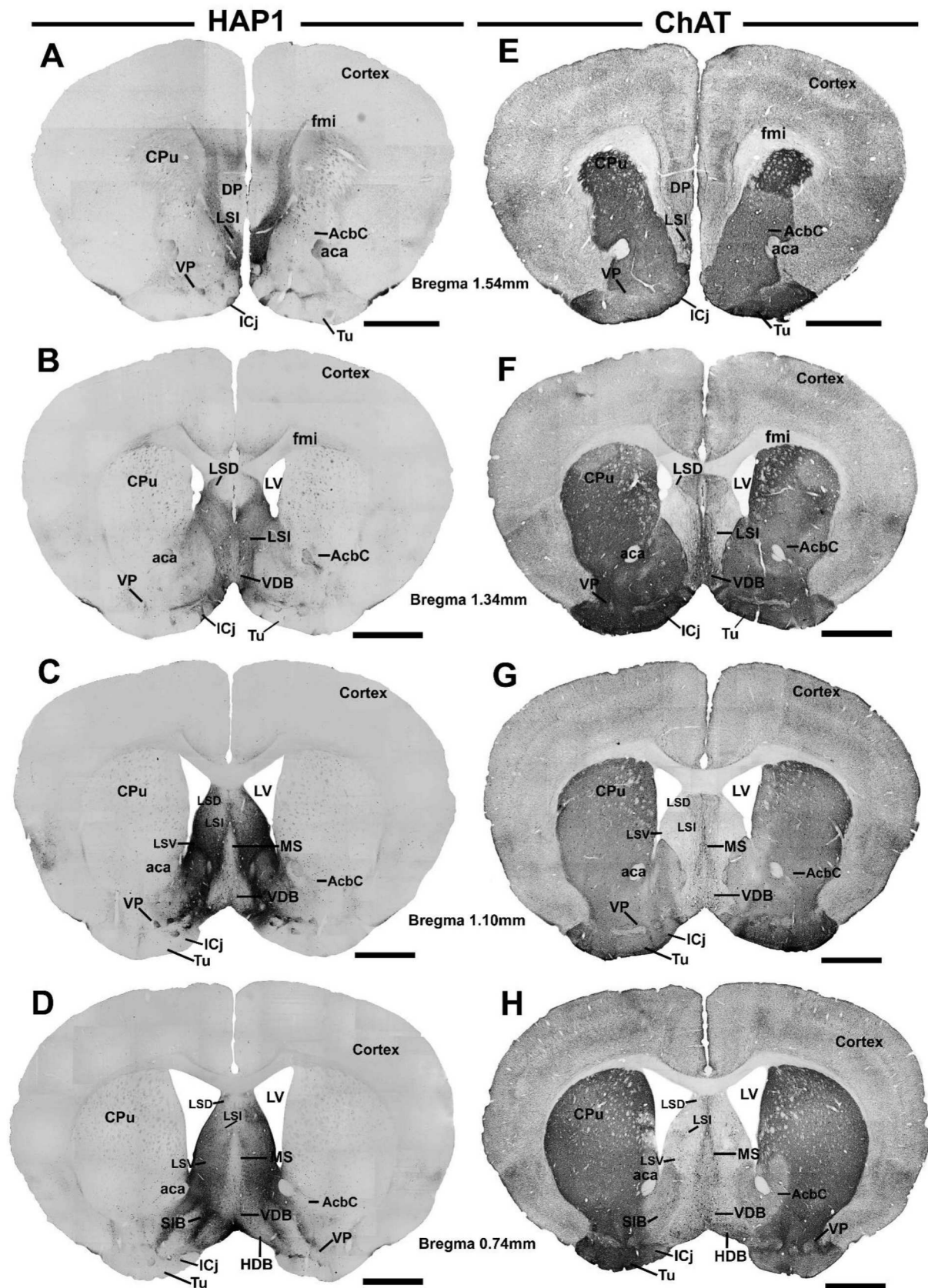


Figure 2.3. HAP1 and ChAT immunoreactivity in the forebrain of the mouse (Bregma 1.54mm to 0.74mm). Photomicrographs showing the HAP1-ir cells (A-D) and ChAT-ir cells (E-H) are distributed throughout the forebrain. Scale bar = 500µm. For details on abbreviations, see Abbreviations.

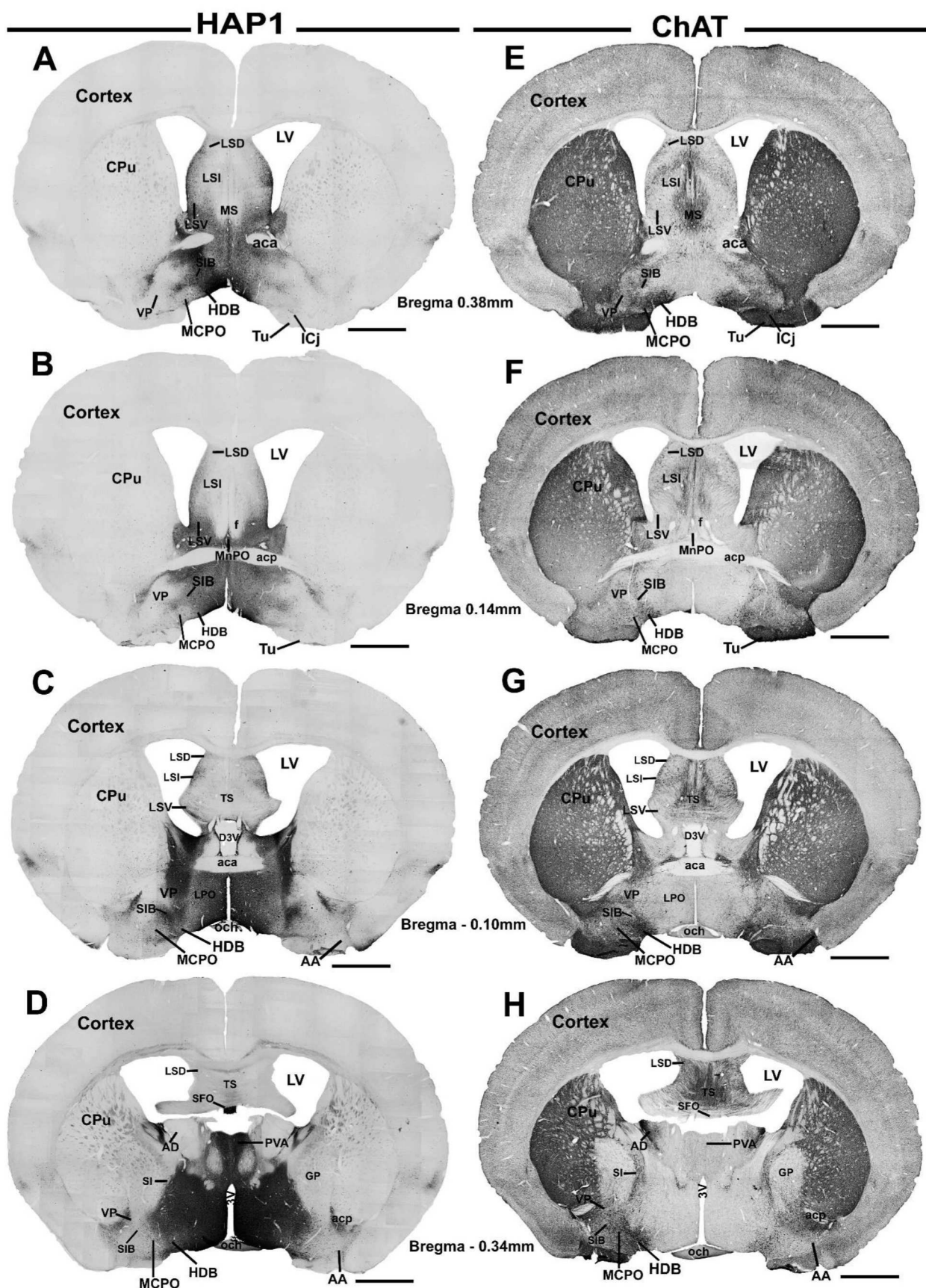


Figure 2.4. HAP1 and ChAT immunoreactivity in the forebrain of the mouse (Bregma 0.38mm to - 0.34mm). Photomicrographs showing the HAP1-ir cells (a-d) and ChAT-ir cells (e-h) are distributed throughout the forebrain. Scale bar = 500µm. For details on abbreviations, see Abbreviations.

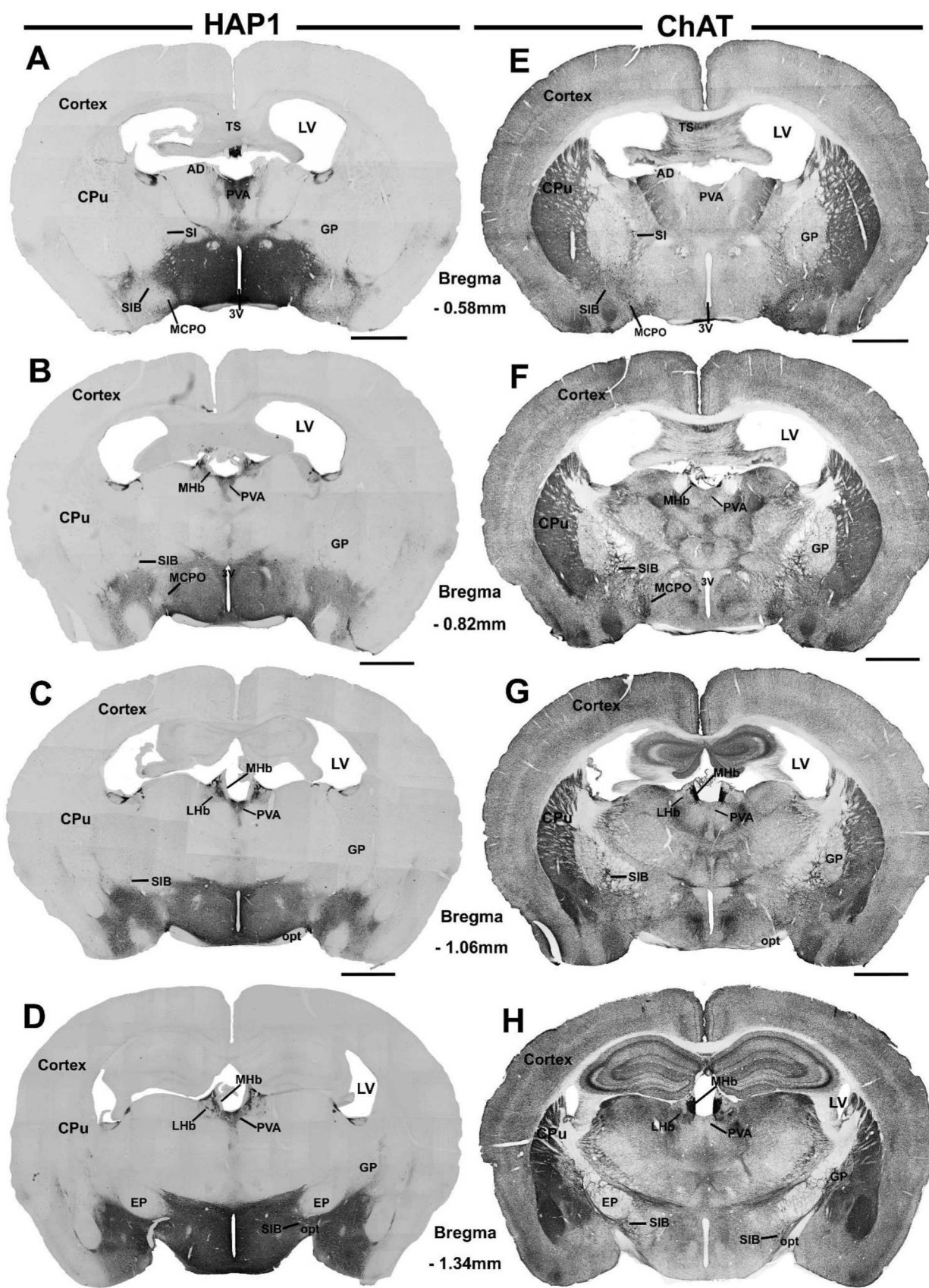


Figure 2.5. HAP1 and ChAT immunoreactivity in the forebrain of the mouse (Bregma -0.58mm to -1.34mm). Photomicrographs showing the HAP1-ir cells (A-D) and ChAT-ir cells (E-H) are distributed throughout the forebrain. Scale bar = 500µm. For details on abbreviations, see Abbreviations.

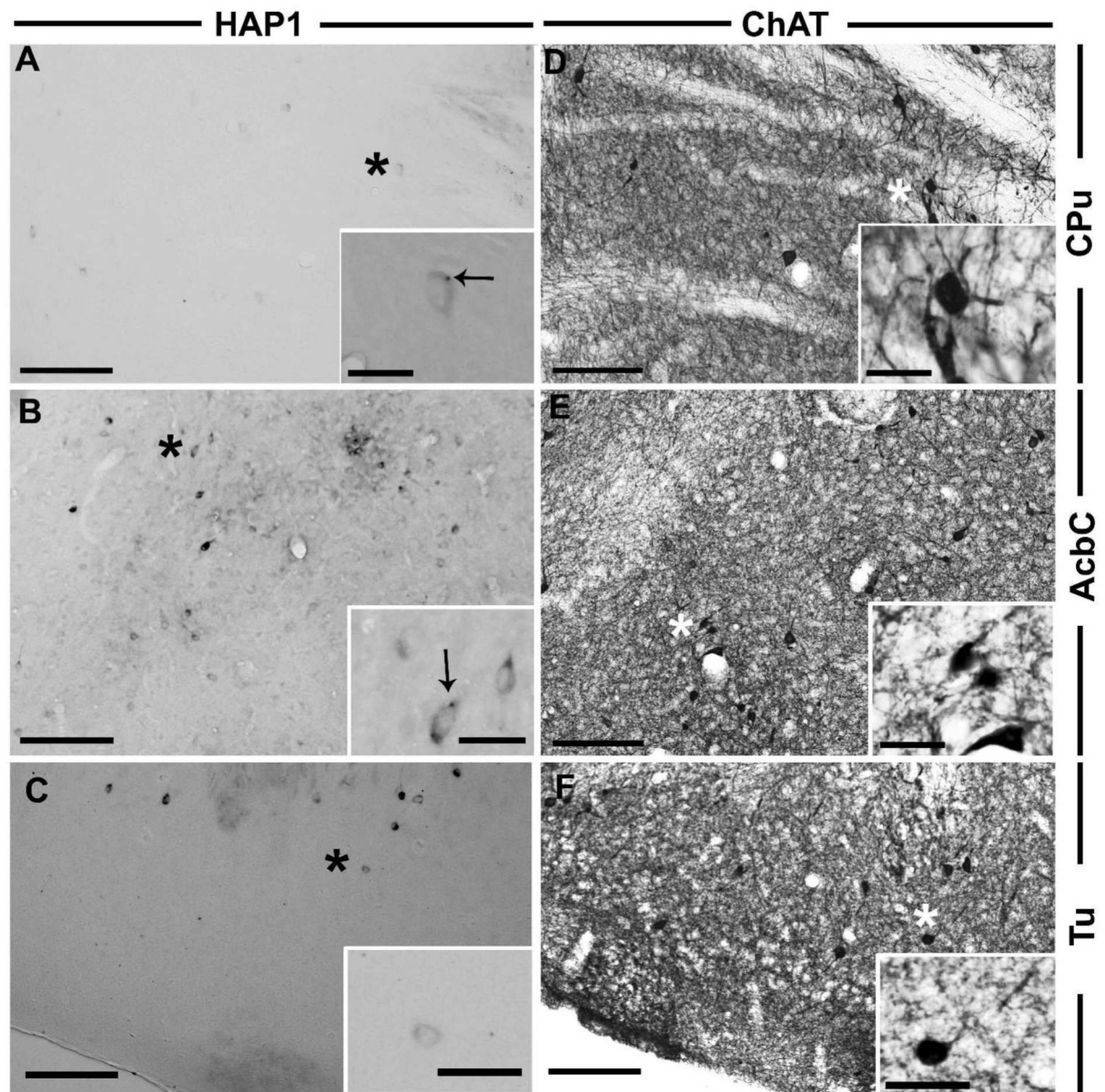


Figure 2.6. HAP1 and ChAT immunoreactivity in the basal ganglia (dorsal and ventral striatal nuclei with associated cell group). “A-C” shows the HAP1-ir cells in CPu, AcbC and Tu. “D-F” indicates the ChAT-ir cells in CPu, AcbC and Tu. Insets are the enlargement of the HAP1-ir and ChAT-ir cells indicated by asterisks, and the black arrow indicates the HAP1-ir STB. Scale bar = 100µm in “A-F” and 20 µm in inset. AcbC, nucleus accumbens core; CPu, caudate putamen; and Tu, olfactory tubercle.

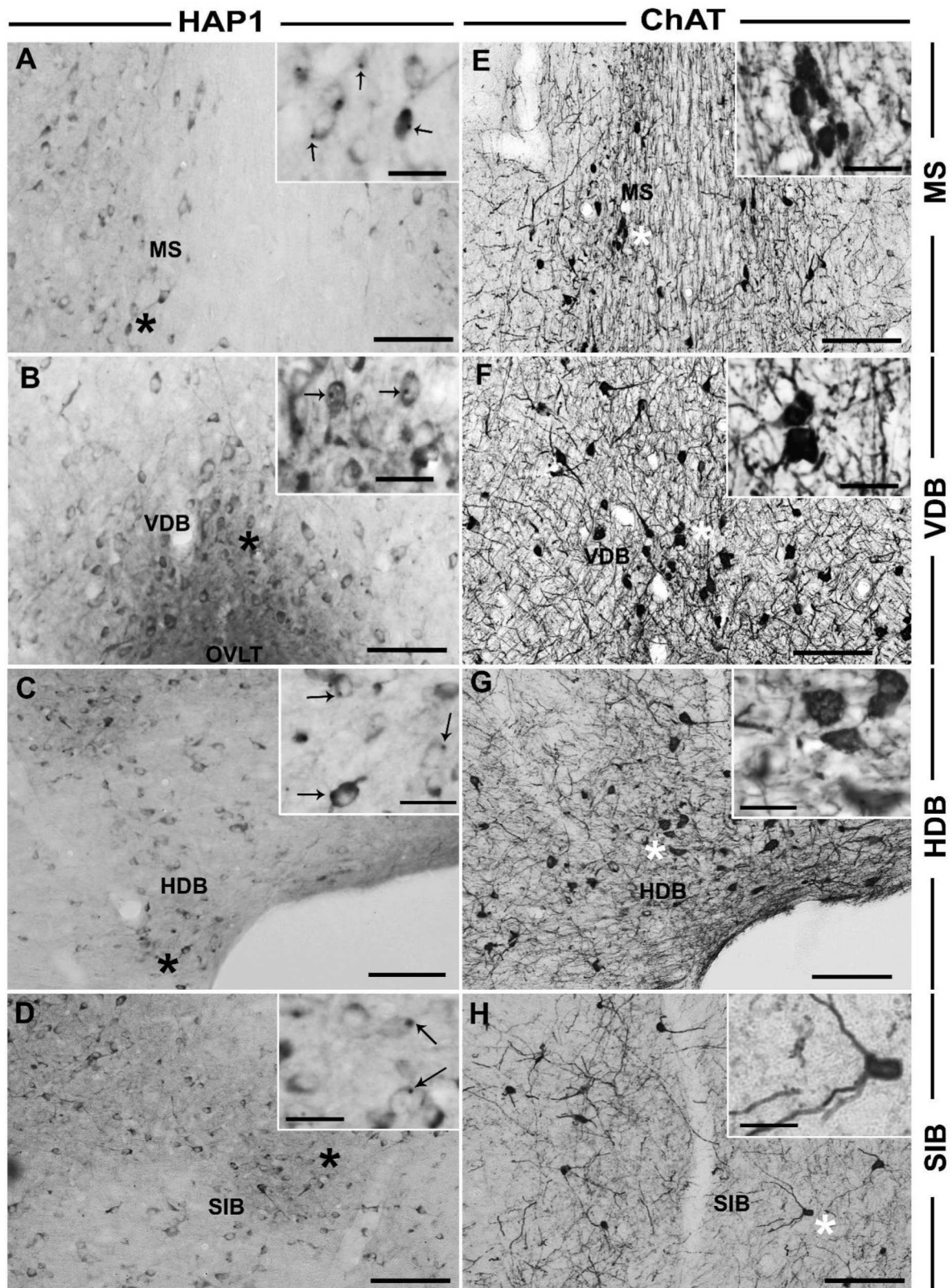


Figure 2.7. HAP1 and ChAT immunoreactivity in the basal forebrain cholinergic nuclei (BFCN). “A-D” indicate the HAP1-ir cells in MS, VDB, HDB, and SIB. “E-H” indicate the ChAT-ir cells in MS, VDB, HDB, and SIB. Insets are the enlargement of the HAP1-ir and ChAT-ir cells indicated by asterisks, and the black arrow indicates the HAP1-ir STB. Scale bar = 100μm in “A-H” and 20 μm in inset. MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band of Broca; HDB, nucleus of the horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part (Meynert nucleus); and OVLT, vascular organ of the lamina terminalis

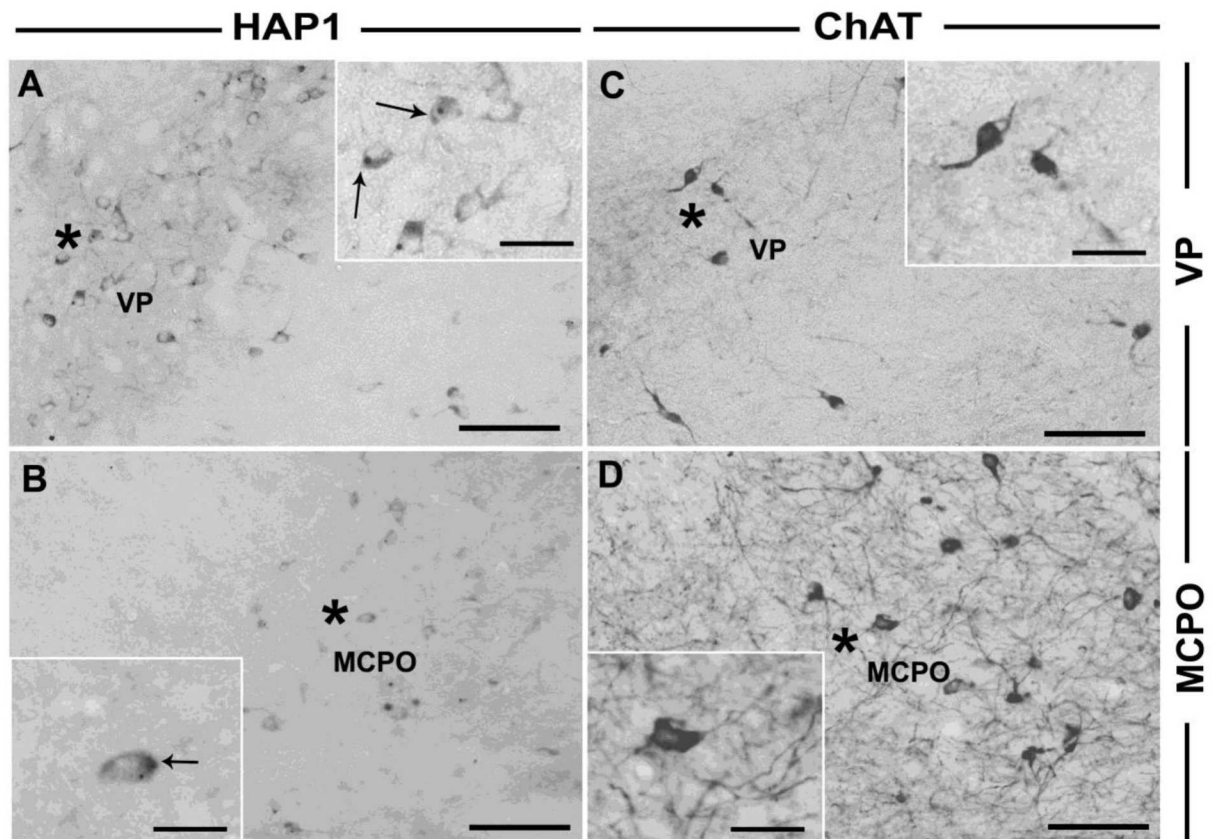


Figure 2.8. HAP1 and ChAT immunoreactivity in the ventral pallidum (VP) and magnocellular preoptic nucleus (MCPO). “A-B” indicates the HAP1-ir cells in VP and MCPO. “C-D” indicates the ChAT-ir cells in VP and MCPO. Insets are the enlargement of the HAP1-ir and ChAT-ir cells indicated by asterisks, and the black arrow indicates the HAP1-ir stigmoid body (STB). Scale bar = 100μm in “A-D” and 20 μm in inset.

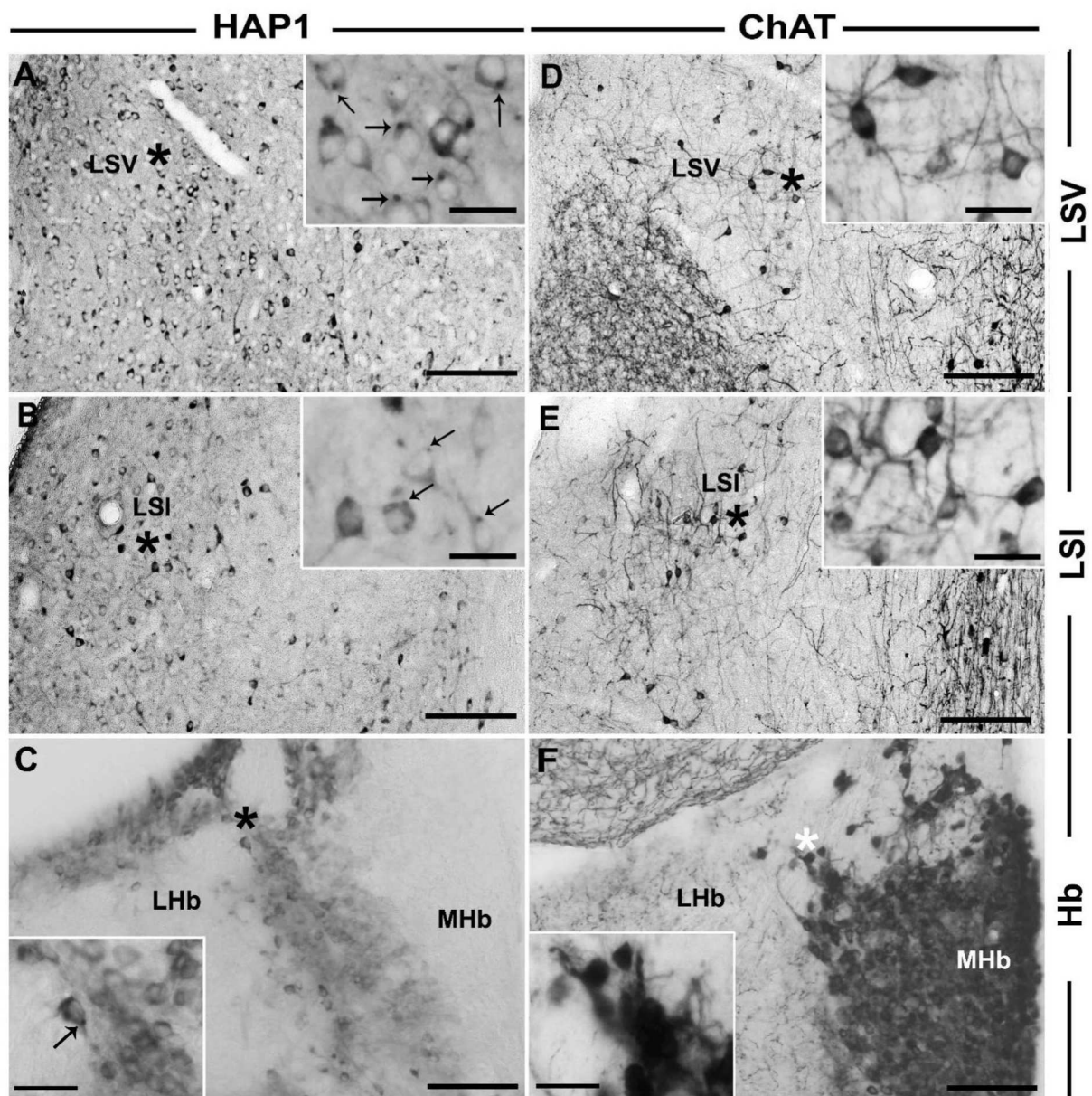


Figure 2.9. HAP1 and ChAT immunoreactivity in the lateral septal nuclei (ventral and intermediate parts), and habenular nucleus. “A-C” indicates the HAP1-ir cells in LSV, LSI, and Hb. “D-F” indicates the ChAT-ir cells in LSV, LSI, and Hb. Insets are the enlargement of the HAP1-ir and ChAT-ir cells indicated by asterisks, and the black arrow indicates the HAP1-ir STB. Scale bar = 100µm in “A-F” and 20 µm in inset. LSV, lateral septal nucleus, ventral part; LSI, lateral septal nucleus, intermediate part; Hb, habenular nucleus; LHb, lateral habenular nucleus; and MHb, medial habenular nucleus.

2.3.3. Immunohistochemical relationships of HAP1 and ChAT in the mouse forebrain cholinergic nuclei

Our double-label immunohistochemistry of HAP1 with ChAT showed that HAP1 immunoreactivity was never present in the ChAT-ir cells of the CPu (**Figure 2.10A-C**), even though the CPu had highly stained ChAT (**Figure 2.6D**) and scattered HAP1-ir neurons (**Figure 2.6A**). A very few HAP1-ir neurons had ChAT immunoreactivity in the AcbC (**Figure 2.10D-F**), while HAP1-ir neurons in Tu never contained ChAT immunoreactivity (**Figure 2.10G-I**). Cell counting results showed that the co-expression ratio of HAP1 in ChAT-ir cells was $14.53 \pm 1.75\%$, and that of ChAT in HAP1-ir cells was $7.37 \pm 0.67\%$ in the AcbC (**Table 4**).

In the BFCN, a considerable number of neurons showed co-expression of HAP1 with ChAT immunoreactivity in the MS (**Figure 2.11A-C**), VDB (**Figure 2.11D-F**), HDB (**Figure 2.11G-I**), and SIB (**Figure 2.11J-L**). Cell counting results revealed that in the MS, the co-expression ratio of HAP1 in ChAT-ir cells was $81.43 \pm 1.73\%$, and that of ChAT in HAP1-ir cells was $49.32 \pm 4.69\%$ (**Table 4**). In the VDB, the co-expression ratio of HAP1 in ChAT-ir cells was $77.44 \pm 1.82\%$, while that of ChAT in HAP1-ir cells was $33.89 \pm 4.19\%$ (**Table 4**). In HDB, the co-expression ratio of HAP1 in ChAT-ir cells was $39.53 \pm 2.36\%$, while that of ChAT in HAP1-ir cells was $19.45 \pm 4.41\%$. In the SIB, the co-expression ratio of HAP1 in ChAT-ir cells was $26.3 \pm 2.52\%$, and that of ChAT in HAP1-ir cells was $4.82 \pm 0.81\%$ (**Table 4**).

There were very few HAP1-ir neurons in VP expressed ChAT immunoreactivity (**Figure 2.12 a-c**). In the VP, the co-expression ratio of HAP1 in ChAT-ir cells was $4.68 \pm 0.59\%$, whereas ChAT in HAP1-ir cells was $7.44 \pm 1.61\%$ (**Table 4**). Besides this, a group of magnocellular neurons in the MCPO exhibited a small number of HAP1 co-expressions with ChAT. (**Figure 2.12D-F**). The MCPO showed a co-expression ratio of $7.65 \pm 0.78\%$ for ChAT in HAP1-ir cells and $24.48 \pm 2.46\%$ for HAP1 in ChAT-ir cells (**Table 4**).

The LS, particularly the LSV (**Figure 2.13A-C**) and the LSI (**Figure 2.13D-F**), had HAP1-ir neurons that contained ChAT immunoreactivity. According to our cell count results, LSV had a co-expression ratio of $8.62 \pm 1.13\%$ for ChAT in HAP1-ir cells but $76.51 \pm 2.28\%$ for HAP1 in ChAT-ir cells (**Table 4**). In the LSI, the ratio of the ChAT in HAP1-ir cells was $9.24 \pm 0.88\%$, while the HAP1 in ChAT-ir cells was $74.43 \pm 3.34\%$ (**Table 4**). Meanwhile, neurons that showed co-expression for HAP1 with ChAT were few in number in the Hb, especially at the edge between the LHb and MHb. (**Figure 2.13G-I**). In the Hb, there was about a $17.33 \pm 2.01\%$ co-expression ratio of ChAT in HAP1-ir cells, whereas HAP1 in ChAT-ir cells was $6.35 \pm 0.37\%$ (**Table 4**).

The statistical analyses show significant regional variation in the co-expression ratios of HAP1 and ChAT in the mouse forebrain. The highest HAP1/ChAT ratios were found in the MS, LSV, and LSI, while no co-expression was detected in the CPu and Tu. Significant differences were confirmed by one-way ANOVA followed by Tukey's test ($p < 0.01$), and specific pairwise comparisons were made using Student's t-test, showing multiple significant results at both 1% and 5% levels of significance.

Table 2.4. Co-expression ratios of HAP1/ChAT or ChAT/HAP1 in the forebrain cholinergic nuclei and the other nuclei in mouse forebrain

Category	Area	Mean $\pm$ SEM		LS (Student t test)
		HAP1/ChAT ratio (%)	ChAT/HAP1 ratio (%)	
Basal ganglia	Caudate Putamen (CPu)	0 [#]	0 [#]	N/A
	Nucleus accumbens core (AcbC)	14.53 ^d $\pm$ 1.75	7.37 ^{cd} $\pm$ 0.67	*
	Olfactory tubercle (Tu)	0 [#]	0 [#]	N/A
Forebrain cholinergi c nuclei	Medial septal nucleus (MS)	81.43 ^a $\pm$ 1.73	49.32 ^a $\pm$ 4.69	**
	Vertical limb of the diagonal band of Broca (VDB)	77.44 ^a $\pm$ 1.82	33.89 ^b $\pm$ 4.19	**
	Horizontal limb of the diagonal band of Broca (HDB)	39.53 ^b $\pm$ 2.36	19.45 ^c $\pm$ 4.41	**
	Substantia innominata basal part (Meynert nucleus; SIB)	26.3 ^c $\pm$ 2.52	4.82 ^d $\pm$ 0.81	**
	Ventral pallidum (VP)	4.68 ^e $\pm$ 0.59	7.44 ^{cd} $\pm$ 1.61	NS
	Magnocellular preoptic nucleus (MCPO)	24.48 ^c $\pm$ 2.46	7.65 ^{cd} $\pm$ 0.78	**
Lateral septal nucleus	Lateral septal nucleus ventral part (LSV)	76.51 ^a $\pm$ 2.28	8.62 ^{cd} $\pm$ 1.13	**
	Lateral septal nucleus intermediate part (LSI)	74.43 ^a $\pm$ 3.34	9.24 ^{cd} $\pm$ 0.88	**
	Habenular nucleus (Hb)	6.35 ^{de} $\pm$ 0.37	17.33 ^c $\pm$ 2.01	**
LS (One way ANOVA followed by Tukey's test)		**	**	

Values represent the mean $\pm$ SEM of co-expression ratios (%) in adult mice ($n = 6$). ^{a,b,c,d,e} Means within same column with different superscript letters differ significantly. **, Significant at 1% ($p < 0.01$); [#] No co-expression; *, Significant at 5% ($p < 0.05$); NS, non-significant; LS, Level of significance; SEM, Standard error of mean; ChAT, Choline acetyltransferase; HAP1, Huntingtin-associated protein 1.

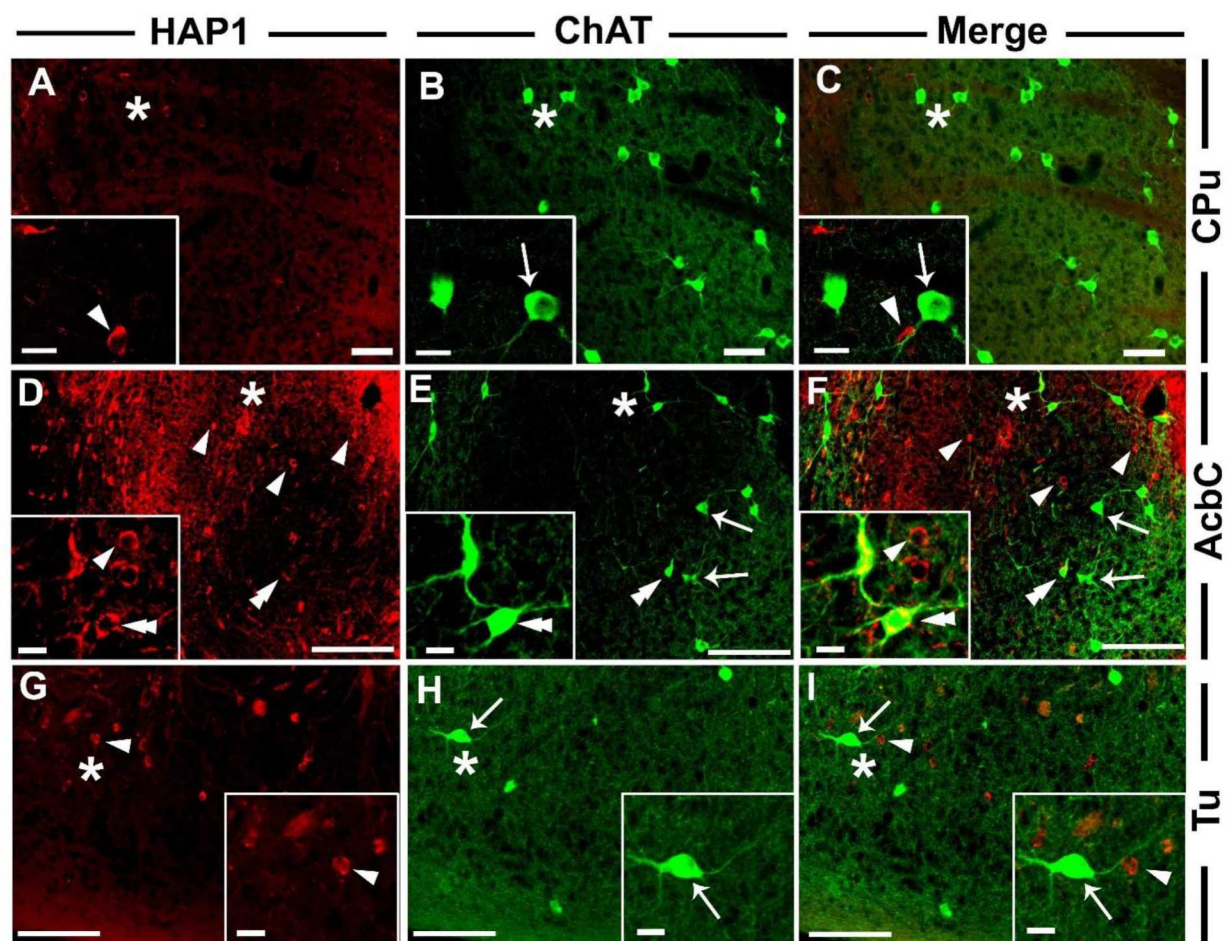


Figure 2.10. Double-label immunofluorescence immunohistochemistry for HAP1 with ChAT in the basal ganglia (dorsal and ventral striatal nuclei with associated cell group). Photomicrographs showing double-label immunofluorescence staining of HAP1 and ChAT in CPu (A-C), AcbC (D-F), and Tu (G-I). Double arrowhead indicates the cells are positive for both HAP1 and ChAT. Single white arrowhead for HAP1-ir cells while the white arrow indicates the cells positive only for ChAT. Insets are the enlargement of the HAP1-ir and ChAT-ir cells indicated by asterisks. Scale bar = 50 μ m in “A-I” and 20 μ m in inset. AcbC, nucleus accumbens core; CPu, caudate putamen; and Tu, olfactory tubercle.

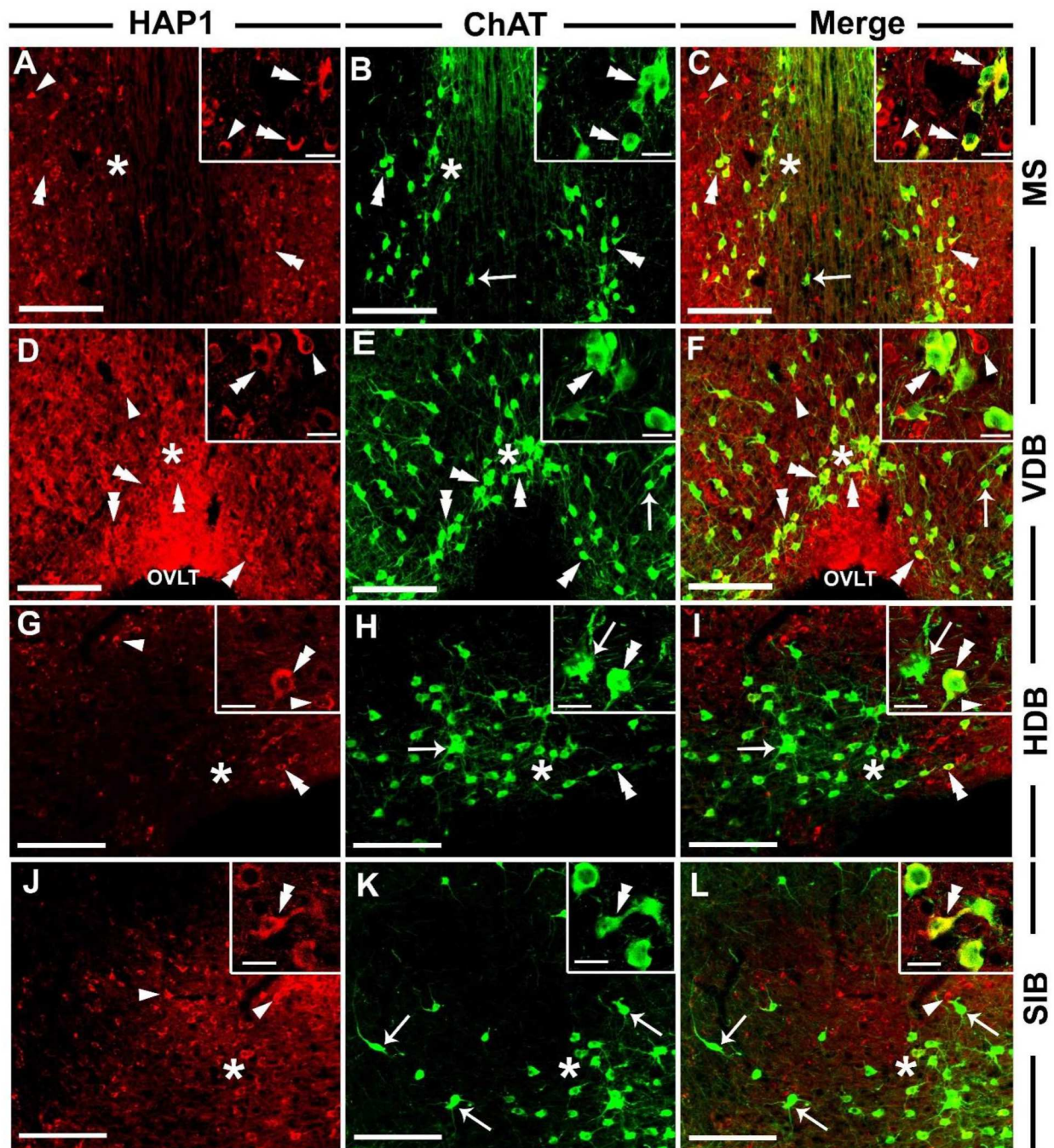


Figure 2.11. Double-label immunofluorescence immunohistochemistry for HAP1 with ChAT in the basal forebrain cholinergic nuclei (BFCN). Photomicrographs showing double-label immunofluorescence staining of HAP1 and ChAT in MS (A-C), VDB (D-F), HDB (G-I), and SIB (J-L). Double arrowhead indicates the cells are positive for both HAP1 and ChAT. Single white arrowhead for HAP1-ir cells while the white arrow indicates the cells positive only for ChAT. Insets are the enlargement of the HAP1-ir and ChAT-ir cells indicated by asterisks. Scale bar = 50µm in “A-L” and 20 µm in inset. MS, medial septal nucleus; OVLT, vascular organ of the lamina terminalis; VDB, nucleus of the vertical limb of the diagonal band of Broca; HDB, nucleus of the horizontal limb of the diagonal band of Broca; and SIB, substantia innominata basal part (Meynert nucleus).

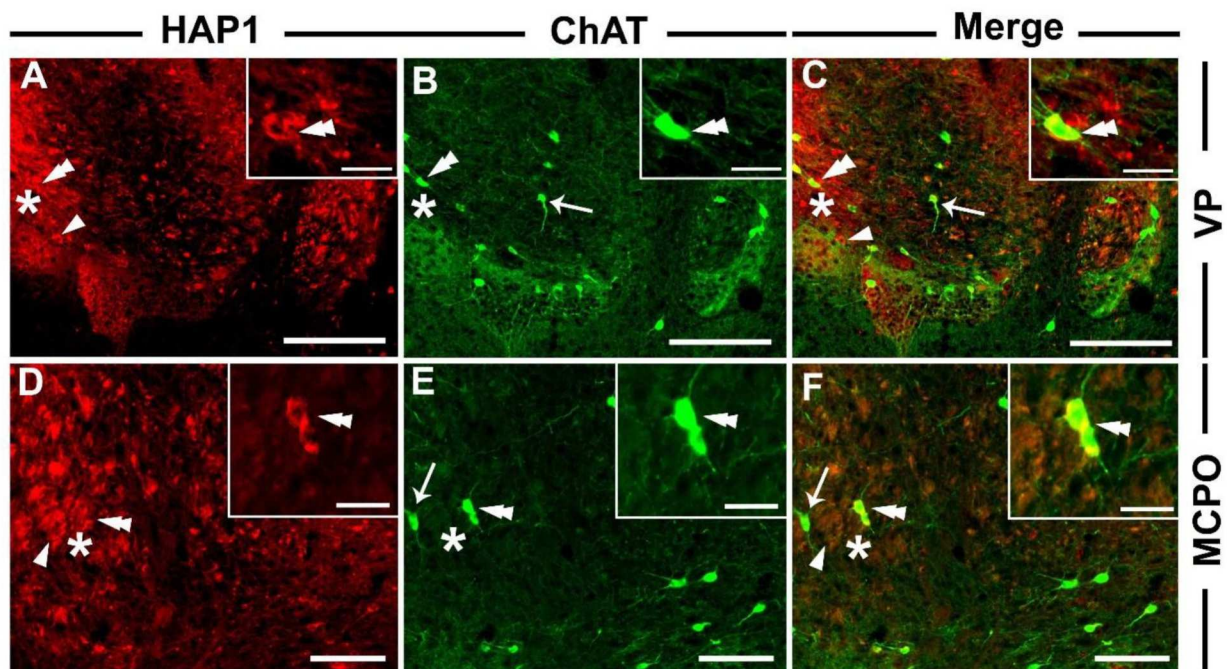


Figure 2.12. Double-label immunofluorescence immunohistochemistry for HAP1 with ChAT in the ventral pallidum (VP) and magnocellular preoptic nucleus (MCPO). Photomicrographs showing double-label immunofluorescence staining of HAP1 and ChAT in VP (**a-c**), and MCPO (**d-f**). Double arrowhead indicates the cells are positive for both HAP1 and ChAT. Single white arrowhead for HAP1-ir cells while the white arrow indicates the cells positive only for ChAT. Insets are the enlargement of the HAP1-ir and ChAT-ir cells indicated by asterisks. Scale bar = 50μm in “**a-f**” and 20 μm in inset.

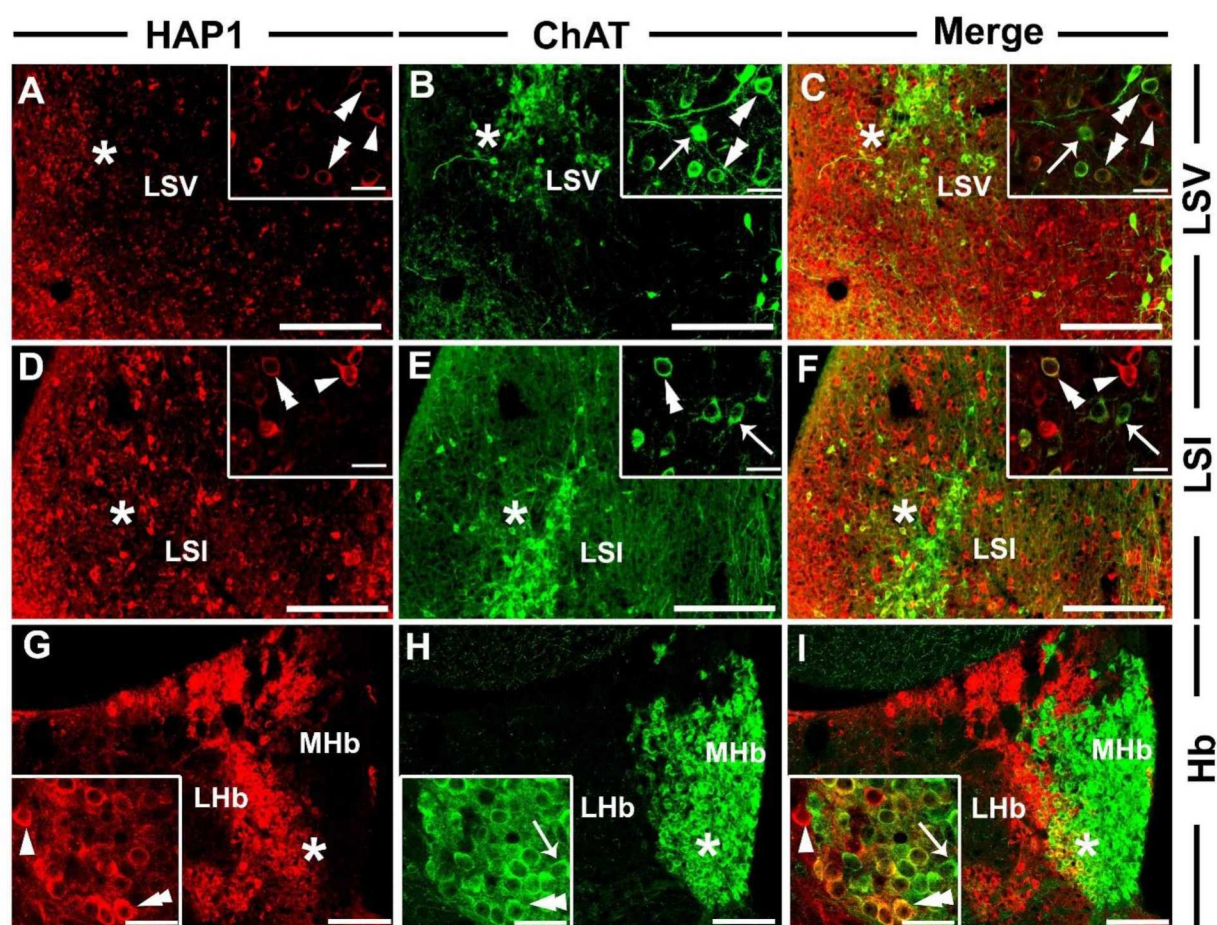


Figure 2.13. Double-label immunofluorescence immunohistochemistry for HAP1 with ChAT in the lateral septal nuclei (ventral and intermediate parts), and habenular nucleus. Photomicrographs showing double-label immunofluorescence staining of HAP1 and ChAT in LSV (**A-C**), LSI (**D-F**), and Hb (**G-I**). Double arrowhead indicates the cells are positive for both HAP1 and ChAT. Single white arrowhead for HAP1-ir cells while the white arrow indicates the cells positive only for ChAT. Insets are the enlargement of the HAP1-ir and ChAT-ir cells indicated by asterisks. Scale bar = 50 μ m in “**A-I**” and 20 μ m in inset. LSV, lateral septal nucleus, ventral part; LSI, lateral septal nucleus, intermediate part; Hb, habenular nucleus; LHb, lateral habenular nucleus; and MHb, medial habenular nucleus.

2.4. Discussion

The present study is the first to identify the expression, comparative distribution, and morphological relationship between HAP1 and ChAT in the forebrain cholinergic nuclei of adult mice. Although HAP1 expression has been investigated using in situ hybridization (Fujinaga et al. 2004), Western blotting (Li et al., 1998), RT-PCR (Li et al., 2000), and immunohistochemistry (Niu et al., 2011; Sheng et al., 2008) across various regions of the rodent brain, its cellular localization, comparative immunohistochemical distribution, and neurochemical associations with ChAT have not been explored in the forebrain.

In the present study, we confirm the existence of two isoforms of HAP1 (HAP1A and HAP1B) in different forebrain regions. These results coincide with earlier research (Li et al. 1998; Gutekunst et al. 1998; Fujinaga et al. 2009; Islam et al. 2017, 2022; Tarif et al., 2021) where HAP1 exists in two isoforms in the rodent brain, spinal cord, or enteric nervous system. It is well known that HAP1A induces cytoplasmic STB, whereas HAP1B gives rise to diffuse cytoplasmic expression (Fujinaga et al., 2007; Li et al., 1998). According to our current research, most HAP1-ir cells exhibit STB (smaller or larger) in their diffusely immunoreactive cytoplasm. However, there are some strongly stained HAP1-ir cells where the STB are challenging to identify under light microscopy despite their existence. Moreover, HAP1 immunoreactivity is present exclusively in neurons but not in the glial cells of the forebrain. These findings correspond with previous observations, which show that HAP1 immunoreactivity is present in neurons rather than glial cells in the adult mouse brainstem, spinal cord, or enteric nervous system (Islam et al., 2022, 2020a; Tarif et al., 2023).

Our findings regarding the immunohistochemical interactions of HAP1 and ChAT in the mouse forebrain cholinergic nuclei reveal region-specific differences in the co-expression of HAP1 and ChAT. Such differences may influence cholinergic signaling or contribute to neurodegenerative processes. From a neuropathological viewpoint, the most remarkable findings of the present study are that cholinergic neurons of the basal ganglia in the forebrain (striatal and pallidal components) mostly lack HAP1 immunoreactivity. Basal ganglia systems have been described previously based on their connections. Two different striatopallidal systems are described: a dorsal part of the basal ganglia involving CPu and pallidum, which is primarily related to motor behavior, and a ventral part of the basal ganglia involving the AcbC and pallidum, related to motivation and reward. However, the CPu indirectly projects to the substantia nigra (SN) through the GP externa (GPe) and subthalamic nuclei (Stanley et al., 2021). It has been shown that both the GPe and SN modulate aversive behaviors (Stanley et al., 2021). Then, SN projects to many areas implicated in the fear response, including the superior colliculus, inferior colliculus, and periaqueductal gray (Castellan-Baldan et al., 2006). These

pathways collectively enable the CPu to integrate sensory and aversive information while regulating aversive responses (Stanley et al., 2021). Significant progress has been made in understanding synaptic plasticity processes in the corticostriatal circuits. ACh has also entered the fray despite dopamine being the primary actor in the development of plasticity at corticostriatal synapses of CPu neurons (Surmeier et al., 2010). Degeneration of neurons throughout the striatum exhibits patterns of standardized time course and spatial topography specific to the tauopathies, AD, PD, and HD (Kawakami et al., 2014). Striatal degeneration patterns are believed to be distinct for each ND (Del Rey and García-Cabezas, 2023). However, the selective vulnerability of striatal neurons is an unresolved and critical question in different NDs. Our current study indicates that HAP1 immunoreactivity is entirely absent in the ChAT-ir CPu neurons (Figure 2.14). Additionally, the expression of HAP1 is very low in the ChAT-ir neurons of the AcbC. It is tempting to speculate that the lack of HAP1 protectivity could render CPu and AcbC cholinergic neurons particularly vulnerable to neurodegeneration (Figure 2.14). Therefore, HAP1 may underlie a potential impact on selective vulnerability across the striatum.

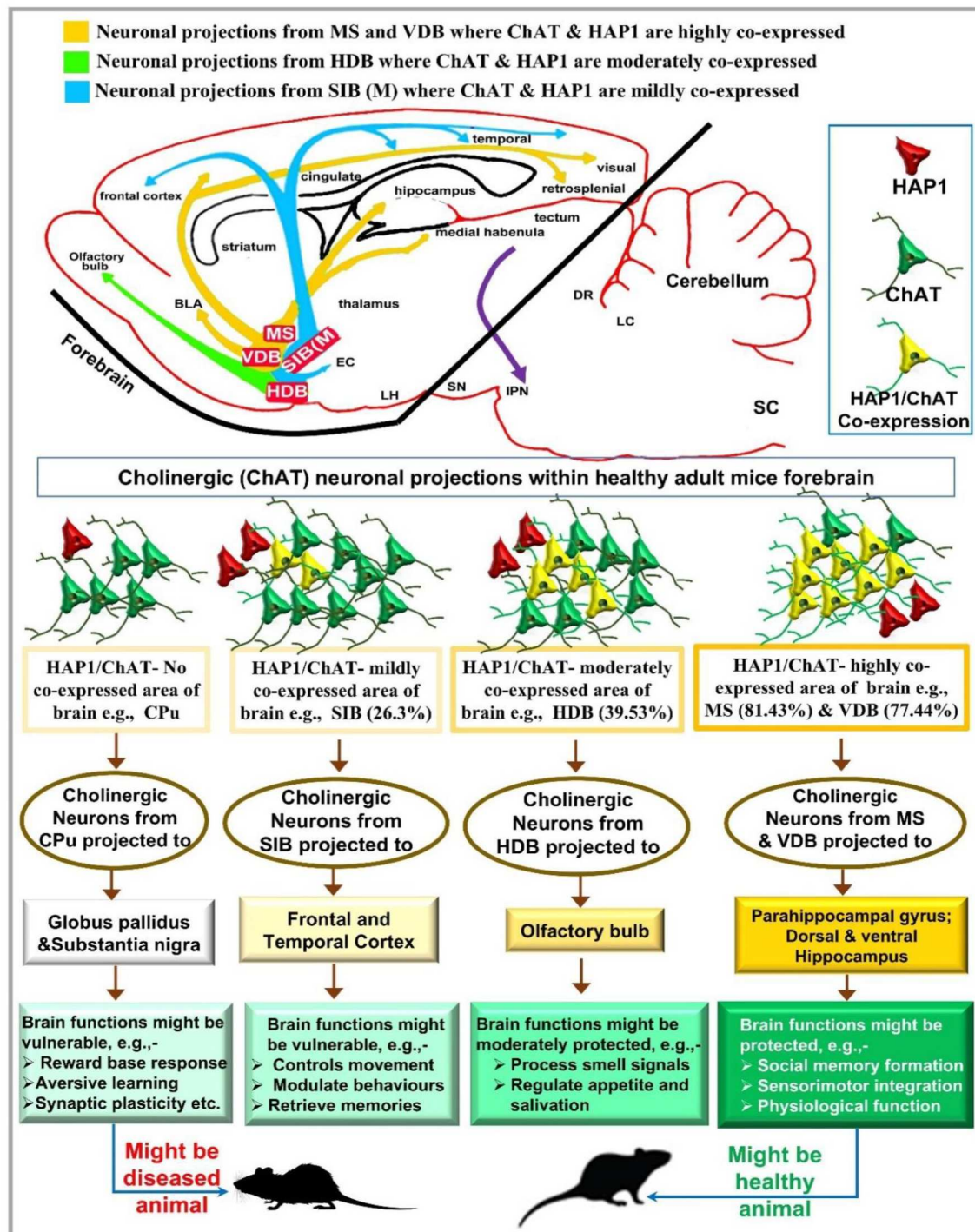


Figure 2.14. Hypothetical models for the HAP1 protection mechanism in the forebrain cholinergic nuclei. We proposed this hypothetical model based on our current results, previous findings, or contributions from other groups. The figure illustrates neuronal projections from forebrain cholinergic nuclei and their correlation with HAP1 and ChAT co-expression in healthy adult mice. The forebrain areas impacted by these projections include the hippocampus, parahippocampal gyrus, olfactory bulb, cortex, and substantia nigra. The bottom panel outlines the varying levels of ChAT and HAP1 co-expression across specific brain regions: no co-expression in the CPu (0%), mild co-expression in the SIB (26.3%), moderate co-expression in the HDB (39.53%), and substantial co-expression in the MS (81.43%) and VDB (77.44%). These HAP1 and ChAT co-expression patterns may influence brain functions, including responses to rewards, memory formation, movement control, and sensory information processing. The graphic illustrates that HAP1 may protect certain brain regions, while a low expression ratio or absence could heighten their vulnerability to neurodegeneration.

Our current immunohistochemical study shows that a significant quantity of ChAT-ir neurons from the BFCN contain STB/HAP1 immunoreactivity, especially in the MS and VDB. It is known that the BFCN are one of the major sources of cholinergic innervation in the mouse brain (Ahmed et al., 2019; Unal et al., 2012; Zaborszky et al., 2015) and are mainly projected to the hippocampus (dorsal and ventral part) and parahippocampal gyrus (Woolf, 1996), which is responsible for social memory formation, emotion, sensory-motor integration to cognition, and other psychological functions (Anand and Dhikav, 2012; Rubin et al., 2014). According to the STB/HAP1 protection hypothesis, due to having putative HAP1-protectivity, the brain cholinergic functions regulated by the MS and VDB might be safeguarded due to the stabilization of neurons against neurodegenerative or psychotic stresses (Fig. 14). Conversely, the cholinergic neurons in the HDB show moderate co-expression of ChAT with HAP1, whereas the cholinergic neurons in the SIB show slightly lower co-expression of ChAT with HAP1. Earlier research has shown that cholinergic modulatory inputs from HDB onto olfactory system neuronal circuits may affect how olfactory stimuli are processed and stored in the olfactory bulb (Linster et al., 2001). By shielding the cholinergic neurons in the HDB, it is interesting to hypothesize that the olfactory bulb may get a moderate degree of STB/HAP1 protection from pathological damages (Figure 2.14). Moreover, cholinergic axons from SIB typically project to the amygdala, limbic cortex, and the neocortex (Heimer et al., 2008; Kim et al., 2011; Zheng et al., 2022). Consequently, the brain functions integrated by cholinergic neurons of the SIB, such as attention, belief, awareness, episodic recall, motivation, feelings, learning, and the processing of rewards (Lodato and Arlotta, 2015; Olds and Milner, 1954; Šimić et al., 2021), might not get sufficient HAP1 protection against neuropsychological stresses (Fig.14). Our current research also reveals that very little numbers of neuron in the VP exhibit co-expression of HAP1 and ChAT. Numerous studies demonstrate that the VP is crucial in regulating adaptive and exploratory behaviors, motivated states, reward-based learning, and hedonic processing (Root et al., 2015). Through its projections to the prefrontal cortex and amygdala, the VP also modulates cognitive processes (Soares-Cunha and Heinsbroek, 2023). According to our current results, activities controlled by VP might be vulnerable to neurodegenerative/psychotic stresses due to a lack of HAP1 putative protectivity. In addition, the cholinergic neurons in MCPO exhibit comparatively lower co-expression of HAP1 with ChAT. The MCPO plays a crucial role in hormonal regulation, thermoregulation, olfactory analysis, and perception of sensory signals (Cousens, 2020; Feng et al., 2021; Murata, 2020; Wang and Swann, 2006). As a result, the brain activities mediated by cholinergic neurons of the MCPO might lack enough HAP1 protection against neurodegenerative stresses.

Substantial evidence demonstrates that the LS plays a critical role in regulating processes associated with mood and motivation, social behavior, and feeding processes through neural

connections with the amygdala, hippocampus, and hypothalamus (Sheehan et al., 2004). The LS may also lessen or enhance fear reaction when its neuronal activity is increased or repressed, respectively, probably through its projections to the hypothalamus (Anthony et al., 2014). Since HAP1 is widely expressed in the hypothalamus (Fujinaga et al., 2004), LS and the hypothalamus might have a reciprocal relationship. Prior research suggests LS dysfunction could disrupt mechanisms crucial to the prevention of schizophrenia and other psychotic spectrum diseases (McCutcheon et al., 2023). LS may also help to regulate the behavioral signs of depression (Sheehan et al., 2004). Our current morphological research shows that cholinergic neurons from the LSI and LSV exhibit strong HAP1 immunoreactivity. Consequently, the physiological and psychological functions controlled by the LS may be safeguarded due to the HAP1 putative protectivity.

Another complex nucleus of the mice forebrain is the Hb, which comprises LHb and MHb subnuclei and is referred to as a link between the limbic forebrain and the midbrain (Lee et al., 2019; Viswanath et al., 2013). Our current study reveals that the medial MHb contained ChAT-ir cells, whereas the marginal area between the LHb and MHb includes HAP1-ir cells. Our current results show that very few ChAT-ir neurons contain HAP1 at the boundary between the LHb and MHb. It is possible that the lack of HAP1 could render the MHb cholinergic functions vulnerable to psychological stresses that have also been associated with various psychiatric syndromes, such as schizophrenia, depression, and drug-induced psychosis (Hikosaka et al., 2008).

The potential mechanism by which HAP1 could prevent neurodegeneration in the forebrain cholinergic nuclei is unknown. It is interesting to speculate that the intracellular trafficking function of HAP1 is vital for normal neuronal function and survival, where dysfunction or absence of HAP1 might be associated with its involvement in NDs. Many NDs, including AD, HD, PD, ALS, SBMA, and SCA, show axonal abnormalities involving aberrant accumulations of proteins and organelles (Chen et al., 2023). Such pathophysiology emphasizes impairment to axonal transport, a critical component in developing NDs. Considering the results of our current study and those from the previous study, we proposed hypothetical models for the HAP1 protection mechanism against neurodegeneration. It has been reported that HAP1 interacts with kinesin-dependent amyloid precursor protein (APP), and lack of HAP1 significantly reduces APP transport in neurons (McGuire et al., 2006). APP is the precursor molecule that, upon proteolysis, forms amyloid beta ($A\beta$), a polypeptide with 37–49 amino acid residues that causes amyloid plaques in the brain of AD patients (Priller et al., 2006; Wang et al., 2021). Nonetheless, HAP1 can lower the amount of $A\beta$ in neurons and control the trafficking of APP to the non-amyloidogenic pathway (Yang et al., 2012). HAP1 can modulate the kinesin-dependent transport of APP vesicles from the cell body to neurites, and it is

known that suppressing or deleting the HAP1 may hinder this process (McGuire et al., 2006). The spontaneously provided microtubule-dependent axonal transportation could potentially inhibit the accumulation of β -amyloid (Yang et al., 2012), and ultimately, the systematic neuronal integrity might be maintained. In these consequents, our results can predict that the cholinergic neuronal functions might be protected by the neurons that are projected from the forebrain area, e.g., MS, VDB, LSI, and LSV, where most of the ChAT-ir neurons have the HAP1 immunoreactivity.

The forebrain area where the ChAT-ir neurons have no HAP1 immunoreactivity (e.g., CPu) or the loss of HAP1 due to age, mutation, or apoptosis (Metzger et al., 2008) may lead to unbound and unregulated APP. The premature processing of this unregulated APP may occur due to the disruption of microtubule-dependent axonal transport (Kamal et al., 2001). Therefore, a blockage to axonal transport may cause β -secretase-1 (cleaving enzyme 1) to break down premature APP, producing the cleaving product amyloid- β (Vassar et al., 1999) and releasing kinesin-1 from the transport vesicle (Kamal et al., 2001). This Kinesin-1 often attaches to APP via cargo protein or receptor proteins (Muresan and Muresan, 2009). Free kinesin-1 may impair axonal transport, potentially leading to NDs, such as AD (Muresan and Muresan, 2009). Furthermore, the halting of axonal transport encourages the breakdown of APP molecules by secretase enzymes, leading to increased accumulation of A β (Stokin et al., 2005). Subsequently, an increase in A β accumulation may cause β amyloid plaque development in the neuronal cells, microtubule disintegration, and Tau protein triangle disintegration, leading to NDs such as AD (Khan et al., 2023). In addition, the destruction of synaptic vesicles leads to disruption of neurotransmission (Lewis, 2021), leading to neurodegeneration that may also be caused by A β . Likewise, an increase in A β accumulation might be one of the causes for the reduction of pyruvate dehydrogenase (Khan et al., 2023). Specifically, these reductions in pyruvate dehydrogenase also cause cholinergic neuron degeneration and alter the ACh transmission (Koukourakis et al., 2005). The altered ACh transmission may cause impaired muscarine M1-like signalling (Scarpa et al., 2020). As a result, cognitive function could be affected, which may lead to AD and also other NDs (Khan et al., 2023). Consequently, one possible preventative measure against NDs might be to improve or restore the function of STB/HAP1-ir neurons expressing with ChAT.

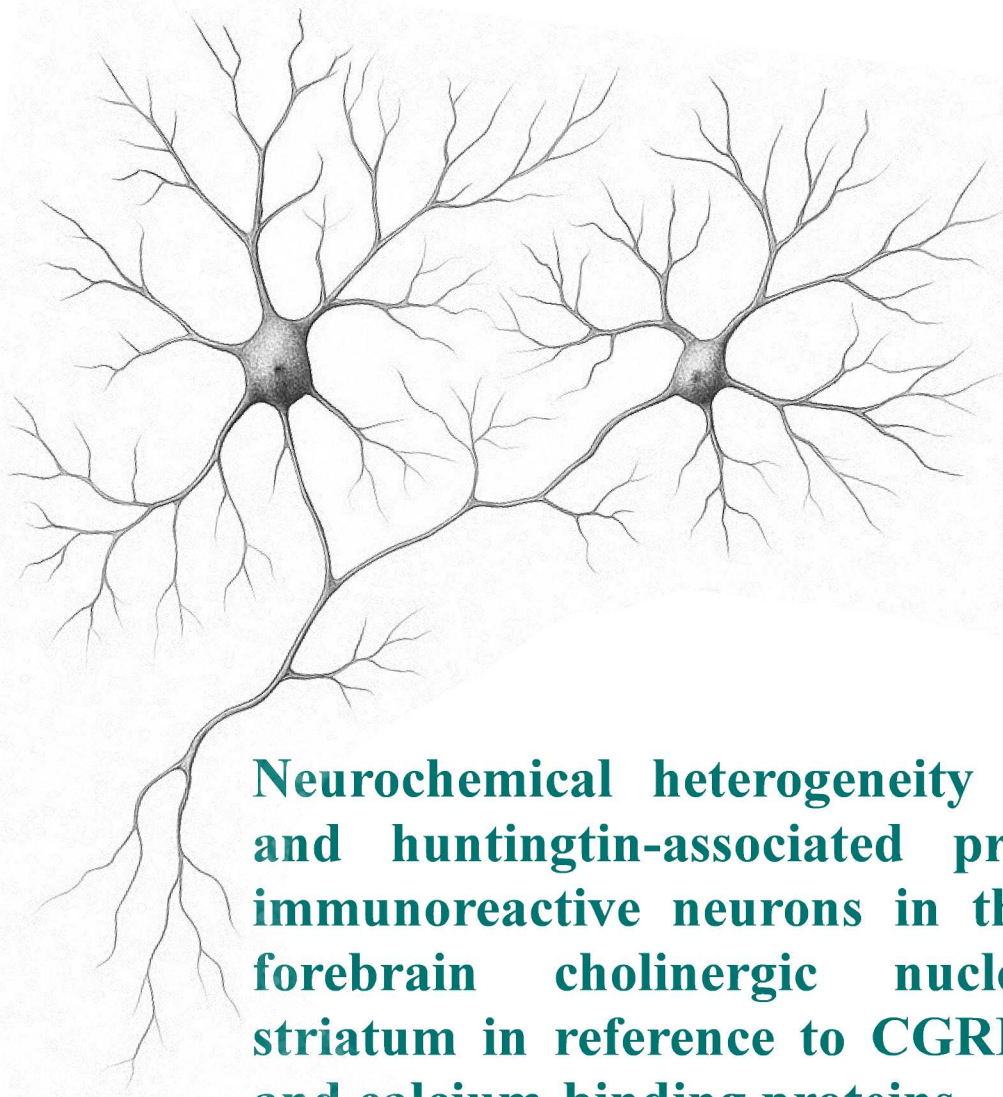
While our current research suggests that HAP1 may provide neuroprotection to cholinergic neurons in certain forebrain regions, a key limitation is that it does not explore the molecular mechanisms involved. Therefore, future studies should concentrate on understanding how HAP1 affects cholinergic signaling or neurodegenerative processes.

2.5. Conclusions

The present study examined the detailed comparative distribution of HAP1 and ChAT-ir neurons in the adult mouse forebrain. HAP1-ir neurons are moderately to highly distributed in the MS, VDB, SIB, LSI, LSV, and LHb. The double-label immunohistochemical analysis reveals that the co-expression ratio of HAP1 with ChAT is substantially higher in the MS, VDB, LSI, and LSV. The cholinergic neurons in these nuclei that are primarily projected to the hippocampus/parahippocampal regions or the amygdala and hypothalamus may be protected to regulate social memory formation, emotion, and other psychological functions during neurodegenerative/psychotic stresses because of their putative HAP1 protectivity. However, the ChAT-ir neurons in the striatum (CPu) never contain HAP1 immunoreactivity. Accordingly, the absence of HAP1 protection may render striatal neurons more susceptible to neurodegeneration in certain NDs.



Chapter 3



Neurochemical heterogeneity of chat and huntingtin-associated protein 1 immunoreactive neurons in the basal forebrain cholinergic nuclei and striatum in reference to CGRP, CCK, and calcium-binding proteins



Abstract

In the basal forebrain cholinergic nuclei (BFCN) and neostriatum (CPu), the cholinergic neuronal populations are critically important in learning, attention and motor control. The loss of cholinergic neurons is one of the major causes of neurodegenerative diseases such as Alzheimer's disease. The Huntingtin-associated protein 1 (HAP1) is a cytoplasmic protein that is important for the intracellular trafficking, neuronal health, vesicle transport, synaptic transmission, and maintenance of neuronal connections. Therefor HAP1 might protect the neuronal damage during the neurodegenerative stress. The purpose of this study was to clarify the molecular diversity of choline acetyltransferase (ChAT) and HAP1 immunoreactive (ir) neurons in the basal forebrain and CPu (Caudate putamen, Neostriatum). We used immunohistochemistry to identify the co-expression of ChAT and HAP1-ir neurons with two neuropeptides, calcitonin gene-related peptide (CGRP) and cholecystokinin (CCK), and three calcium-binding proteins (calbindin, calretinin and parvalbumin) in the adult mouse brain. The results showed that ChAT, calbindin, CGRP and CCK were strongly expressed in the BFCN, including medial septal nucleus (MS), nucleus of vertical limb and horizontal limb of the diagonal band of Broca (VDB and HDB), substantia innominata basal part (SIB), and in the caudate putamen (CPu). HAP1 was strongly expressed in the BFCN (except HDB where moderate to strongly expressed). CGRP and CCK showed a high immunoreactive co-expression with ChAT, especially in the HDB and CPu. Calbindin immunoreactivity was widely present and coincided with ChAT in the VDB, HDB, and CPu. However, calretinin immunoreactivity showed a selective co-expression with ChAT in the VDB, SIB, and CPu. Although parvalbumin immunoreactivity was observed throughout the BFCN and CPu, but there was no co-expression between ChAT and parvalbumin. On the other hand, HAP1 showed selective and region-dependent co-expression with both neuropeptide and calcium-binding protein-expressing neurons. Moderate proportion of HAP1 was co-localized with CGRP-ir and CCK-ir neurons in the MS, VDB, and HDB while no co-expression with CGRP-ir and CCK-ir neurons was detected in the SIB. In CPu, very little proportion of HAP1 was co-expressed with CGRP. As for calcium-binding proteins, HAP1 co-expressed mildly with calbindin and calretinin only in the MS, VDB, HDB and SIB, but did not co-localize with parvalbumin. In the CPu, though the slight proportion of HAP1 was co-expressed with calretinin but calbindin and PV were not co-expressed. Taken together, the neurochemical diversity of ChAT-ir neurons in the BFCN and neostriatum suggests the specialized functions of cholinergic neurons in different circuits, in particular by modulating CGRP, CCK, or calbindin. However, the results also suggest that HAP1 is specifically and selectively coupled with individual populations of neuropeptidergic and calcium-buffering neurons in the basal forebrain, and can have a moderate modulatory effect on synaptic or intracellular signaling pathways. These results could provide new insight into cholinergic and HAP1 neuronal modulation and their effects throughout the BFCN and striatum.

Keywords: *Cholinergic neurons; HAP1; Basal forebrain; Striatum; Neuropeptide; Calcium-binding proteins*

3.1. Introduction

Acetylcholine (ACh) is the key neurotransmitter of cholinergic neurons. This ACh is generated by choline acetyltransferase (ChAT), which is an excellent indicator of cholinergic neurons. In mammalian brains, ACh is found in the thalamus, striatum, limbic system and neocortex and is critically involved in higher cognitive functions such as memory formation, learning, attention mechanisms, and executive functions (Hampel et al., 2018). In addition to these well-defined cognitive functions, cholinergic transmission is also being increasingly appreciated for the role that it plays in overall brain homeostasis and in modulation of neural plasticity (Hampel et al., 2018; Nagori et al., 2024). Indeed, the cholinergic system exerts a powerful modulatory effect on both physiological and behavioral states (Haam and Yakel, 2017). In addition to crucial role of cholinergic system in cognition, it is also integral to the control of motor functions (Bohnen et al., 2022). Furthermore, cholinergic system involvement in the pathophysiology of neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD), is becoming increasingly evident (Eickhoff et al., 2022a). The cholinergic hypothesis is the oldest theory to explain the complicated and yet unknown pathophysiology of AD. Age-related neurodegenerative diseases like AD exacerbate cognitive impairment and cholinergic atrophy (Chen et al., 2022), which also exhibit psychotic symptoms (Eickhoff et al., 2022a). Another widely accepted theory has suggested that the development and progression of AD are caused by the activation of the amyloidogenic pathway, which is regulated by basal forebrain's cholinergic signaling (Hampel et al., 2021). This activation affects neuronal Ca^{2+} homeostasis and the mechanisms responsible for learning and memory (Cascella and Cecchi, 2021). Cholinergic deficits are further involved in the multifaceted spectrum of cognitive and behavioral symptoms of patients with Parkinson's disease (PD) and Lewy body dementia, which are emphasizing a common vulnerability of cholinergic neurons in different neurodegenerative diseases (Eickhoff et al., 2022a). The dense and complex network of cholinergic fiber projections create elaborate neural circuits to modulate the activity of various brain nuclei by the specific secretion of neurotransmitters (Eickhoff et al., 2022a; He et al., 2023). Cholinergic neurons were disseminated extensively throughout the forebrain regions. A significant population of basal forebrain cholinergic neurons (BFCN) includes the area of medial septum (MS), vertical and horizontal limbs of the diagonal band of Broca (VDB and HDB), and the substantia innominata basal part (SIB) (Meher et al., 2025b). In the central nervous system, BFCN serves as the primary output site for cholinergic energy (Shekari and Fahnstock, 2021). It has been reported that nicotinic acetylcholine receptors are abundant in the hippocampus (Café-Mendes et al., 2017). As a major cholinergic circuit, BFCN plays an important role in memory, learning, and cognitive function (Hu et al., 2016) by dominating the cortical and hippocampus regions (Latina et al., 2018). However, BFCN degeneration is a constant in AD patients, which would impair learning, memory, attention,

and sleep regulation (Gamage et al., 2020). Synaptic loss between the target tissues of the hippocampus, cortex, and basal forebrain is believed to be the main source of memory decline and is associated with the severity of dementia (Fahnestock and Shekari, 2019). In particular, the loss of cholinergic neurons in the nucleus basalis of Meynert (NBM) is closely associated with the degree of cognitive impairment that is often seen in AD and PD (Bohnen et al., 2022; He et al., 2023). Although the precise mechanism of action is yet unknown, aberrant central cholinergic alterations may also cause abnormal tau protein phosphorylation, inflammation of nerve cells, cell death, and other pathogenic events. Another important group of cholinergic neurons is located in the neostriatum (caudate-putamen, CPu) (He et al., 2023). These cholinergic interneurons of the striatum have unique morphological and electrophysiological properties. They are known to play a key role in motor control, reward processing, social behaviors, formation of habits, and learning (Ahmed et al., 2019). Impairment of these striatal cholinergic neurons has been implicated in neurological and psychiatric disorders including PD, Huntington's disease, dystonia and schizophrenia (Ahmed et al., 2019). Although the striatum has the highest density of cholinergic markers in the brain, role of cholinergic neurons in striatal function is yet to be thoroughly investigated (Bohnen et al., 2022; Tanimura et al., 2018). However, they are the only neurons synthesizing ACh in the striatum (Woolf and Butcher, 1981).

The HAP1 is an intra cytoplasmic protein regarded as a definite marker of stigmoid bodies (STB) (Shinoda et al., 1993, 1992). The STB are the spherical to oval in shape, size is 0.5 to 3µm in diameter non-membranous cytoplasmic inclusions. STB have a granular or a fuzzy texture with low-to-moderate electron density (Chen et al., 2020; Fujinaga et al., 2009, 2007, 2004; Islam et al., 2020b, 2017, 2012; Li et al., 1995; Sheng et al., 2006; Shinoda et al., 1992, 1993; Wroblewski et al., 2018). HAP1 was first identified as an interactor of huntingtin (htt) which is a polyglutamine (polyQ) length-dependent substrate. The htt is the protein that encoded by the gene mainly responsible for Huntington's disease (Li et al., 1995). Subsequently, numerous studies have revealed that HAP1/STB plays a crucial neuroprotective role. Particularly in opposition to htt-induced apoptosis and cell death correlated with expanded polyQ sequences (Fujinaga et al., 2004; Li et al., 2003; Metzger et al., 2008). Beyond its interaction with htt, HAP1 can also bind to other polyQ-expanded proteins. For example, the HAP1 can bind with the androgen receptor in spinal and bulbar muscular atrophy (SBMA), where its overexpression suppresses the apoptosis induced by the receptor (Takeshita et al., 2006). Additional studies have reported that the HAP1 has interactions with several proteins that might be implicated in other neurodegenerative disorders (e.g., Abelson helper integration site 1 in Joubert syndrome, TATA-binding protein in spinocerebellar ataxia type 17, and ataxin-3 in Machado–Joseph disease) (Prigge and Schmidt, 2007; Sheng et al., 2006; Takeshita et al., 2011).

Immunohistochemical investigations have demonstrated that HAP1 is abundantly expressed in the limbic and hypothalamic regions as well as in the dorsal horn of the spinal cord in rodents (Chen et al., 2020; Fujinaga et al., 2009, 2004; Islam et al., 2020a, 2017, 2012; Sheng et al., 2006; Takeshita et al., 2011; Wroblewski et al., 2018). Interestingly, HAP1 expression enriched regions likely to be invulnerable to neurodegeneration, whereas regions with sparse or no HAP1 (such as the neocortex, striatum, thalamus, cerebellum, and spinal motoneurons) are more vulnerable in various neurodegenerative diseases (Fujinaga et al., 2004; Islam et al., 2017). This correlation has given rise to the “STB/HAP1 protection hypothesis.” The hypothesis proposes that STB/HAP1 might enhance neuronal stability and raises the protection to neurodegenerative apoptosis (Fujinaga et al., 2004; Islam et al., 2020a, 2017, 2012; Metzger et al., 2008; Takeshita et al., 2011; Wroblewski et al., 2018). Therefore, the HAP1 neurochemical diversified characteristics could be important to predict the HAP1 protective role over the other neuronal functions.

In the brain, certain neuropeptides like calcitonin gene-related peptide (CGRP) and cholecystokinin (CCK) are involved in neuromodulation. CGRP is involved in pain pathways and vasodilation (Iyengar et al., 2019), whereas CCK plays a role in regulating food intake, learning processes, anxiety levels, and fear responses (Arzani et al., 2020). On the other hand, calcium binding proteins (CBPs) like calbindin, calretinin and parvalbumin are known to be expressed in specific neuronal populations where they are critically involved in calcium buffering and intracellular signaling (Pace et al., 2020; Parent et al., 1996). These expression patterns often help to define different subtypes of interneurons in different brain regions, including the striatum (Pace et al., 2020; Parent et al., 1996; Qi et al., 2022a). Parvalbumin is found in a subset of inhibitory interneurons and the PV is known as fast-spiking, non-adapting neurons that are vital to control the balance of excitatory and inhibitory signals between the brain's cortex (Udakis et al., 2020; Wagatsuma et al., 2023). In the context of ChAT immunoreactivity, it will be necessary to examine whether and how these additional markers are expressed. However, the previous studies were restricted to individual cell markers or random regions, and it is hard to deeply understand the neurochemical heterogeneity of ChAT-ir neurons in mice. Even probable ChAT-induced neurodegenerative diseases and neurobehavioral disorders lack efficient prevention and treatment strategies due to their complicated and unclear pathophysiology. Moreover, the HAP1 relationship with these molecular markers (peptidergic and CBPs) may help to clarify HAP1 functional role and potential neuroprotective importance in BFCN and CPu. Although, the HAP1 neuronal diversity was determined in the submucosal plexuses of rodent small intestine (Tarif et al., 2023, 2021), but never been studied in the rodent brain. As far as we are aware, a comprehensive and simultaneous approach to explore the neurochemical relationship of neuropeptidergic and CBP cell markers with ChAT and HAP1-ir

neurons in the specific BFCN (MS, VDB, HDB, SIB) and neostriatum of adult mice has never been studied before. Thus, the co-expression pattern of neuropeptides or CBPs in ChAT-positive neurons could reflect the impacts of developmental origin, connectivity, action potential, firing pattern, synaptic modulation, pathogenic cascade of neurodegenerative diseases, and responsiveness to stimuli. Therefore, the present study aims to investigate the neurochemical heterogeneity of ChAT-ir and HAP1-ir neurons in the adult mouse forebrain by determining the co-expression patterns of neuropeptides and CBPs in BFCN and neostriatum.

3.2. Materials and methods

3.2.1. Animals

In this study, eight-week-old adult male C57BL/6J mice were purchased from Japan SLC Inc., located in Shizuoka, Japan. The animals were housed in standard cages under controlled environmental conditions, with the temperature maintained between 22°C and 24°C. Mice were provided with a regular pellet diet and given free access to water. They were kept under a 12h light/dark cycle (lights on from 8:00 AM to 8:00 PM). All experimental procedures were approved by the Committee on the Ethics of Animal Experimentation at the Yamaguchi University School of Medicine. The study followed the guidelines for the care and use of laboratory animals set by the Government of Japan (Law No. 105, Notification No. 6). A total of 18 adult male mice were used in the experiments. Efforts were made to minimize animal suffering and reduce the number of animals used.

3.2.2. Tissue Preparation for Immunohistochemistry

For immunohistochemistry, mice were anesthetized using a mixture known as MMB (medetomidine, midazolam, and butorphanol) as described in the previous studies ([Islam et al., 2025](#); [Kiriha et al., 2013](#)). The mixture included medetomidine (Domitor®, Orion Pharma, Espoo, Finland; 0.3 mg/kg), midazolam (Midazolam®, Sandoz, Basel, Switzerland; 4.0 mg/kg), and butorphanol (Vetorphale®, Meiji Seika Pharma, Tokyo, Japan; 5.0 mg/kg). Each mouse received an intraperitoneal injection of 0.25–0.30 ml of MMB. Then mice were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). After perfusion, the brains were carefully removed and placed in 4% paraformaldehyde (in 0.1 M PB, pH 7.4) for overnight fixation. Following this, the brains were transferred to a 30% sucrose solution in 0.1 M PB and stored for five to seven days for cryoprotection. Once the brains were cryoprotected, they were frozen using powdered dry ice. The frozen tissues were then coronally sectioned at a thickness of 30 µm using a cryostat (Leica CM1900, Germany).

3.2.3. Antibodies

Details of the primary and secondary antibodies used in this study are listed in Tables 1 and Table 2, respectively. All antibodies were commercially purchased for this investigation.

Table 1. Detailed information on the primary antibodies used in the present study

Antibody	Immunogen	Host/ Clonality	Code	Source	Dilution	Reference
Anti-ChAT	Human placental ChAT	Goat polyclonal	Cat# AB144P, RRID: AB_11214092	Millipore, Billerica, MA, USA	1: 3,000	(Meher et al., 2025b)
Anti-ChAT	Synthetic peptide within Pig Choline Acetyltransferase aa 150–250	Rabbit monoclonal	Cat# ab178850, RRID: AB_2721842	Abcam, Cambridge, UK	1: 5,000	(Meher et al., 2025b)
CGRP	Synthetic CGRP (rat) conjugated to KLH	Rabbit polyclonal	Cat# C8198, RRID: AB_259091	Sigma-Aldrich, St. Louis, MO	1:1,000	Tarif et al., 2023
CCK	Synthetic sulfated CCK-8 bound to KLH	Rabbit polyclonal	Cat# C2581 RRID: AB_258806	Sigma-Aldrich, St. Louis, MO	1:1,000	Islam et al., 2012
Calretinin	Recombinant human calretinin a 6-his tag at the N-terminal	Rabbit polyclonal	Cat# CR7697, RRID: AB_2619710	Swant, Marly, Switzerland	1:2000	Tarif et al., 2023
Calbindin	Recombinant rat calbindin D-28 K (CB)	Rabbit polyclonal	Cat# CB-38a, RRID: AB_2721225	Swant, Marly, Switzerland	1:25,000	Tarif et al., 2023
Parvalbumin	Purified carp. muscle	Mouse monoclonal	Cat # 235; RRID: AB_10000343	Swant, Marly, Switzerland	1:1,000	(Magno et al., 2021)
Anti-HAP1 (R19)	Rat HAP1 C-terminus	Goat polyclonal	Cat# sc-8770, RRID: AB_647322	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:20,000	Tarif et al., 2021

Table 2. Detailed information on the secondary antibodies and other reagents used in the present study

Antibody	Immunogen	Host	Code/Source	Application	Dilution	References
Biotinylated goat anti-rabbit	IgG, isolated from rabbit serum	Goat	Cat# AP180B, RRID: AB_92569 Millipore, Darmstadt, Germany.	Immunoperoxidase staining	1:1,000	(Afrin et al., 2025)
Biotinylated polyclonal goat anti-mouse	Purified mouse IgG	Goat	Cat# E0433, RRID: AB_2687905, Dako, Denmark	Immunoperoxidase staining	1:1,000	(Köhler et al., 2025)
Biotinylated donkey anti-goat	IgG, isolated from goat serum	Donkey	Cat# AP180B, RRID: AB_92569 Millipore, Darmstadt, Germany	Immunoperoxidase staining	1:1,000	Islam et al., 2022
Alexa Fluor 488 donkey anti-goat	IgG (H + L) goat	Donkey	Cat# A11055, RRID: AB_2534102 Invitrogen, Eugene, OR, USA	Immunofluorescence staining	1:1,000	Yanai et al., 2020
Alexa Fluor 594 donkey anti-rabbit	IgG (H + L) goat	Donkey	Cat# A21207, RRID: AB_141637 Invitrogen, Eugene, OR, USA	Immunofluorescence staining	1:1,000	Yanai et al., 2020
Alexa Fluor 594 donkey anti-goat	IgG, isolated from goat serum	Donkey	Cat# A-11058, RRID: AB_2534105 Invitrogen, Eugene, OR, USA	Immunofluorescence staining	1:1,000	Yanai et al., 2020
HRP-conjugated streptavidin	N/A	N/A	Cat# P0397, Agilent Dako, Glostrup, Denmark	Immunoperoxidase staining	1:1,000	Islam et al., 2022

3.2.4. Single immunoperoxidase histochemical staining

A single immunoperoxidase immunohistochemical protocol was used, which was adapted from previous studies (Islam et al., 2023, 2022, 2017; Jahan et al., 2015; Tarif et al., 2023; Yanai et al., 2020). In summary, all adjacent free-floating forebrain sections (n=6) had been processed in parallel using same identical conditions. The forebrain sections were blocked with 0.3% Triton X-100-containing 10% normal goat serum or normal donkey serum and then incubated for 2h at 4°C on a shaker. After that, a pretreatment of 1.5% hydrogen peroxide and 50% methanol was applied to the free-floating sections in order to get rid of endogenous peroxidase. Next, rabbit anti-ChAT (1:5,000), rabbit anti-CGRP (1:1,000), rabbit anti-CCK (1:1000), rabbit anti-calbindin (1:25,000), rabbit anti-calretinin (1:2,000), mouse anti-parvalbumin (1:1,000), and goat anti-HAP1 R19 (1:20,000) primary antibodies were added in the successive forebrain sections respectively, which were then incubated for 5 days at 20°C (Islam et al., 2020a, 2017; Tarif et al., 2023). After three washes, the brain sections were incubated for 3h at 20°C with either biotinylated goat anti-rabbit or biotinylated goat anti-mouse or biotinylated donkey anti-goat secondary antibodies. Following this, the sections were incubated for 2h at 20°C with a streptavidin-biotin peroxidase complex (P0397, Dako, Glostrup, Denmark). To visualize the signal, a nickel-enhanced diaminobenzidine (DAB) reaction was performed for 10 to 20 min at 4°C. After staining, the sections were mounted on glass slides and air-dried for 30 min. They were then dehydrated through a graded series of alcohol and xylene, and finally cover-slipped using Entellan New (Merck KGaA, Darmstadt, Germany).

3.2.5. Dual-labeling immunofluorescence immunohistochemistry

The immunofluorescence immunohistochemistry approach was adapted from previously published works (Islam et al., 2022, 2017; Jahan et al., 2015; Tarif et al., 2023; Yanai et al., 2020) and all forebrain sections were processed in parallel using same identical conditions. In summary, 10% NDS or 10% NGS containing 0.3% Triton X-100 was applied to all adjacent free-floating forebrain sections (n=6) for 2h to 3h at 20°C. Next, the successive series sections were incubated with goat anti-ChAT (1:3,000) or goat anti-HAP1 R19 (1:20,000) in combination with one of the following primary antibodies: rabbit anti-CGRP (1:1,000), rabbit anti-CCK (1:1,000), rabbit anti-calbindin (1:20,000), rabbit anti-calretinin (1:2,000), or mouse anti-parvalbumin (1:1,000) antibody at 20°C for 4 days (Table 1 for detail about primary antibodies). Here 1% NDS or 1% NGS in PBS with 0.3% Triton X-100 was used to dilute the primary antibodies. The brain tissue sections were washed again and then incubated for 3h at 20°C with appropriate species-specific secondary antibodies of Alexa Fluor 488-conjugated donkey anti-goat IgG and Alexa Fluor 594-conjugated donkey anti-rabbit IgG (Table 2). PBS with 1% NDS or 1% NGS was used to dilute the secondary antibodies. Subsequently, all forebrain sections were incubated with 4',6-diamidino-2-phenylindole

(DAPI; Dojindo, Kumamoto, Japan) for nuclear staining. For this, DAPI (1 µg/ml) was diluted 1:1000 in PBS and let to sit at room temperature for 20 min. The sections were then washed with PBS and mounted on glass slides, air-dried for 30 min, and finally cover-slipped using Fluoromount/Plus (K048, Diagnostic Biosystems, Pleasanton, CA, USA).

3.2.6. Photomicrographs

Photomicrographs of immunoperoxidase-stained sections were captured using a Lumenera USB 2.0 colour digital camera (Lumenera Corporation, Ottawa, Canada) attached to a Nikon Eclipse E80i photomicroscope. For immunofluorescence imaging, an inverted confocal microscope (Leica Stellaris-8 CRS, Wetzlar, Germany) equipped with a ×40 oil immersion objective and a BZ-X810 fluorescent microscope (KEYENCE Corporation, Osaka, Japan) equipped with a ×20 objective was used. The captured images were then imported into Adobe Photoshop Elements 2021 (Adobe Systems, Inc., San Jose, CA, USA), where brightness and contrast were adjusted to enhance image quality.

3.2.7. Tissue analyses and cell counting

Nissl (cresyl violet) staining method was adopted from previous research ([Bjorkli et al., 2022](#)). Nissl stain was employed on adjacent section, the precise locations of each nucleus included within BFCN and neostriatum in the forebrain were identified (Fig. 1A-B). Additionally, the distribution of ChAT, HAP1, CGRP, CCK, or CBPs-ir cells in the mouse forebrain (BFCN and neostriatum) were ascertained by employing immunoperoxidase staining (n=6) of the neighbouring sections. The intensity of expression from these immune-positive neurons of BFCN have been measured in the medial septal nucleus (MS), the vertical limb of the diagonal band of Broca (VDB), the horizontal limb of the diagonal band of Broca (HDB), and substantia innominata basal part (SIB) throughout the forebrain. Using a x20 objective, immunofluorescence images were captured and imported into the ImageJ program (NIH, Bethesda, MD, USA). A total of twelve mice were used for cell counting. The actual number of ChAT, HAP1, CGRP, CCK, or CBPs-ir cells, as well as those neurons double labelled for ChAT or HAP1 with either CGRP, CCK, or CBPs were used to determine the co-expression proportions, in accordance with the counting method outlined in previous work ([Islam et al., 2022, 2017; Jahan et al., 2015](#)). For each nucleus, at least four sections from a single mouse were utilized, with a gap of never more than 60µm between the sections in every immunofluorescent staining.

3.3. Results

3.3.1. Comparative expression pattern of ChAT-ir or HAP1-ir cells with CGRP, CCK, and CBPs in the BFCN and neostriatum

First, we studied the expression of ChAT in order to explain the comparative distribution pattern of cholinergic neurons in the adult mice's BFCN and neostriatum (CPu). The comparative neuroanatomical distribution pattern of ChAT (**Figure 3.1C**), CGRP (**Figure 3.1D**), CCK (**Figure 3.1E**) and CBPs-ir cells including, calbindin (**Figure 3.1F**), calretinin (**Figure 3.1G**), and parvalbumin (**Figure 3.1H**) was summarized in a successive series of forebrain sections throughout the BFCN and neostriatum (CPu) of the adult mouse. Since quantitative analysis revealed no appreciable differences in cell numbers throughout the rostral-caudal extent, but the representative staining images of discrete nuclei were provided for demonstration of regional localization (**Figure 3.1A-E**).

Strong ChAT immunoreactivity was consistently observed in the MS, VDB, HDB, SIB and CPu (**Figure 3.2A-E; Table 3**). All these forebrain regions had intense ChAT immunoreactivity, which suggested dense cholinergic neuronal populations throughout the BFCN and CPu (**Figure 3.2A-E; Table 3**). The BFCN is well known of ChAT-expressing neurons projected to discrete anatomical regions and associated at different levels. ChAT-ir neurons in the MS are mostly large or medium-sized multipolar neurons. These neurons have long, sparsely arborescent dendrites (**Figure 3.2A**). Within the MS, cholinergic neurons were localized mainly medially and few scattered centrally but they differed in their larger soma size and distribution pattern (**Figure 3.1C; 2A**). The ChAT-ir neurons were densely positioned in and adjacent to the MS proper. A minor number of these cells were also outspread in the VDB (**Figure 3.1C; 2A**). The VDB houses medium- to large-sized ChAT-ir cells that had round to oval or fusiform somata, and the dendrites were sparsely branched and densely distributed along the midline (**Figs. 1C and 2B**). Instead, HDB had large multipolar ChAT-ir neurons with long dendrites and polygonal or oval soma (**Figure 3.2C**). In the HDB, ChAT positive neurons were prominently situated medially to the olfactory tubercle (**Figure 3.1C**). HDB was a horizontally oriented band of cholinergic neurons and was ventrolateral to the vertical limb (**Figure 3.1C; 2C**). ChAT-ir neurons in the SIB were usually large, multi-polar and arranged in a sheet-like pattern (**Figure 3.1C; 2D**). It was located lateral to the HDB and caudal to the VDB (**Figure 3.1C**). These neurons could be seen in the coronal sections just medial to ventral pallidum and nucleus accumbens (**Figure 3.1C**). There were instances when these cells sometimes extended into the ventral pallidum (**Figure 3.1C**). CPu cholinergic neurons were mostly oval and a few were polygonal in shape (**Figure 3.2E**). They are the largest interneurons in the striatum as identified by

immunolabeling with ChAT. Their scattered distribution throughout the CPU is almost uniform, with the dorsomedial and central areas having the highest density (**Figure 3.1C; 2E**).

Neuropeptide-expressing neurons across the BFCN (MS, VDB, HDB, and SIB) as well as in the CPu had strong immunoreactivity for CGRP (**Figure 3.2F-J; Table 3**) and CCK (**Figure 3.2K-O; Table 3**). Both CGRP and CCK exhibited strong expression intensities, indicating widespread neuropeptidergic signaling in these forebrain areas. Notably, CGRP (**Figure 3.1D; 2F**) and CCK (**Figure 3.1E; 2K**) -ir cells were abundantly disseminated throughout the MS (medially and centrally), but cholinergic cells had a topographically distinct distribution in MS (**Figure 3.1C; 2A**).

Calcium-binding protein expressing neurons were expressed in the BFCN and CPu regions of the mouse forebrain, with distinct regional variation in their expression pattern. Calbindin was highly expressed in all the analyzed brain regions, including MS, VDB, HDB, SIB and CPu (**Figure 3.3A-E; Table 3**). Conversely, the expression of calretinin was moderate to strong in the MS (**Figure 3.3F; Table 3**) or VDB (**Figure 3.3G; Table 3**), and calretinin expression was consistently strong in the HDB, SIB and CPu (**Figure 3.3H-J; Table 3**). However, parvalbumin showed more restrictive expression with weak to moderate immunoreactive intensity in the VDB, whereas moderate to strong levels of immunoreactivity were found in the MS, HDB, and SIB (**Figure 3.3K-N; Table 3**). Intriguingly, strong parvalbumin immunoreactivity was observed in the CPu (**Figure 3.3O; Table 3**). In comparison to the cholinergic cell localization (**Figure 3.1C; 2A**), calbindin (**Figure 3.1F; 3A**) and calretinin-ir neurons (**Figure 3.1G; 3F**) were almost medially resided in MS, but parvalbumin-ir neurons were both medially and centrally positioned (**Figure 3.1H; 3K**).

The HAP-1 expression in the basal forebrain revealed that the strong expression was present in the MS, VDB and SIB except the HDB where moderate to strong co-expression was present (**Fig. 3.4A-E; Table 3**). In the CPu, HAP1 immunoreactivity was weak (**Fig. 3.4A, E; Table 3**).

Table 3. Expression of ChAT and other neuronal markers in the basal fore brain cholinergic nuclei and caudate putamen in adult mouse

Area	Intensity of expression						
	ChAT	CGRP	CCK	Calbindin	Calretinin	PV	HAP1
Medial septal nucleus	+++	+++	+++	+++	++	++	+++
Vertical limb of the diagonal band of Broca	+++	+++	+++	+++	++	+	+++
Horizontal limb of the diagonal band of Broca	+++	+++	+++	+++	+++	++	++
Substantia innominata basal part (Meynert nucleus)	+++	+++	+++	+++	+++	++	+++
Caudate Putamen (Neostriatum)	+++	+++	+++	+++	+++	+++	+/-

(- indicates no expression, +/- indicates weak, + indicates weak to moderate, ++ indicates moderate to strong, and +++ indicates strong expression). For detail on abbreviations, see Abbreviations.

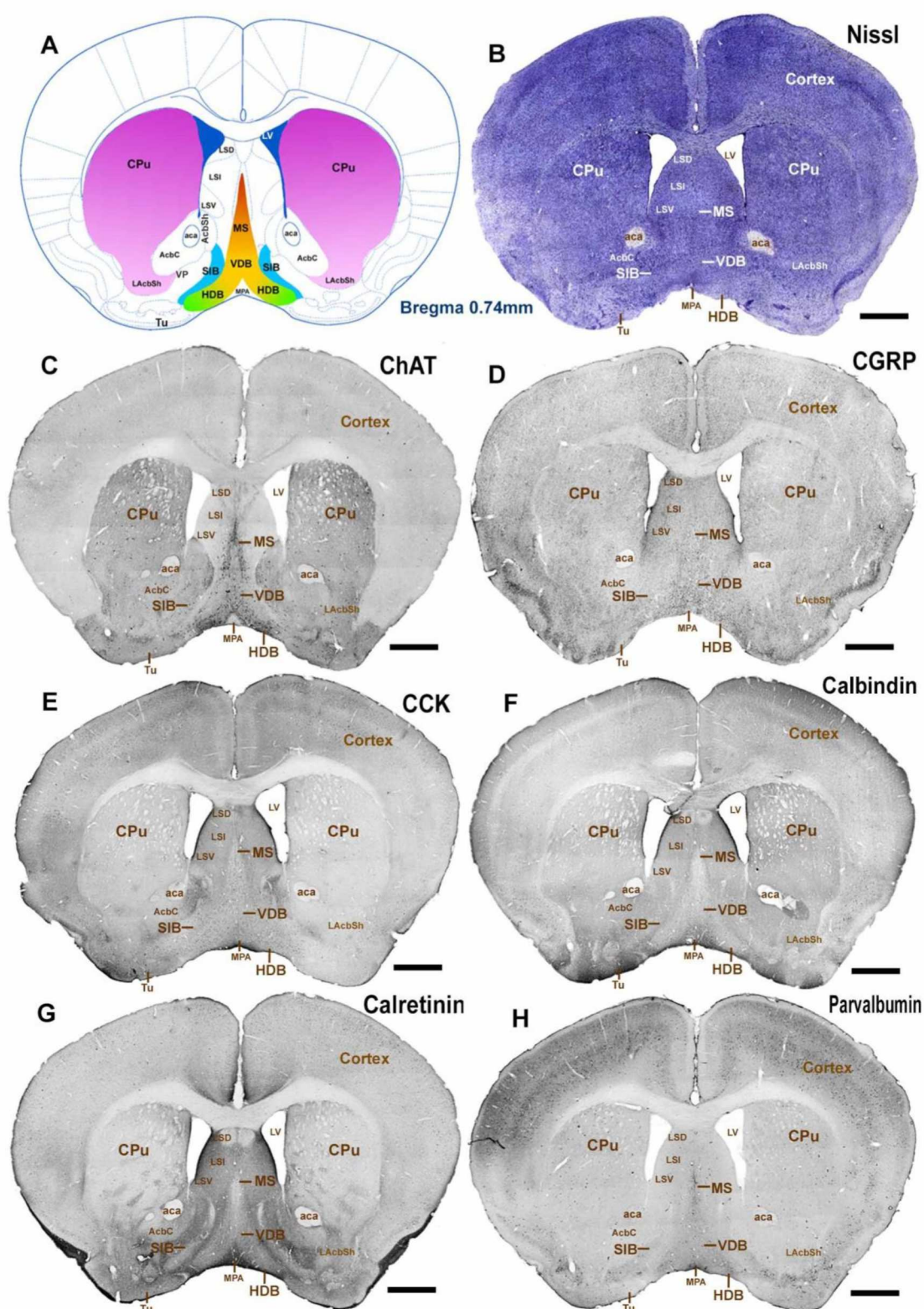


Figure 3.1. Nissle (cresyl violet) and immunoperoxidase immunohistochemistry for ChAT and neuropeptide or calcium-binding protein expressing cell markers. (A-B) representative forebrain section was examined employing Nissle staining for delineating basal forebrain cholinergic nuclei (BFCN) and neostriatum (CPu). (C-H) distribution of ChAT, CGRP, CCK, calbindin, calretinin, and parvalbumin immunoreactivity across the BFCN and CPu in the adult mouse forebrain. For topographic reference, a few preferred structures are noted. Scale bar = 500µm. ChAT, Choline acetyltransferase; CGRP, Calcitonin gene-related peptide; CCK, Cholecystokinin; MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band of Broca; HDB, nucleus of the horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part, and CPu, caudate putamen. For detail on abbreviations, see Abbreviations.

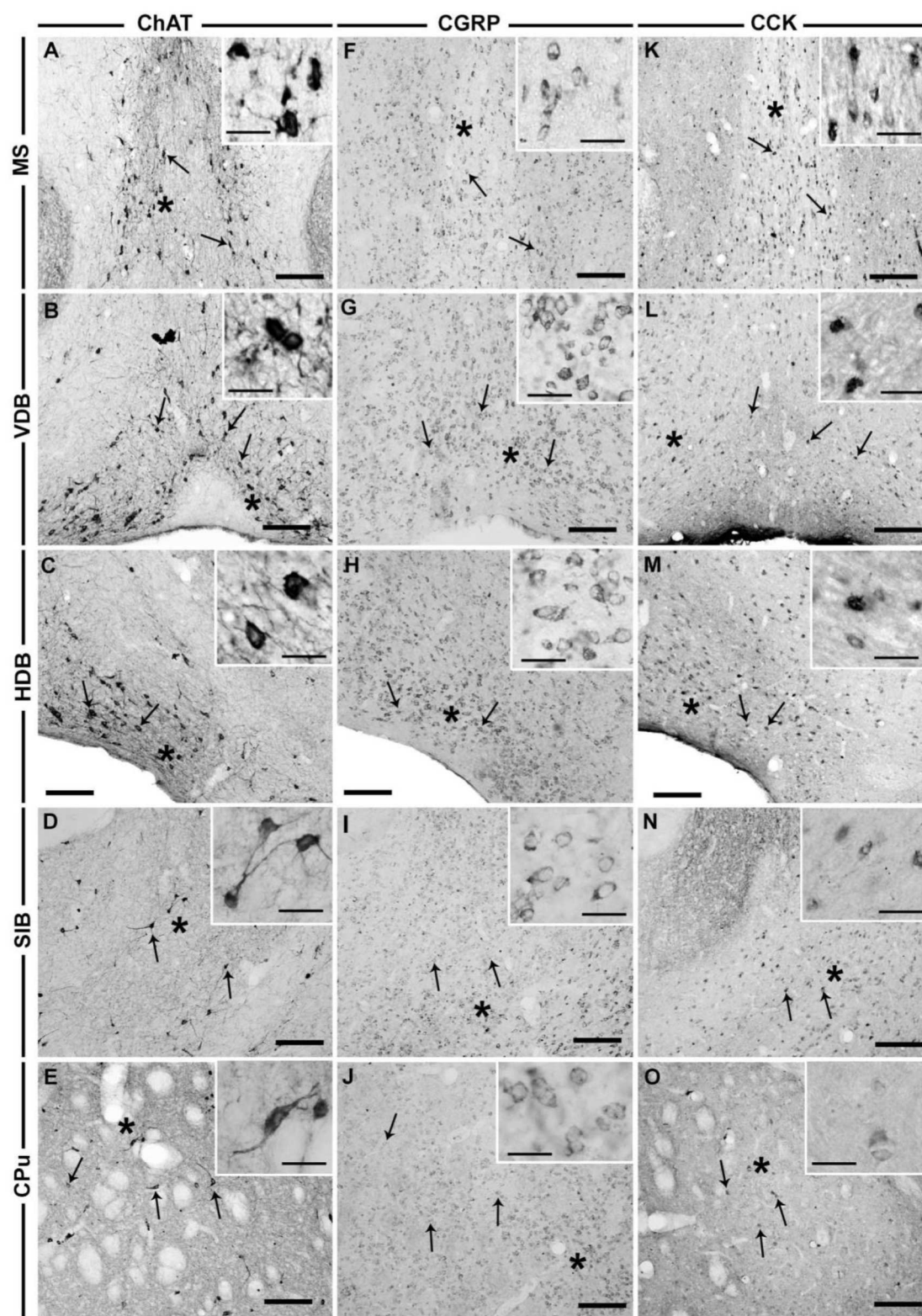


Figure 3.2. ChAT, CGRP, and CCK immunoreactivity in the MS, VDB, HDB, SIB, and CPu in the adult mice. “A-E” indicates the ChAT-ir cells in MS, VDB, HDB, SIB, and CPu. “F-J” indicates the CGRP-ir cells in MS, VDB, HDB, SIB, and CPu. “K-O” indicates the CCK-ir cells in MS, VDB, HDB, SIB, and CPu. Arrow indicates the immunoreactive cells and insets are the enlargement of the ChAT, CGRP, and CCK immunoreactive cells indicated by asterisks. Scale bar = 100µm in “A-O” and 20µm in inset. ChAT, Choline acetyltransferase; CGRP, Calcitonin gene-related peptide; CCK, Cholecystikinin; MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band of Broca; HDB, nucleus of the horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part, and CPu, caudate putamen (neostriatum).

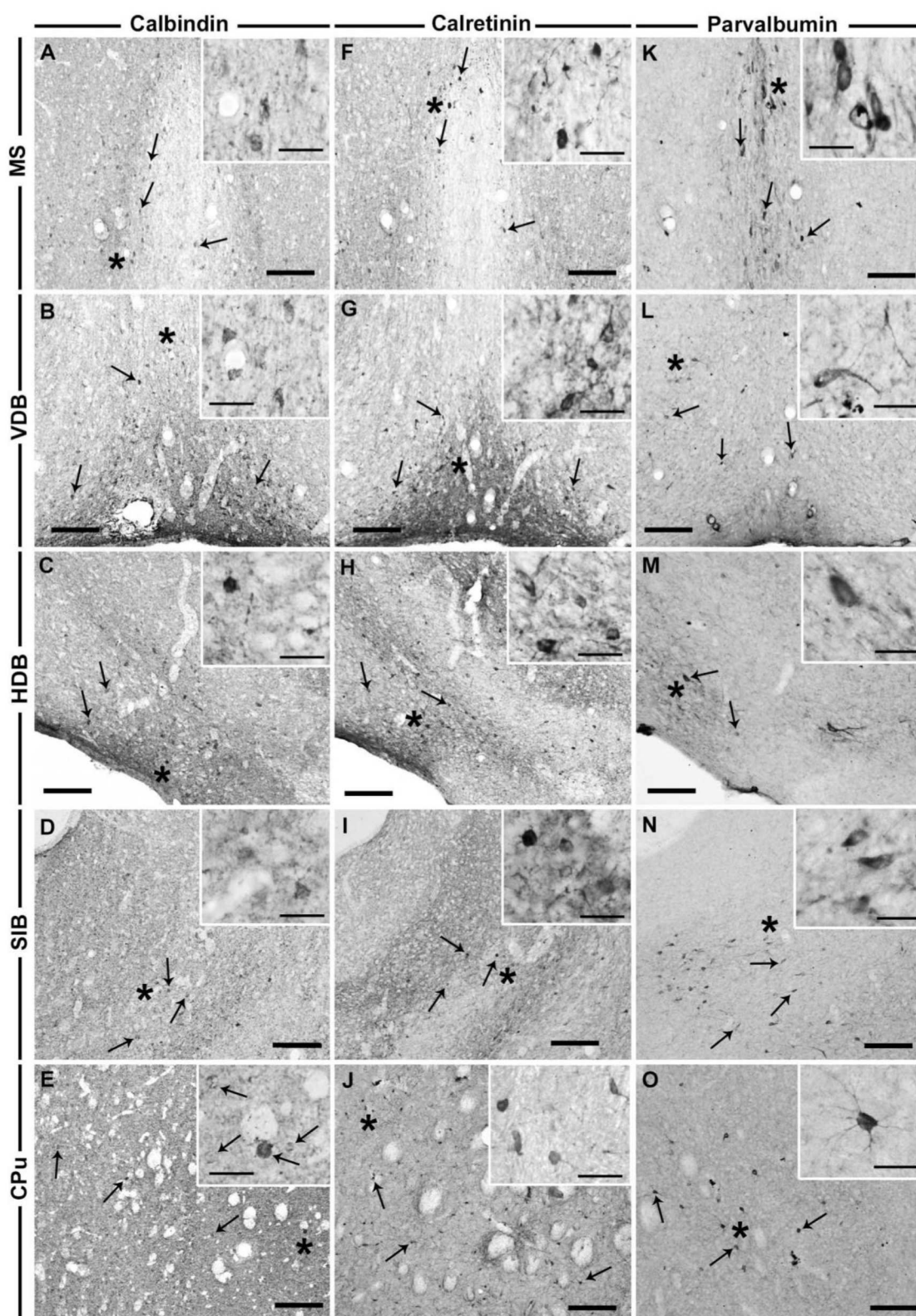


Figure 3.3. Calcium binding proteins (Calbindin, Calretinin, and Parvalbumin) immunoreactivity in the MS, VDB, HDB, SIB, and CPu in the adult mice. “A-E” indicates the calbindin-ir cells in MS, VDB, HDB, SIB, and CPu. “F-J” indicates the calretinin-ir cells in MS, VDB, HDB, SIB, and CPu. “K-O” indicates the parvalbumin-ir cells in MS, VDB, HDB, SIB, and CPu. Arrow indicates the immunoreactive cells and insets are the enlargement of the calbindin, calretinin, and parvalbumin immunoreactive cells indicated by asterisks. Scale bar = 100μm in “A-O” and 20μm in inset. MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band of Broca; HDB, nucleus of the horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part, and CPu, caudate putamen (neostriatum).

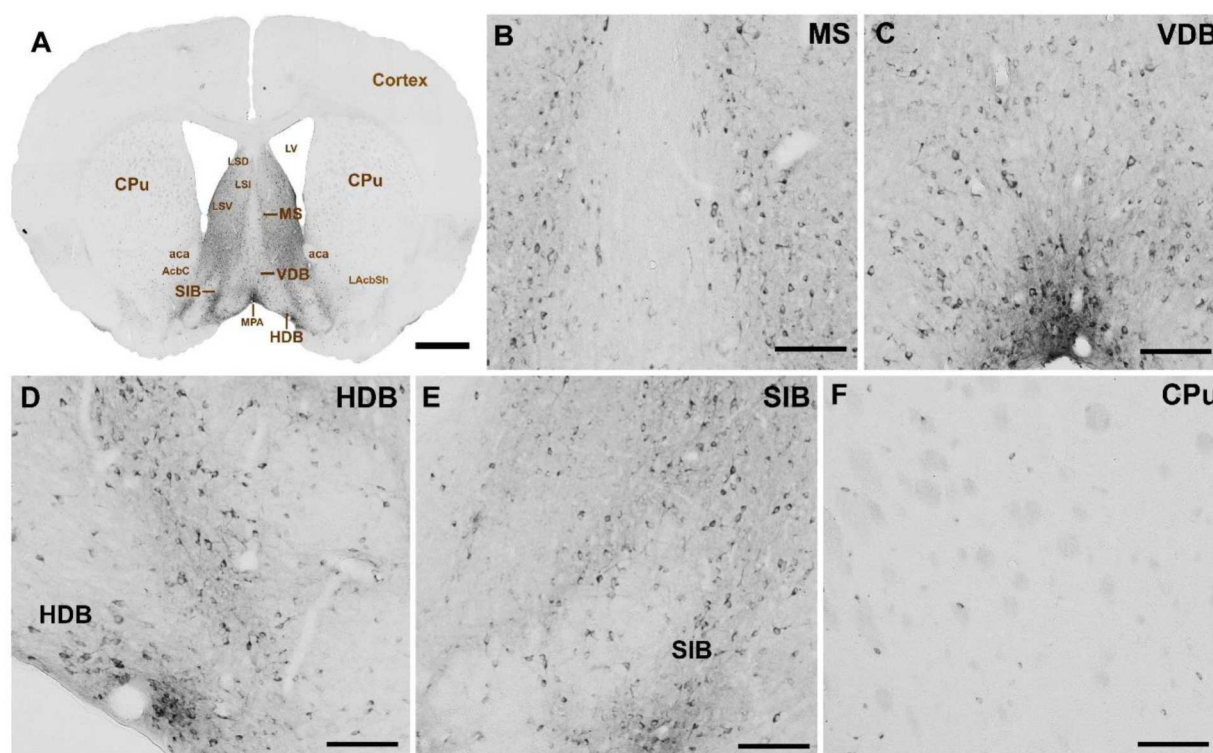


Figure 3.4. HAP1 immunoreactivity in the MS, VDB, HDB, SIB, and CPu in the adult mice. “A-F” indicates the HAP1-ir cells in MS, VDB, HDB, SIB, and CPu. Scale bar = 100 μ m in “A-F”. HAP1, Huntingtin Associated Protein 1; MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band of Broca; HDB, nucleus of the horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part, and CPu, caudate putamen (neostriatum).

3.3.2. Immunohistochemical relationship of ChAT with neuropeptide-expressing neurons

This approach allowed us to identify subsets of peptidergic neurons with cholinergic phenotype, providing insight into the potential modulatory complexity of cholinergic projections. In the MS, 71.27% of ChAT-ir neurons contained the CGRP, while 33.32% of CGRP-ir neurons had ChAT immunoreactivity (**Figure 3.4A-D; Table 4**). CCK-ir neurons were even more prominently co-localized, 73.67% of ChAT-ir neurons expressed CCK, and 37.36% of CCK-ir neurons showed ChAT immunoreactivity (**Figure 3.5A-D; Table 4**). In the VDB, 76.06% of ChAT-ir neurons contained the CGRP, and 43.3% of CGRP-ir neurons had ChAT immunoreactivity (**Figure 3.4E-H; Table 4**). The co-expression of CCK with ChAT was also prominent in VDB, 67.03% of ChAT-ir neurons expressed CCK, and 38.21% of CCK-ir neurons had ChAT immunoreactivity (**Figure 3.5E-H; Table 4**). In the HDB, there was a substantial population of ChAT-ir neurons (85.97%) that contained CGRP, where $31.12 \pm 0.79\%$ of CGRP-ir neurons had ChAT-immunoreactivity (**Figure 3.4I-L; Table 4**). In contrast, CCK-ir neurons co-expression was relatively lower in HDB, 52.70% of ChAT-ir neurons expressed CCK and 16.89% of CCK-ir neurons had ChAT immunoreactivity (**Figure 3.5I-L; Table 4**). In the SIB, 68.51% of ChAT-ir neurons were CGRP positive, while 28.18% of CGRP-ir neurons had ChAT immunoreactivity (**Figure 3.4M-P; Table 4**). A moderate proportion ChAT neurons had CCK immunoreactivity in SIB, where 46.34% of ChAT-ir neurons showed co-expression with CCK. On the other hand, $7.84 \pm 0.48\%$ of CCK-ir neurons showed co-expression with ChAT (**Figure 3.5M-P; Table 4**). In the CPu, a high proportion of ChAT-ir neurons expressed CGRP (75.68%), and 30.45% of CGRP-ir neurons were also expressing ChAT immunoreactivity (**Figure 3.4Q-T; Table 4**). Similarly, a considerable proportion of ChAT-ir neurons (75.70%) had CCK, and 24.71% of CCK-ir neurons contained ChAT immunoreactivity in CPu (**Figure 3.5Q-T; Table 4**).

In the basal forebrain and neostriatum, peptidergic neurons (CGRP or CCK) showed significant differences in different nuclei. Across all of the study areas, a significant proportion of ChAT-ir cells co-expressed either CGRP or CCK; notably, the HDB had the highest CGRP co-expression (85.97%), whereas the CPu had the same percentage of either co-expression (75.69%). In contrast, around one-third of the cell population of CGRP, or sometimes less than that in the case of CCK-ir neurons, co-expressed ChAT. This suggests that although many cholinergic neurons were likely to express either of these neurotransmitters, fewer CGRP or CCK-ir neurons were cholinergic. The neurochemical and potential functional variability that characterizes the cholinergic neurons is therefore shown by our results.

Table 4. Co-expression ratios of ChAT and different neuronal markers in the basalfore brain cholinergic nuclei and caudate putamen in adult mice.

Brain areas	Co-expression ratios (%)									
	Mean $\pm$ SEM									
	CGRP / ChAT	ChAT / CGRP	CCK / ChAT	ChAT / CCK	Calbindin / ChAT	ChAT / Calbindin	Calretinin / ChAT	ChAT / Calretinin	PV / ChAT	ChAT / PV
MS	71.27 $\pm$ 4.49	33.32 $\pm$ 1.02	73.67 $\pm$ 2.9	37.36 $\pm$ 1.91	00	00	00	00	00	00
VDB	76.06 $\pm$ 5.18	43.3 $\pm$ 3.39	67.03 $\pm$ 4.25	38.21 $\pm$ 1.85	27.34 $\pm$ 2.35	38.26 $\pm$ 2.68	8.52 $\pm$ 1.41	22.62 $\pm$ 2.37	00	00
HDB	85.97 $\pm$ 4.9	31.12 $\pm$ 0.78	52.7 $\pm$ 1.98	16.89 $\pm$ 1.15	32.75 $\pm$ 1.48	26.16 $\pm$ 0.86	00	00	00	00
SIB	68.51 $\pm$ 8.54	28.18 $\pm$ 2.91	46.34 $\pm$ 3.21	7.84 $\pm$ 0.81	00	00	15.25 $\pm$ 3.07	8.10 $\pm$ 1.74	00	00
CPu	75.68 $\pm$ 3	30.45 $\pm$ 1.02	75.70 $\pm$ 2.34	24.71 $\pm$ 0.95	59.74 $\pm$ 1.79	25.23 $\pm$ 0.68	6.06 $\pm$ 0.54	2.56 $\pm$ 0.17	00	00

Values represent the mean $\pm$ SEM of co-expression ratios (%) in adult mice (n = 6). SEM, standard error of mean; MS, medial septal nucleus; VDB, vertical limb of the diagonal band of Broca; HDB, horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part; CPu, caudate putamen (neostriatum); ChAT, choline acetyltransferase; CGRP, calcitonin gene-related peptide; CCK, cholecystokinin; PV, parvalbumin.

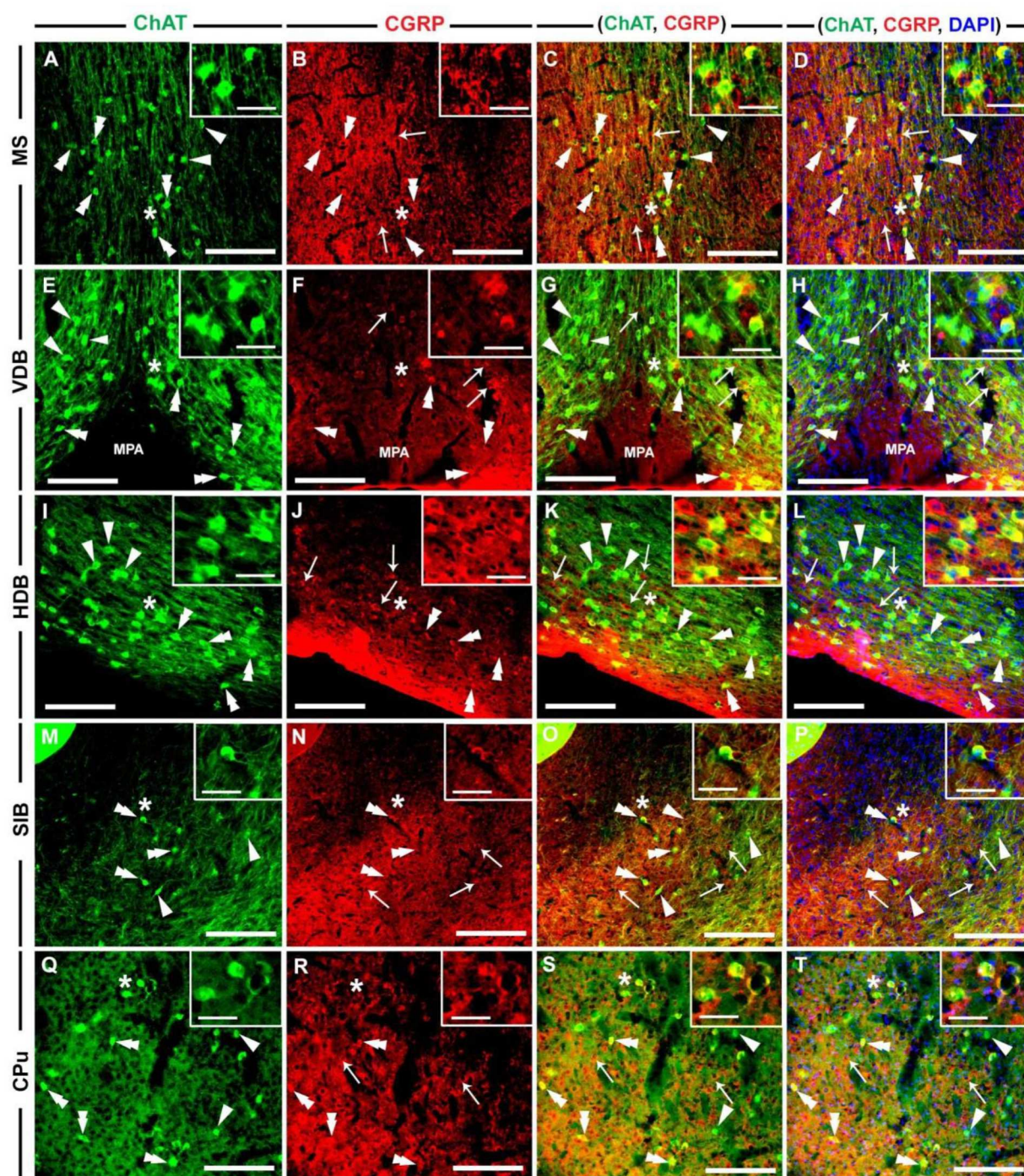


Figure 3.5. Double-label immunofluorescence immunohistochemistry for ChAT with CGRP. Photomicrographs showing immunolabeling of ChAT with CGRP in the MS (A-D), VDB (E-H), HDB (I-L), SIB (M-P), and CPu (Q-T) in the adult mice. DAPI: Nuclear marker 4',6-diamidino-2-phenylindole; blue. Single white arrowheads indicate the cells positive only for ChAT, long white arrows indicate the cells positive only for CGRP, and double white arrowheads indicate the cells positive for both ChAT and CGRP. Insets are the enlargement of the CGRP immunoreactive cells indicated by asterisks. Scale bar = 50μm in “A-T” and 20μm in inset. ChAT, Choline acetyltransferase; CGRP, Calcitonin gene-related peptide; MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band of Broca; HDB, nucleus of the horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part, and CPu, caudate putamen (neostriatum).

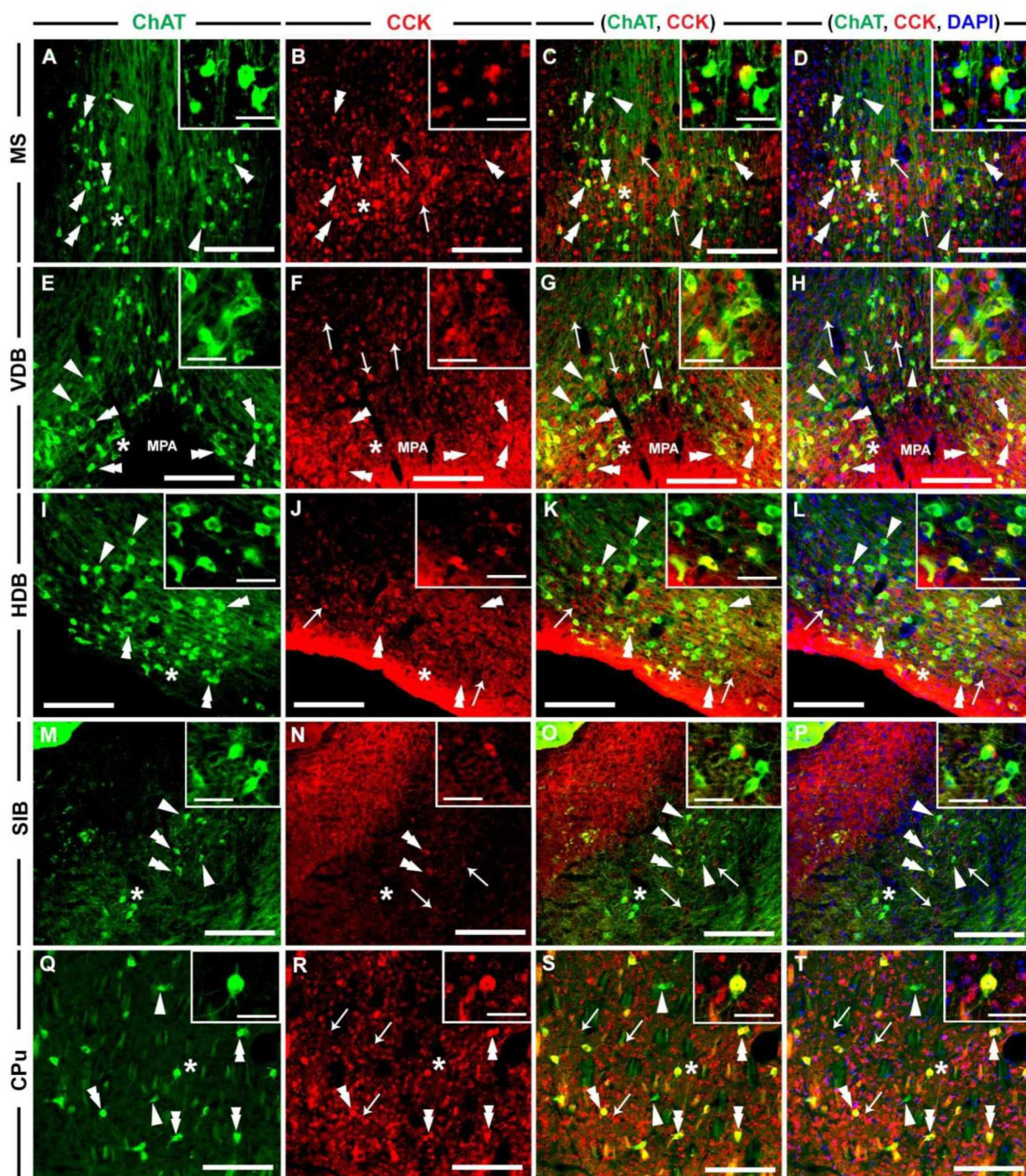


Figure 3.6. Double-label immunofluorescence immunohistochemistry for ChAT with CCK. Photomicrographs showing immunolabeling of ChAT with CCK in the in the MS (A-D), VDB (E-H), HDB (I-L), SIB (M-P), and CPu (Q-T) in the adult mice. DAPI: Nuclear marker 4',6-diamidino-2-phenylindole; blue. Single white arrowheads indicate the cells positive only for ChAT, long white arrows indicate the cells positive only for CCK, and double white arrowheads indicate the cells positive for both ChAT and CCK. Insets are the enlargement of the CCK immunoreactive cells indicated by asterisks. Scale bar = 50μm in “A-T” and 20μm in inset. ChAT, Choline acetyltransferase; CCK, Cholecystokinin; MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band of Broca; HDB, nucleus of the horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part, and CPu, caudate putamen (neostriatum).

3.3.3. Immunohistochemical relationship of ChAT with calcium-binding protein expressing neurons

We next investigated the degree of ChAT-ir neurons co-expression with the CBPs (calbindin, calretinin and parvalbumin) in order to further characterize cholinergic subtypes according to their calcium-buffering capacity and interneuron classification. In the MS, we did not find any co-expression between the ChAT and CBPs-immunoreactivity (**Figure 3.5A-D; Figure 3.7A-D; Figure 3.8A-D; Table 4**). In the VDB, 27.34% of ChAT-ir neurons co-expressed with calbindin, and 38.26% of calbindin-ir neurons had ChAT immunoreactivity (**Figure 3.5E-H; Table 4**). Co-expression ratio of calretinin with ChAT was also very low in VDB, 8.52% of ChAT-ir neurons contained calretinin and 22.62% of calretinin-ir neurons expressed with ChAT (**Figure 3.7E-H; Table 4**). However, there was no co-expression of parvalbumin with ChAT in VDB (**Figure 3.8E-H; Table 4**). In the HDB, 32.75% of ChAT-ir neurons co-expressed with calbindin, and 26.16% of calbindin-ir neurons co-expressed with ChAT (**Figure 3.5I-L; Table 4**). However, no co-expression of ChAT with both calretinin (**Figure 3.7I-L; Table 4**) and parvalbumin (**Figure 3.8I-L; Table 4**) was observed in this brain region. In the SIB, 15.25% of ChAT-ir neurons contained calretinin, while 8.10% of calretinin-ir neurons had ChAT immunoreactivity (**Figure 3.7M-P; Table 4**). Neither calbindin (**Figure 3.5M-P; Table 4**) nor parvalbumin (**Figure 3.8M-P; Table 4**) co-expression was observed with ChAT in the SIB. In the CPu, 59.74% of ChAT-ir neurons co-expressed with calbindin, and 25.23% of calbindin-ir neurons had ChAT immunoreactivity (**Figure 3.5Q-T; Table 4**). Approximately, 6.06% of ChAT immunoreactivity also had calretinin expression, whereas 2.56% of calretinin-ir neurons contained ChAT immunoreactivity in CPu (**Figure 3.7Q-T; Table 4**). In this brain area, there was no co-expression observed between the parvalbumin and ChAT immunoreactivity (**Figure 3.8Q-T; Table 4**).

Region-specific mappings of cholinergic neurons were thoroughly examined where ChAT-ir neurons co-expressed with CBPs. It was found that there was no co-localization with any of the three CBPs and ChAT at MS. The VDB and HDB nuclei, on the other hand, had almost one-third co-expression with calbindin (27.34% and 32.75%, respectively); nevertheless, the ChAT-ir neurons in both regions lacked calretinin and parvalbumin immunoreactivity. Notably, parvalbumin and calbindin did not co-express with ChAT in the SIB, and only calretinin (15.25%) had limited co-expression. However, CPu had the highest co-expression of calbindin (59.74%), the least amount of co-localization to calretinin, and no identified parvalbumin co-expression. Overall, the results indicated that the cholinergic subtypes identified by CBPs were region-specific and likely to exhibit variation in the plausible functional role of calcium-buffering characteristics.

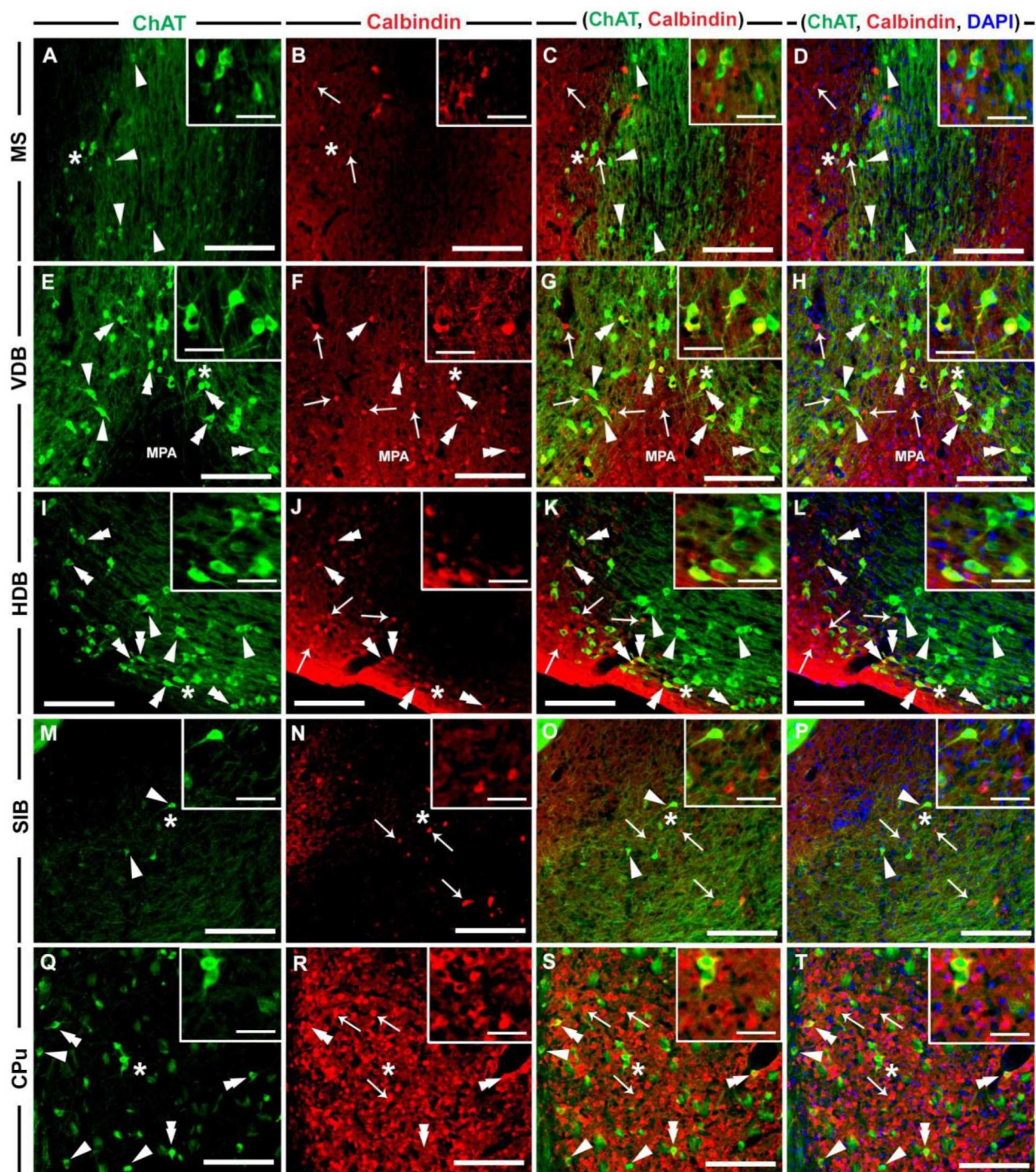


Figure 3.7. Double-label immunofluorescence immunohistochemistry for ChAT with calbindin. Photomicrographs showing immunolabeling of ChAT with calbindin in the MS (A-D), VDB (E-H), HDB (I-L), SIB (M-P), and CPu (Q-T) in the adult mice. DAPI: Nuclear marker 4',6-diamidino-2-phenylindole; blue. Single white arrowheads indicate the cells positive only for ChAT, long white arrows indicate the cells positive only for calbindin, and double white arrowheads indicate the cells positive for both ChAT and calbindin. Insets are the enlargement of the calbindin immunoreactive cells indicated by asterisks. Scale bar = 50μm in "A-T" and 20μm in inset. ChAT, Choline acetyltransferase; MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band of Broca; HDB, nucleus of the horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part, and CPu, caudate putamen (neostriatum).

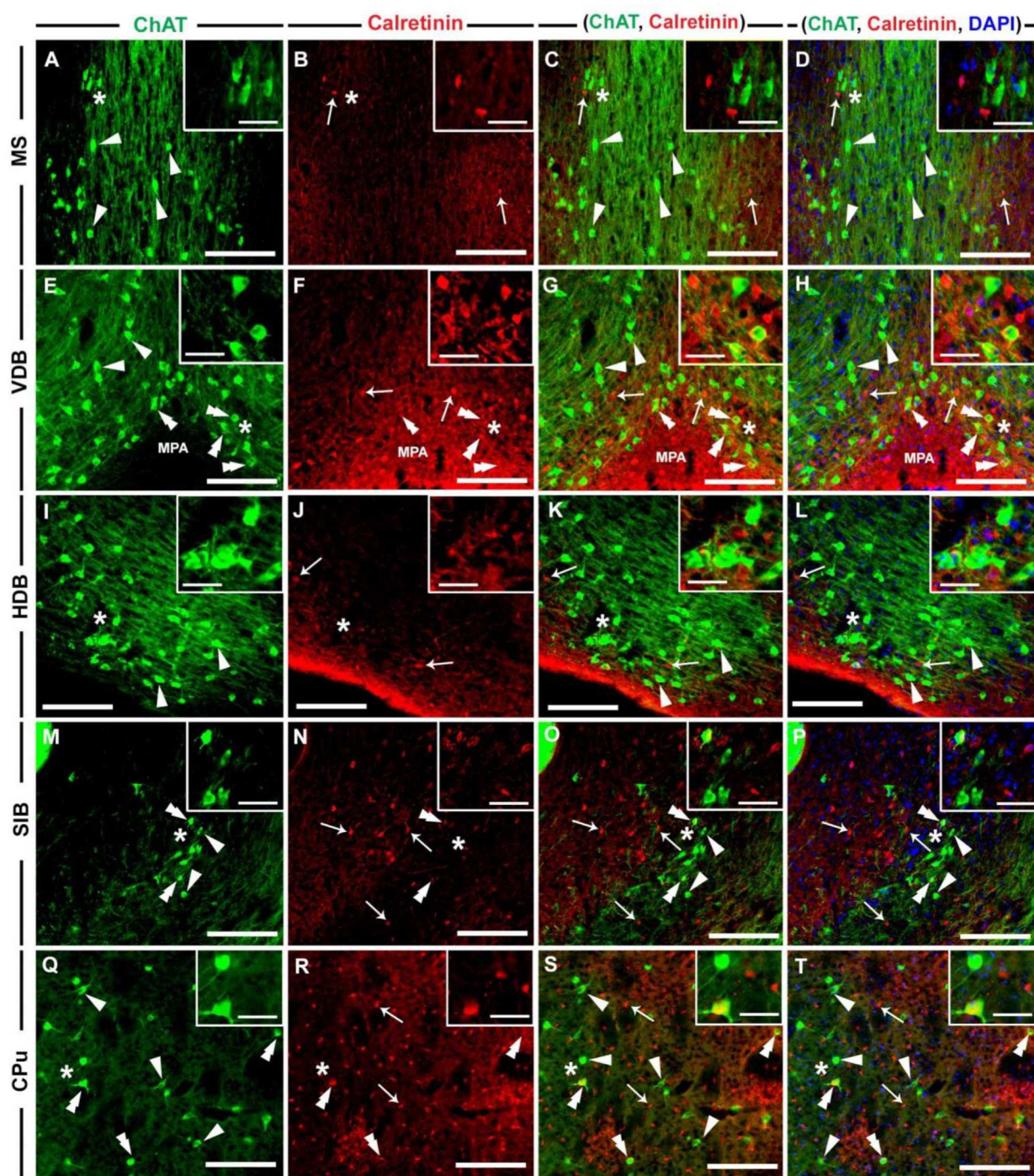


Figure 3.8. Double-label immunofluorescence immunohistochemistry for ChAT with calretinin. Photomicrographs showing immunolabeling of ChAT with calretinin in the MS (A-D), VDB (E-H), HDB (I-L), SIB (M-P), and CPu (Q-T) in the adult mice. DAPI: Nuclear marker 4',6-diamidino-2-phenylindole; blue. Single white arrowheads indicate the cells positive only for ChAT, long white arrows indicate the cells positive only for calretinin, and double white arrowheads indicate the cells positive for both ChAT and calretinin. Insets are the enlargement of the calretinin immunoreactive cells indicated by asterisks. Scale bar = 50µm in "A-T" and 20µm in inset. ChAT, Choline acetyltransferase; MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band of Broca; HDB, nucleus of the horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part, and CPu, caudate putamen (neostriatum).

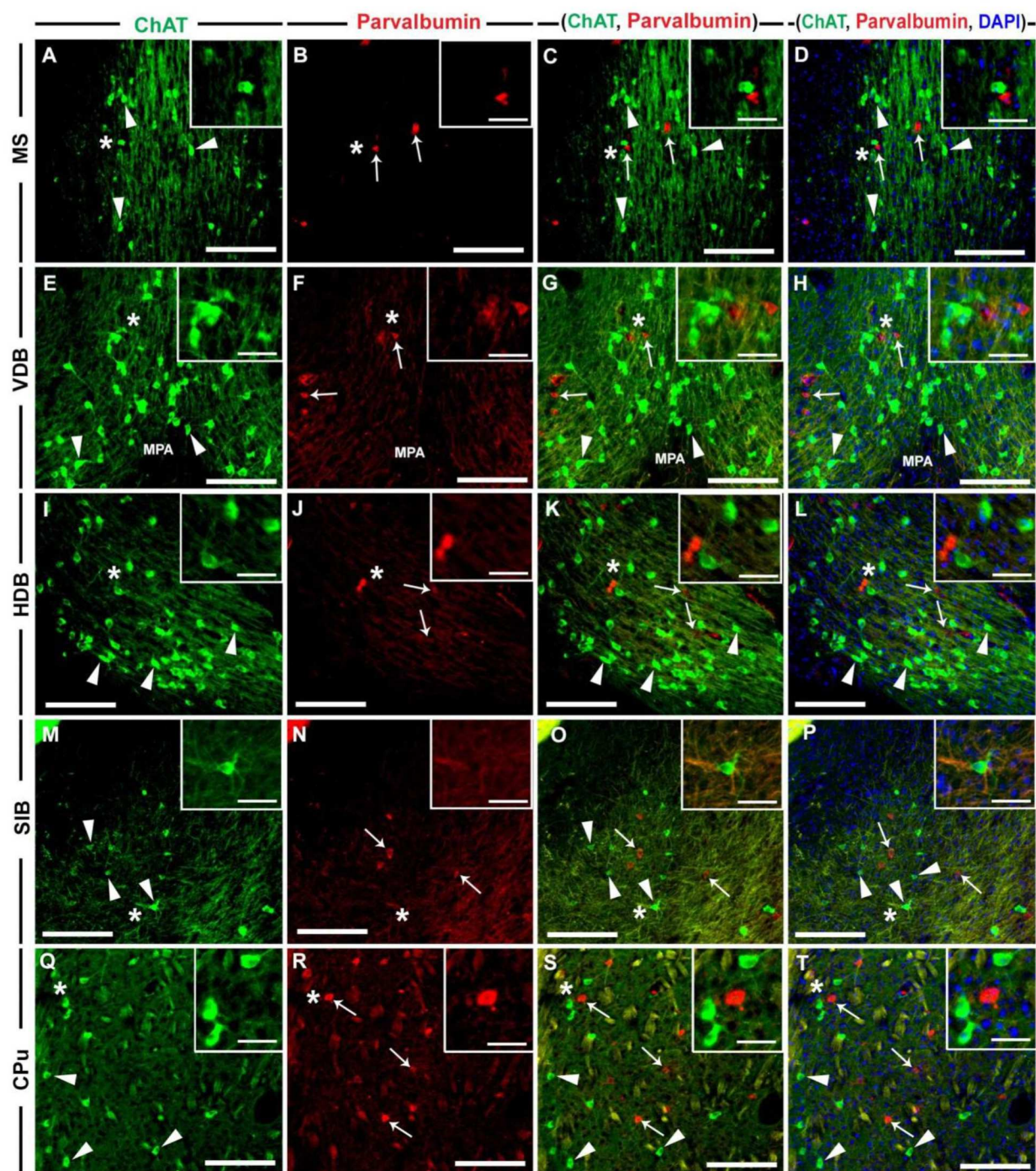


Figure 3.9. Double-label immunofluorescence immunohistochemistry for ChAT with parvalbumin. Photomicrographs showing immunolabeling of ChAT with parvalbumin in the MS (A-D), VDB (E-H), HDB (I-L), SIB (M-P), and CPu (Q-T) in the adult mice. DAPI: Nuclear marker 4',6-diamidino-2-phenylindole; blue. Single white arrowheads indicate the cells positive only for ChAT, long white arrows indicate the cells positive only for parvalbumin, and double white arrowheads indicate the cells positive for both ChAT and parvalbumin. Insets are the enlargement of the parvalbumin immunoreactive cells indicated by asterisks. Scale bar = 50µm in "A-T" and 20µm in inset. ChAT, Choline acetyltransferase; MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band of Broca; HDB, nucleus of the horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part, and CPu, caudate putamen (neostriatum).

3.3.4. Immunohistochemical relationship of HAP1 with neuropeptide-expressing neurons

This method made it possible to locate subpopulations of neurons that expressed neuropeptide and were immunoreactive to HAP1, which showed region-specific interactions between HAP1 and the peptidergic neurons. In the MS, $47.07 \pm 3.99\%$ of HAP1-ir had the CGRP neurons, while $28.77 \pm 0.86\%$ of CGRP-ir neurons expressed HAP1 (**Figure 3.10A–D; Table 5**). Almost similar proportions of co-localization were found between HAP1 and CCK, in which $41.3 \pm 3.78\%$ of HAP1-ir neurons contained CCK and $23.78 \pm 3.23\%$ of CCK-ir neurons were HAP1 positive (**Figure 3.11A–D; Table 5**). About $48.83 \pm 1.9\%$ of HAP1-ir cells expressed CGRP in the VDB and CGRP -ir cells contained HAP1 immunoreactivity were $36.16 \pm 1.26\%$ (**Figure 3.10E–H; Table 5**). The co-expression between HAP1 and CCK was present in the VDB. About $36.23 \pm 4.34\%$ of HAP1-ir neurons expressed CCK, while $22.87 \pm 2.14\%$ of CCK-ir neurons were positive for HAP1 (**Figure 3.11E–H; Table 5**). The association between HAP1 and CGRP was also perceived in the HDB. About $35.46 \pm 1.09\%$ of HAP1-ir neurons were CGRP positive, and $17.06 \pm 0.86\%$ of CGRP-ir neurons contained HAP1 (**Figure 3.10I–L; Table 5**). Likewise, $26.18 \pm 2.42\%$ of HAP1-ir neurons exhibited CCK co-localization as well as $12.39 \pm 1.06\%$ of CCK-ir neurons expressed HAP1 (**Figure 3.11I–L; Table 5**). No co-expression between HAP1 and either CGRP or CCK was observed in the SIB (**Figure 3.10M–P, 3.11M–P; Table 5**). In the CPu, a small population of HAP1-ir neurons ($7.69 \pm 1.77\%$) contained CGRP, whereas only $0.68 \pm 0.11\%$ of CGRP-ir neurons expressed HAP1 (**Figure 3.10Q–T; Table 5**). Co-localization of HAP1 with CCK was not detected in the CPu (**Figure 3.11Q–T; Table 5**).

Table 5. Co-expression ratios of HAP1 and different neuronal markers in the basal fore brain cholinergic nuclei and caudate putamen in adult mice.

Brain areas	Co-expression ratios (%)									
	Mean $\pm$ SEM									
	CGRP / HAP1	HAP1 / CGRP	CCK / HAP1	HAP1 / CCK	Calbindin / HAP1	HAP1 / Calbindin	Calretinin / HAP1	HAP1 / Calretinin	PV / HAP1	HAP1 / PV
MS	47.07 $\pm$ 3.99	28.77 $\pm$ 0.86	41.3 $\pm$ 3.78	23.78 $\pm$ 3.23	15.8 $\pm$ 1.5	16.71 $\pm$ 1.25	13.87 $\pm$ 2.7	17.14 $\pm$ 3.45	0	0
VDB	48.83 $\pm$ 1.9	36.16 $\pm$ 1.26	36.23 $\pm$ 4.34	22.87 $\pm$ 2.14	26.24 $\pm$ 1.96	36.25 $\pm$ 4.56	19.47 $\pm$ 1.52	31.53 $\pm$ 1.79	0	0
HDB	35.46 $\pm$ 1.09	17.06 $\pm$ 0.86	26.18 $\pm$ 2.42	12.39 $\pm$ 1.06	21.23 $\pm$ 0.89	23.59 $\pm$ 1.7	10.76 $\pm$ 1.36	18.76 $\pm$ 2.99	0	0
SIB	0	0	0	0	12.16 $\pm$ 0.99	19.47 $\pm$ 1.69	10.44 $\pm$ 0.89	28.37 $\pm$ 2.65	0	0
CPu	7.69 $\pm$ 1.77	0.68 $\pm$ 0.11	0	0	0	0	8.09 $\pm$ 1.16	0.9 $\pm$ 0.17	0	0

Values represent the mean $\pm$ SEM of co-expression ratios (%) in adult mice (n = 6). SEM, standard error of mean; MS, medial septal nucleus; VDB, vertical limb of the diagonal band of Broca; HDB, horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part; CPu, caudate putamen (neostriatum); HAP1, Huntingtin associated protein 1; CGRP, calcitonin gene-related peptide; CCK, cholecystokinin; PV, parvalbumin.

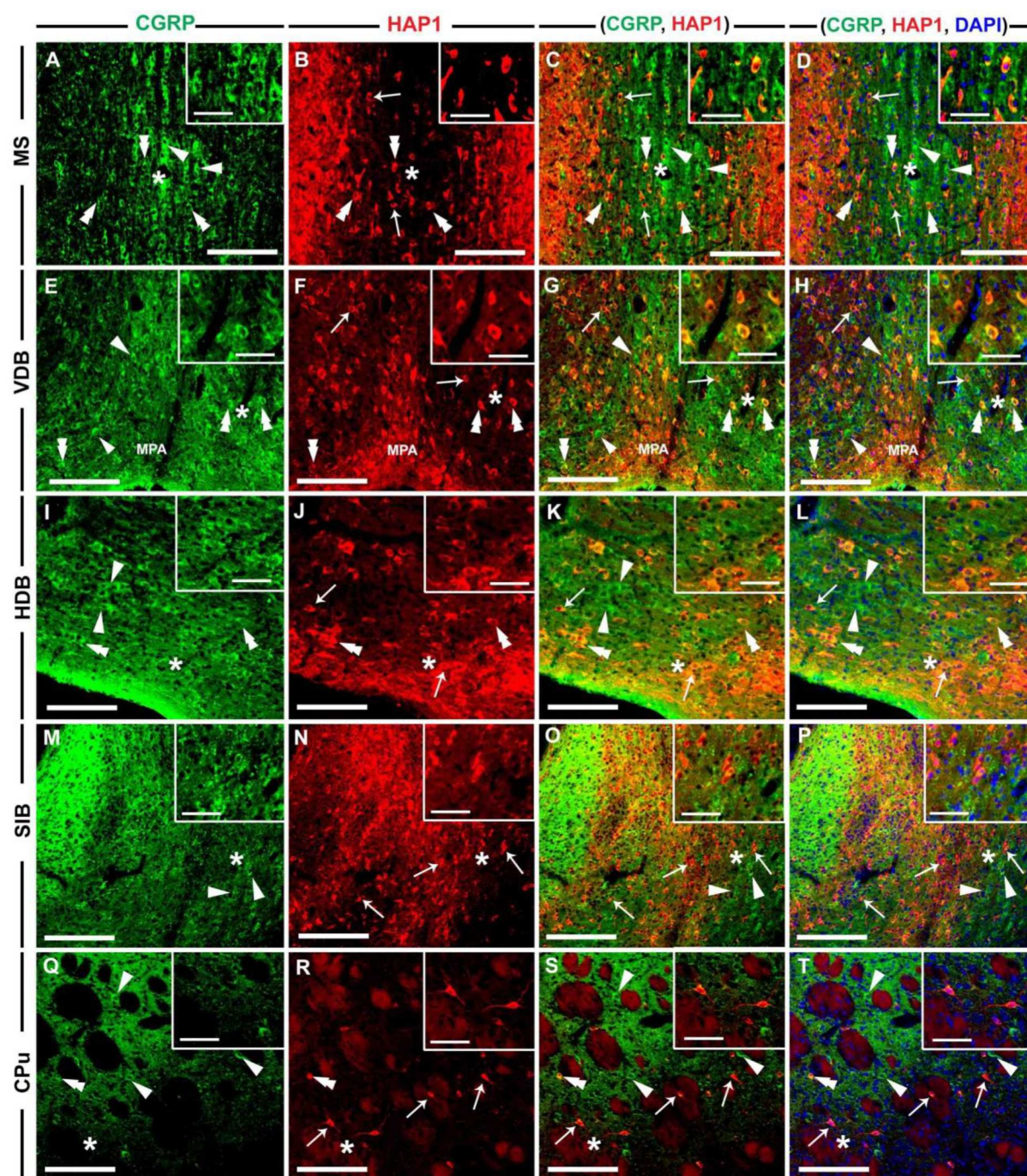


Figure 3.10. Double-label immunofluorescence immunohistochemistry for HAP1 with CGRP. Photomicrographs showing immunolabeling of HAP1 with CGRP in the MS (A-D), VDB (E-H), HDB (I-L), SIB (M-P), and CPu (Q-T) in the adult mice. DAPI: Nuclear marker 4',6-diamidino-2-phenylindole; blue. Single white arrowheads indicate the cells positive only for HAP1, long white arrows indicate the cells positive only for CGRP, and double white arrowheads indicate the cells positive for both HAP1 and CGRP. Insets are the enlargement of the CGRP immunoreactive cells indicated by asterisks. Scale bar = 50µm in "A-T" and 20µm in inset. HAP1, Huntingtin Associated Protein 1; CGRP, Calcitonin gene-related peptide; MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band of Broca; HDB, nucleus of the horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part, and CPu, caudate putamen (neostriatum).

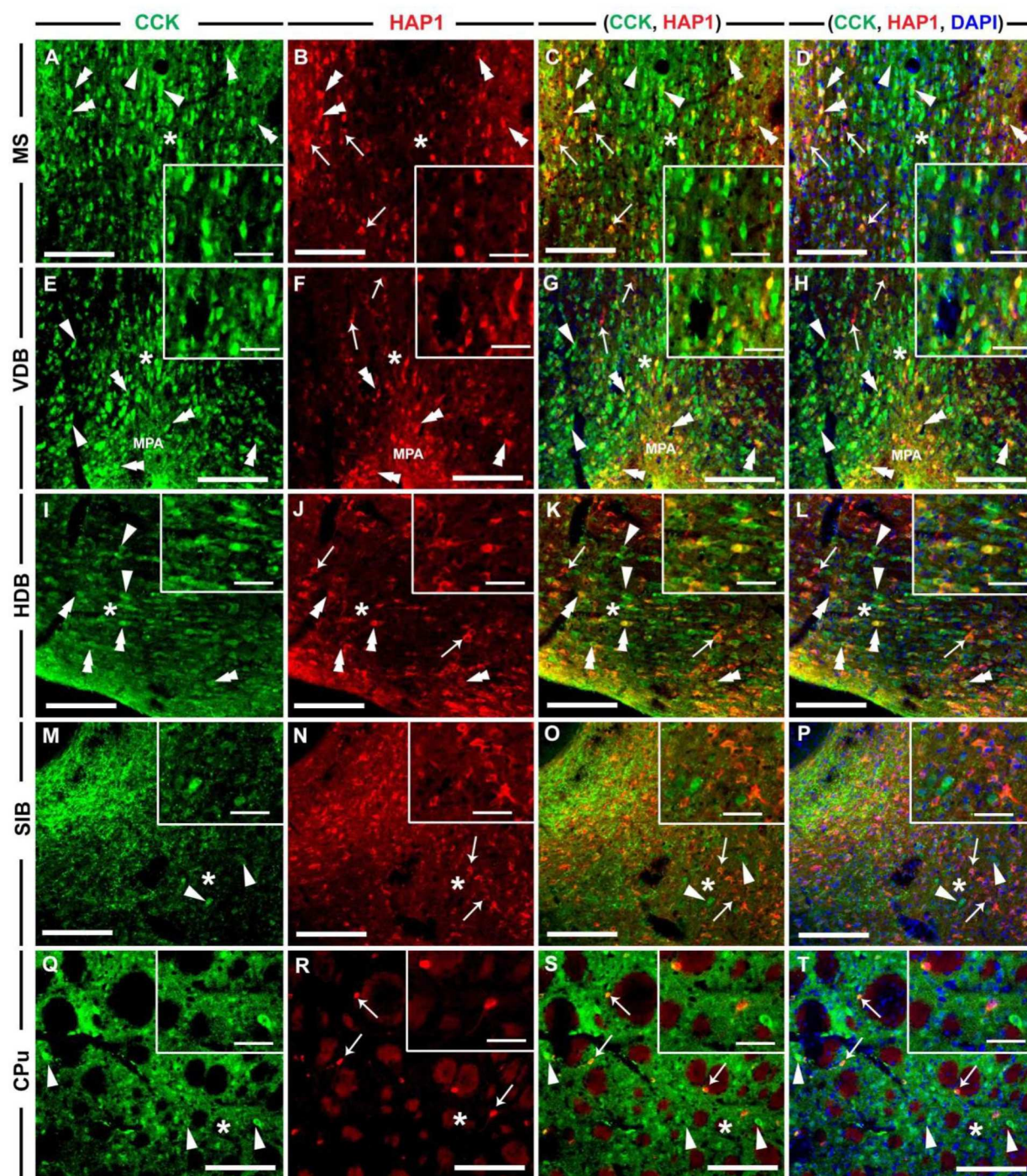


Figure 3.11. Double-label immunofluorescence immunohistochemistry for HAP1 with CCK. Photomicrographs showing immunolabeling of HAP1 with CCK in the in the MS (A-D), VDB (E-H), HDB (I-L), SIB (M-P), and CPu (Q-T) in the adult mice. DAPI: Nuclear marker 4',6-diamidino-2-phenylindole; blue. Single white arrowheads indicate the cells positive only for HAP1, long white arrows indicate the cells positive only for CCK, and double white arrowheads indicate the cells positive for both HAP1 and CCK. Insets are the enlargement of the CCK immunoreactive cells indicated by asterisks. Scale bar = 50µm in "A-T" and 20µm in inset. HAP1, Huntingtin Associated Protein 1; CCK, Cholecystokinin; MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band of Broca; HDB, nucleus of the horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part, and CPu, caudate putamen (neostriatum).

3.3.5. Immunohistochemical relationship of HAP1 with calcium-binding protein expressing neurons

We then looked at the degree of co-expression of HAP1-ir neurons with the calcium-binding proteins (calbindin, calretinin, and parvalbumin) to further describe HAP1-expressing neuronal subtypes in terms of calcium-buffering capacity and interneuron identity. In the MS, about $15.8 \pm 1.5\%$ of HAP1-ir neurons contained calbindin where $16.71 \pm 1.25\%$ of calbindin-ir neurons expressed HAP1 (**Figure 3.12A–D; Table 5**). Co-expression between HAP1 and calretinin was also evident in this region. The HAP1-ir neurons ($13.87 \pm 2.7\%$) showed the calretinin expression and $17.14 \pm 3.45\%$ of calretinin-ir neurons showed HAP1 immunoreactivity (**Figure 3.13A–D; Table 5**). However, no co-expression of HAP1 with PV was observed in the MS (**Figure 3.14A–D; Table 5**). In another basal forebrain area VDB, $26.24 \pm 1.96\%$ of HAP1-ir neurons expressed calbindin and $36.25 \pm 4.56\%$ of calbindin-ir neurons contained HAP1 immunoreactivity (**Figure 3.12E–H; Table 5**). A moderate level of co-localization was observed between HAP1 and calretinin, where $19.47 \pm 1.52\%$ of HAP1-ir neurons expressed calretinin and $31.53 \pm 1.79\%$ of calretinin-ir neurons were HAP1 positive (**Figure 3.13E–H; Table 5**). Similar to the MS, no PV/HAP1 co-expression was found in the VDB (**Figure 3.14E–H; Table 5**). In the HDB, calbindin positive were $21.23 \pm 0.89\%$ of HAP1-ir neurons and average $23.59 \pm 1.7\%$ of calbindin-ir neurons expressed HAP1 (**Figure 3.12I–L; Table 5**). Co-expression between HAP1 and calretinin was present. The $10.76 \pm 1.36\%$ of HAP1-ir neurons were expressing calretinin and $18.76 \pm 2.99\%$ of calretinin-ir were containing HAP1 (**Figure 3.13I–L; Table 5**). No PV/HAP1 co-expression was detected in this region (**Figure 3.14I–L; Table 5**). In the SIB, $12.16 \pm 0.99\%$ of HAP1-ir neurons expressed calbindin while $19.47 \pm 1.69\%$ of calbindin-ir neurons were HAP1 positive (**Figure 3.12M–P; Table 5**). A comparable level of co-localization was found between HAP1 and calretinin in SIB. The $10.44 \pm 0.89\%$ of HAP1-ir neurons contained calretinin, and $28.37 \pm 2.65\%$ of calretinin-ir neurons expressed HAP1 (**Figure 3.13M–P; Table 5**). As like in other brain regions, HAP1 did not co-localize with PV in the SIB (**Figure 3.14M–P; Table 5**). In the CPu, HAP1 showed co-localization only with calretinin, where $8.09 \pm 1.16\%$ of HAP1-ir neurons expressed calretinin, and $0.9 \pm 0.17\%$ of calretinin-ir neurons were HAP1 positive (**Figure 3.13Q–T; Table 5**). No co-expression was found between HAP1 and either calbindin or PV in this region (**Figure 3.12Q–T; Figure 3.14Q–T; Table 5**).

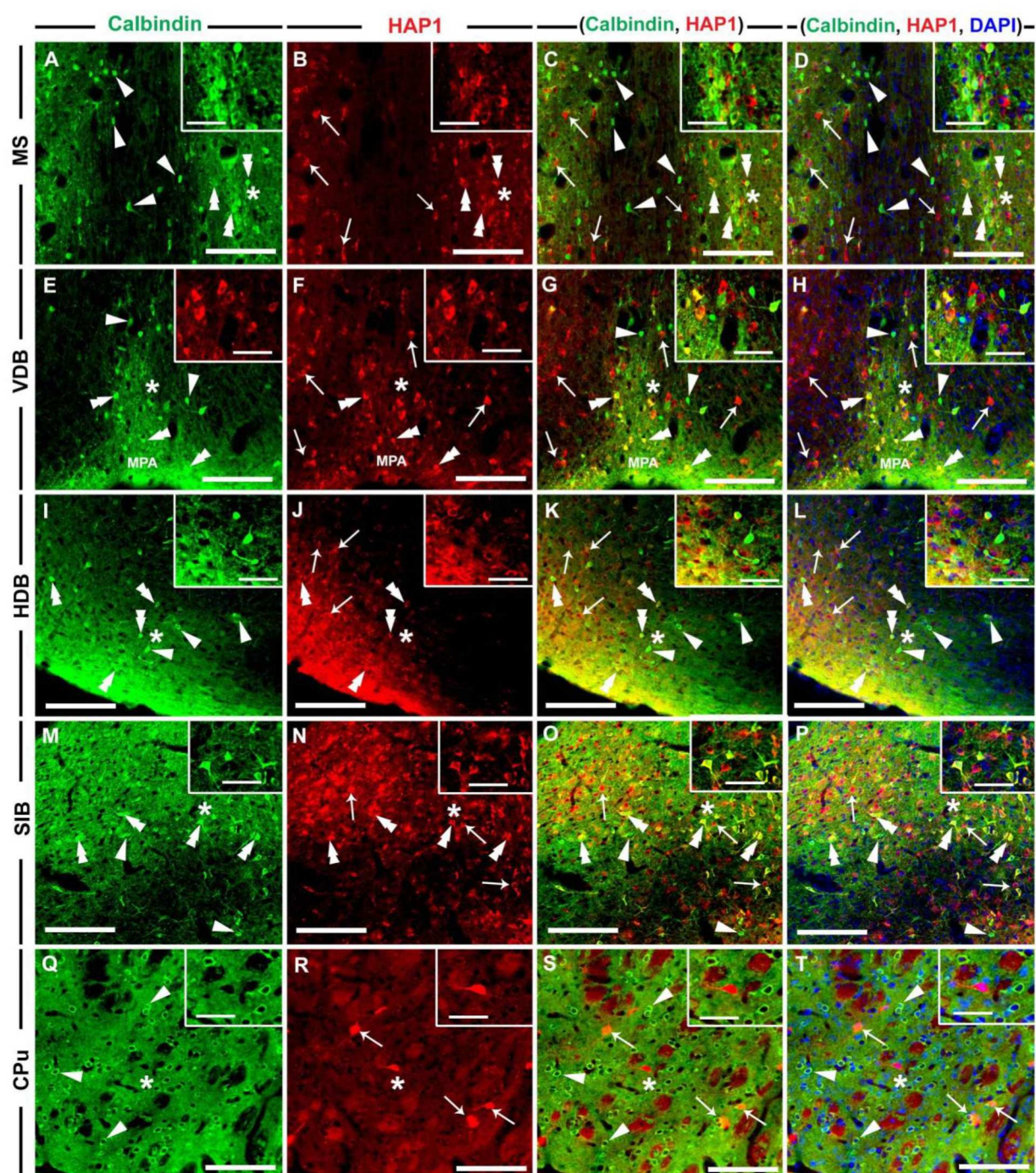


Figure 3.12. Double-label immunofluorescence immunohistochemistry for HAP1 with calbindin. Photomicrographs showing immunolabeling of HAP1 with calbindin in the MS (A-D), VDB (E-H), HDB (I-L), SIB (M-P), and CPu (Q-T) in the adult mice. DAPI: Nuclear marker 4',6-diamidino-2-phenylindole; blue. Single white arrowheads indicate the cells positive only for HAP1, long white arrows indicate the cells positive only for calbindin, and double white arrowheads indicate the cells positive for both HAP1 and calbindin. Insets are the enlargement of the calbindin immunoreactive cells indicated by asterisks. Scale bar = 50µm in "A-T" and 20µm in inset. HAP1, Huntingtin Associated Protein 1; MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band of Broca; HDB, nucleus of the horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part, and CPu, caudate putamen (neostriatum).

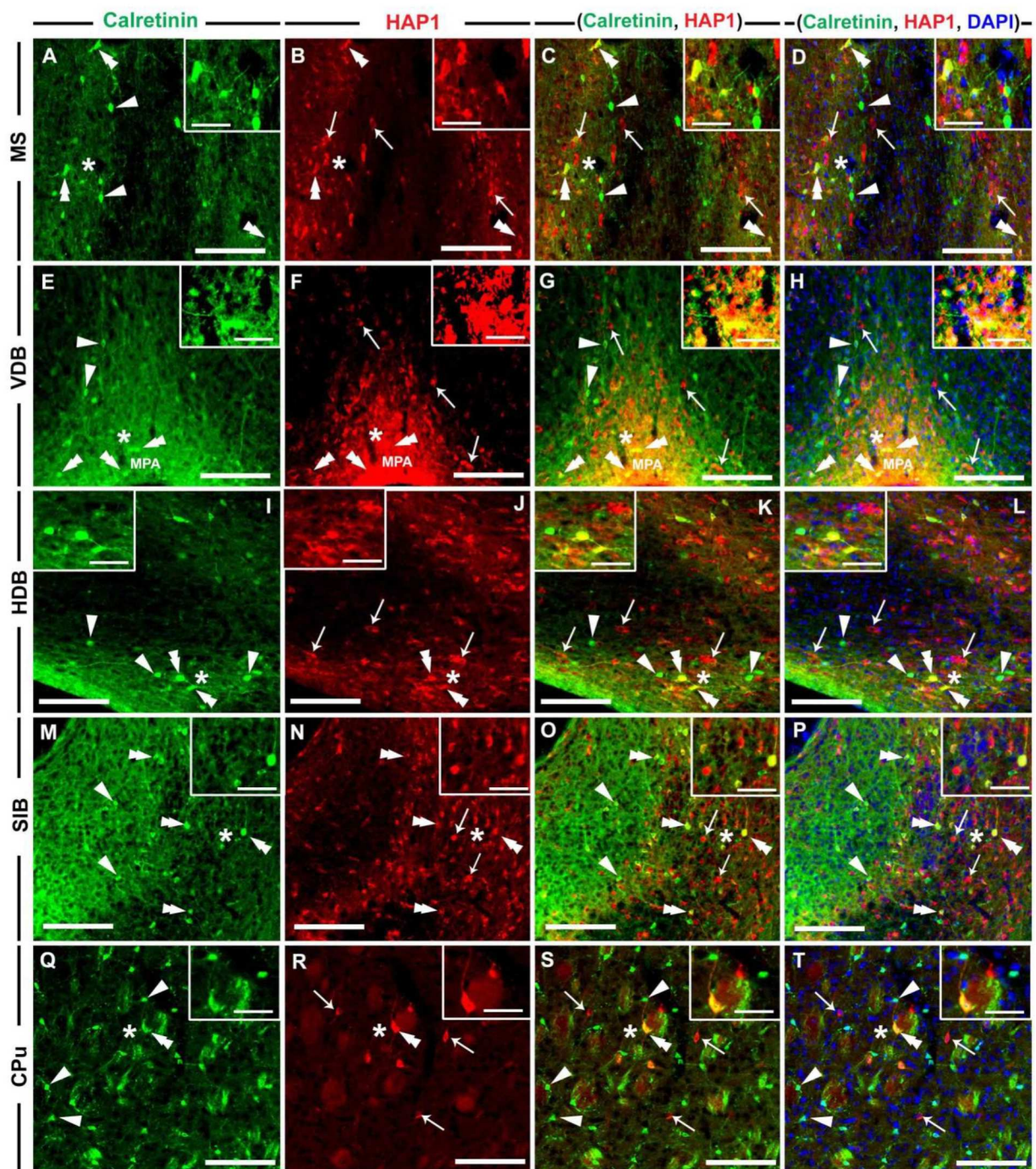


Figure 3.13. Double-label immunofluorescence immunohistochemistry for HAP1 with calretinin. Photomicrographs showing immunolabeling of HAP1 with calretinin in the MS (A-D), VDB (E-H), HDB (I-L), SIB (M-P), and CPu (Q-T) in the adult mice. DAPI: Nuclear marker 4',6-diamidino-2-phenylindole; blue. Single white arrowheads indicate the cells positive only for HAP1, long white arrows indicate the cells positive only for calretinin, and double white arrowheads indicate the cells positive for both HAP1 and calretinin. Insets are the enlargement of the calretinin immunoreactive cells indicated by asterisks. Scale bar = 50µm in "A-T" and 20µm in inset. HAP1, Huntingtin Associated Protein 1; MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band of Broca; HDB, nucleus of the horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part, and CPu, caudate putamen (neostriatum).

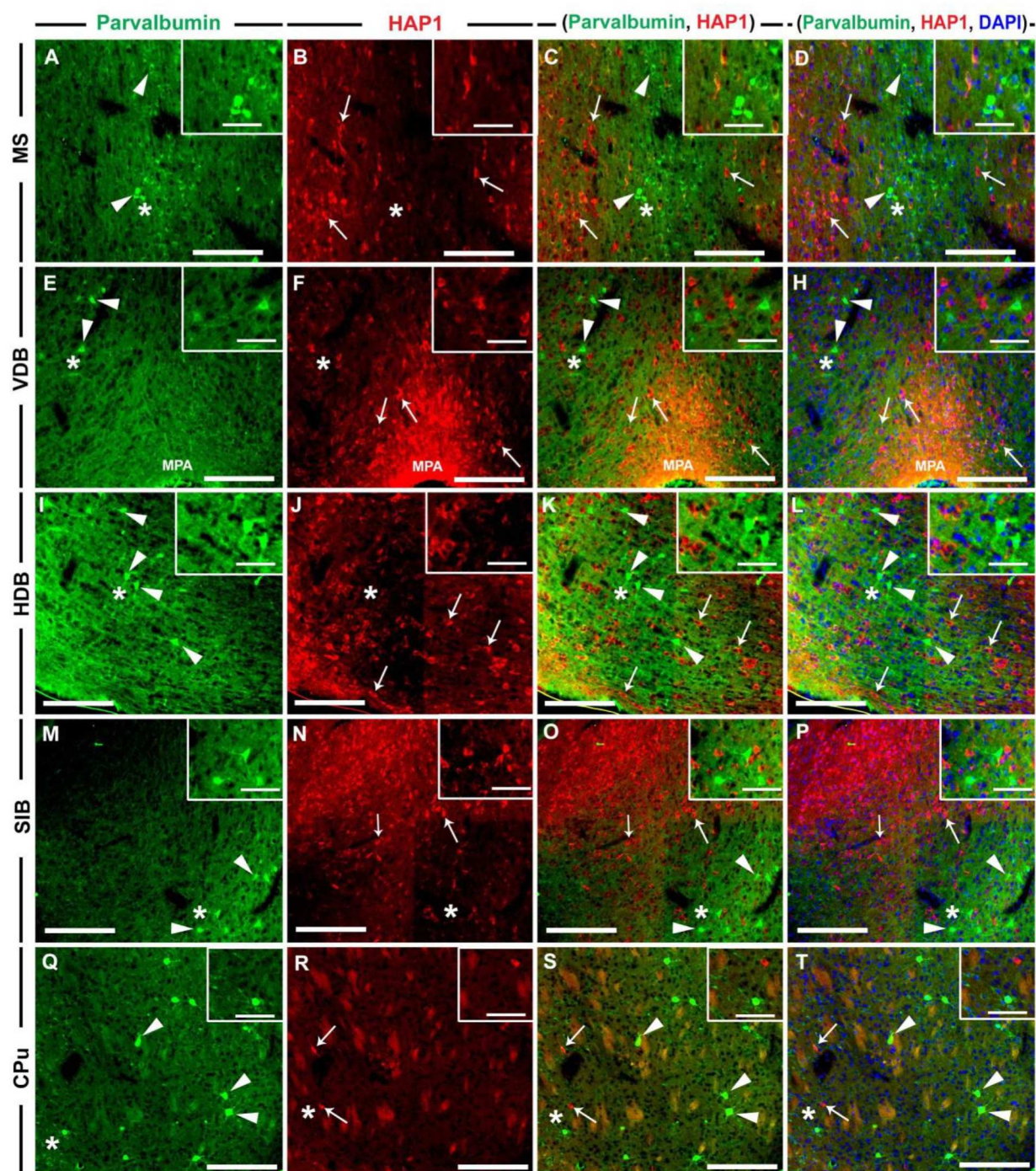


Figure 3.14. Double-label immunofluorescence immunohistochemistry for HAP1 with parvalbumin. Photomicrographs showing immunolabeling of HAP1 with parvalbumin in the MS (A-D), VDB (E-H), HDB (I-L), SIB (M-P), and CPu (Q-T) in the adult mice. DAPI: Nuclear marker 4',6-diamidino-2-phenylindole; blue. Single white arrowheads indicate the cells positive only for HAP1, long white arrows indicate the cells positive only for parvalbumin, and double white arrowheads indicate the cells positive for both HAP1 and parvalbumin. Insets are the enlargement of the parvalbumin immunoreactive cells indicated by asterisks. Scale bar = 50µm in "A-T" and 20µm in inset. HAP1, Huntingtin Associated Protein 1; MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band of Broca; HDB, nucleus of the horizontal limb of the diagonal band of Broca; SIB, substantia innominata basal part, and CPu, caudate putamen (neostriatum).

3.4. Discussion

In this study, we demonstrate that cholinergic neuronal heterogeneity exists in the BFCN and neostriatum (CPu). Specifically, we found that ChAT-ir neurons in subsets contained neuropeptides such as CGRP and CCK, CBPs such as calbindin and calretinin, but not parvalbumin. These results support the idea that cholinergic neurons are heterogeneous in that they contain different subtypes that are highly diverse in terms of their molecular markers and may have different physiological characteristics or functional roles. A previous study on cholinergic neurons in the arcuate nucleus of the hypothalamus revealed remarkable distinctions in gene expression patterns among individual cholinergic neurons (Jeong et al., 2016). Some of these neurons co-express tyrosine hydroxylase, while others are GABAergic or glutamatergic, demonstrating their neurochemical heterogeneity (Jeong et al., 2016). This heterogeneous population of cholinergic neurons may be part of the neural circuitries that are essential for fine-tuning brain functions including the cognitive mechanisms, sensory processing, motor activity, and arousal (Ahmed et al., 2019).

Strong and widespread distribution of ChAT-ir neurons with elongated, fusiform and multiple processes were found throughout the BFCN and CPu which is similar to the findings of other research (Northington et al., 2022). This findings of strong ChAT immunoreactivity in the BFCN is inconsistent with the established understanding that ChAT neurons characterize a primary source of cholinergic signaling throughout the mouse brain (Ahmed et al., 2019; Zaborszky et al., 2015). Anatomically, the MS and VDB are the major cholinergic source to the hippocampus (Bohnen et al., 2022; Eickhoff et al., 2022a; He et al., 2023). Moreover, the cholinergic neurons in the MS are crucial for sensorimotor gating (Jin et al., 2019) and play a vital role in the generation of theta waves (essential for learning and memory processes) within the hippocampus (Király et al., 2023). The nerve fibers of VDB project to the fornix and fimbria via the MS, and then projection fibers reach the hippocampus through the amygdala (He et al., 2023; Woolf, 1991). However, most of the cholinergic inputs to the hippocampus may come from VDB through the MS. Cholinergic neuronal projections from VDB mediate decision-making, planning, and ascribing salience, while projections to the hippocampus and amygdala influence attention, memory, fear, and stress (Picciotto et al., 2012). The HDB has cholinergic neurons which project to the olfactory bulb and piriform cortex, affecting olfactory behavior, including the ability to discriminate between odors as well as memory of olfactory information (Bohnen et al., 2022; He et al., 2023). SIB and especially NBM is a major source of extensive cholinergic innervation to the frontal, temporal, occipital and parietal cortex or amygdala (Bohnen et al., 2022). These neurons are crucially involved in functions like arousal, attention, vision and plasticity within the cortex (He et al., 2023). Neuronal homeostasis deviation is the decisive factor in both irreversible degeneration and neuronal apoptosis. The quantity of

cholinergic neurons generally declines when the cholinergic system is malfunctioning (Chen et al., 2022). Memory and cognitive deficits are associated with a decline in acetylcholine transferase activity and defective ACh receptors (Chen et al., 2022). Particularly in frontal cortex and hippocampus, neuronal apoptosis and gray matter loss relate to the significant loss of memory and cognitive function (Wu et al., 2020). Furthermore, cholinergic neurons of the CPu project indirectly into the substantia nigra pars compacta (SNpc). Then specific brain regions receive axonal efferent from this nucleus, each of which has distinct functional ramifications (Stanley et al., 2021). The knowledge of synaptic plasticity mechanisms in the corticostriatal circuits has advanced significantly in recent years, and ACh has joined the hypothesis along with dopamin (Surmeier et al., 2010). Neuronal degeneration throughout the striatum displays spatial topography and standardized time course patterns, unique to tauopathies, AD, PD, and HD (Kawakami et al., 2014). It is thought that each neurodegenerative disease has unique patterns of striatal degeneration (Del Rey and García-Cabezas, 2023). However, the selective vulnerability of striatal neurons compared to other neuron structures has remained as a vague and critical question in most neurodegenerative disorders (Meher et al., 2025b).

The subsets of peptidergic neurons including CGRP and CCK are the prominent neuromodulators; perhaps they also modulate the function of the basal forebrain and striatal circuits (Asim et al., 2024; Russo and Hay, 2023). The strong expression of CGRP and CCK in both the BFCN and CPu confirms their prominent roles as neuromodulators. The pronounced expression of CGRP and CCK neuronal marker in the both BFCN and CPu confirms prominent role of those neuromodulators. The expression of CGRP may reflect its role in the modulation of vascular tone and in nociceptive processing (Durham, 2004), whereas the expression of CCK demonstrated that BFCN and CPu in the brain may be involved in the regulation of arousal and cortical activation (Ma and Giardino, 2022). In other reports, the CGRP was also found in neurons and nerve fibers throughout the cerebral cortex, thalamus, and hypothalamus, even in the cerebellum, hippocampus, amygdala, and basal ganglia (Iyengar et al., 2019). Accumulating evidence revealed that, CGRP enhances the expression of acetylcholine receptors, inhibits the release of acetylcholinesterase, and produce the Ca^{2+} -induced intracellular Ca^{2+} mobilization. Moreover, exogenous CGRP treatment has been anticipated to lower the amplitude of inflammation in AD by preventing macrophage infiltration and the expression of many inflammatory mediators and cell adhesion molecules (Singh et al., 2017). Remarkably, we found the highest proportion of ChAT-ir neurons contained CGRP in the VDB, HDB. These co-expressive neurons in VDB and HDB are usually long-range projection neurons that target the cortex and hippocampus via ACh for cholinergic modulation, vascular tone or synaptic plasticity (Iyengar et al., 2019; Tian et al., 2022). The high co-expression ratio of CGRP with ChAT in CPu,

suggests that these neurons are most likely striatopallidal cholinergic interneurons that modulate basal ganglia circuitry (Gonzales and Smith, 2015). However, this co-expression may enhance dynamic regulation of the cortical arousal, sensory gating, or motor function. Such neurons could also contribute to pathological states such as migraine, chronic pain or neuroinflammation, to which CGRP signaling is involved.

CCK is known to regulate cortical arousal and anxiety-related behaviors (Ma and Giardino, 2022). Additionally, cortical CCK can enhance audio-visual processing, and alter recognition memory. The Neuroprotective effects of CCK have already been explained concerning certain ND e.g. AD and somewhat PD (Reich and Hölscher, 2024). In our current study, CCK-ir neurons were also highly co-expressed with ChAT. The utmost co-expression rates of ChAT with CCK were observed in the MS and CPu, which supports the hypothesis that CCK plays a role in regulating cholinergic output to the cortex and striatum (Figure 3.14). These CCK co-expressing cholinergic neurons are of particular interest due to the hippocampal projections originating from the MS. Significantly, activation of nigral or hippocampal CCK-2Rs is contemplated neuroprotective by inducing autophagy, mitochondrial fusion modulator expression, and AMPK activation. Therefore, pertinent to AD, CCK intensifies BDNF signaling while altering hippocampus neurotrophin receptors and decreasing neural growth factors and apoptosis (Reich and Hölscher, 2024). Therefore, neurons of MS with dual cholinergic and peptidergic phenotypes may modulate hippocampal circuits with a greater aspect of functional specificity. However, striatal cholinergic interneurons are known to regulate local cortico-striatal and thalamo-striatal transmission (Tanimura et al., 2018). The co-expression of CCK with these cholinergic neurons suggests additional layers of modulatory influence over the basal ganglia circuit. The explicit loss of dopaminergic neurons in the SNpc is believed to be a hallmark of PD that regulate movements by traveling down the nigrostriatal pathway into the neostriatum (Dickson, 2012). Through the mesolimbic pathway, dopamine may cause the release of CCK. Furthermore, high sulphated CCK-8 dosages have been shown to stimulate striatal dopamine release, which may elicit axonal dopamine release in the striatum *in vivo* by activating CCK-1 receptors, hence immediately improving motor function (You et al., 1996).

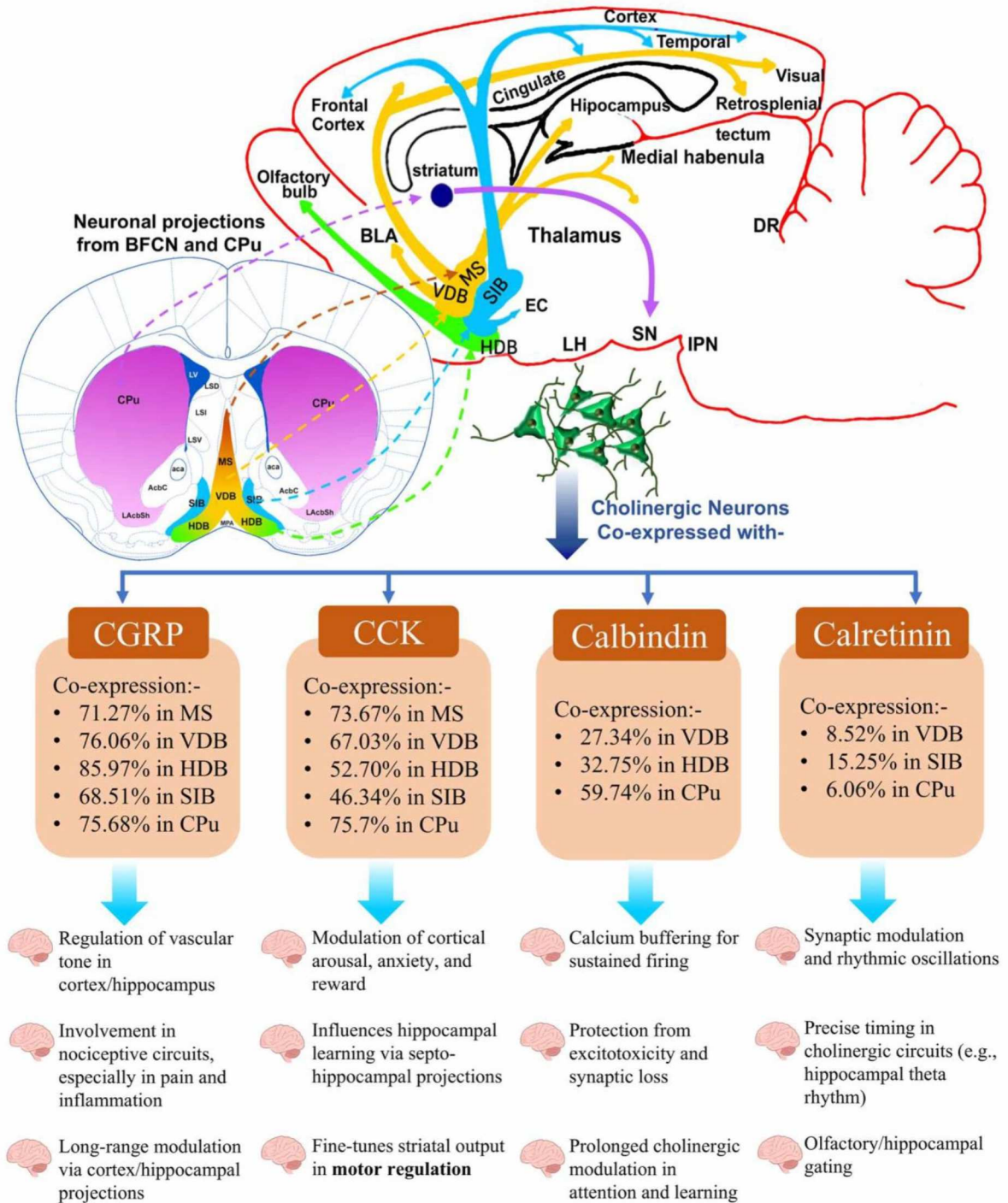


Figure 3.15. Schematic representation of the conjectural functional implications of CGRP, CCK, calretinin, and calbindin co-expression in cholinergic neurons of the BFCN and CPu. This graphic suggests functional variability within the cholinergic neurons projected to particular locations by showing different subpopulations of co-expressing neurons. Neuropeptides (CCK and CGRP) co-expression with ChAT has a neuromodulatory profile that may control limbic-cortical communication, stress, pain, arousal, motor control, and emotional behavior. Calcium-binding proteins, such as calbindin or calretinin, are co-expressed with cholinergic neurons of the BFCN and most likely have improved calcium-buffering capabilities. This would support neuronal excitability, guard against excitotoxic effects and synaptic loss. Co-expression of these markers by ChAT-positive striatal interneurons may help integrate local circuits in the CPu and control dopaminergic, thalamostriatal, and corticostriatal transmission. All of these results highlight the cholinergic system's new functional variability and identify discrete subpopulations that could have played specific roles in regulating behavioral states, physiological homeostasis, and cognitive function.

Compared to previous studies, this research gives quantitative insight into the proportion of peptidergic neurons that are cholinergic, which indicates that neuropeptidergic modulation is a common feature among BFCN and thus not a specialized exception. Importantly, in the different brain areas the proportion of co-expression of CGRP and CCK with ChAT was dissimilar. For example, nearly all ChAT-ir neurons in the HDB expressed CGRP while CCK co-expression was relatively low. In contrast, the cholinergic neurons in the MS and VDB showed high co-expression rates for both neuropeptides (CGRP and CCK). These findings indicate a possibly broader spectrum of cholinergic modulation in these regions (MS and VDB). Moreover, not all the CGRP or CCK-expressing neurons were cholinergic. This is consistent with previous research, in which the results indicated that these neuropeptides (CGRP and CCK) were also present in non-cholinergic populations, such as in the case of the GABAergic interneurons (Arrigoni et al., 2010). These differences suggest that cholinergic neurons are not functionally homogeneous, but also may support distinct physiological outputs through selective neuropeptide co-expression.

The observed robust expression of CBP (calbindin) and region-specific expression of a CBP (calretinin), and the more restricted expression of another CBP (parvalbumin) suggest their important, but different role in the regulation of intracellular calcium homeostasis in different neuronal subtypes. Notably, previous studies of the pedunculopontine nucleus (PPN) had reported that calbindin and calretinin-positive neurons are as numerous as cholinergic neurons and show a rostro-caudal gradient (Martinez-Gonzalez et al., 2012). On the other hand, comparatively fewer number of neurons was expressed parvalbumin in the PPN (Martinez-Gonzalez et al., 2012). Moreover, the strong expression of CBPs among GABAergic and glutamate signaling neurons, as described in the PPN (Martinez-Gonzalez et al., 2012), may help to explain the consistent and robust expression patterns in the brain area that we studied such as the CPu, HDB, and SIB where cholinergic signaling is prominent. In contrast, the parvalbumin that is better expressed in areas such as the VDB, further link the evidence that parvalbumin is a marker that is associated with more specialized interneuron subpopulations such as the GABAergic, fast-spiking interneurons (Hijazi et al., 2023). Together, these findings reinforce the diversity of function and neurochemical composition of basal forebrain cholinergic and striatal regions. And these observations are in line with the idea of a more general CBP-based molecular heterogeneity that is apparent in complex brain structures such as the PPN (Martinez-Gonzalez et al., 2012).

In addition, the ratio of the co-expression of CBPs with cholinergic neurons differed depending on the brain region, particularly for the calbindin and calretinin. We found that calbindin was the most common CBP in the CPu and HDB that co-expressed with nearly 60% and 30% of cholinergic

neurons respectively. These co-expressive neurons comprise a molecularly heterogeneous subpopulation of the striatal cholinergic interneurons that suggest the heterogeneity in calcium buffering mechanisms inside the striatum. This widespread distribution is consistent with the role of calbindin in calcium buffering and protection from excitotoxicity stress (Rintoul et al., 2001). The presence of calbindin properties in cholinergic neurons may protect from ND and assist for their normal function in cognitive processes like attention and learning (Riascos et al., 2011). Furthermore, calbindin containing neurons are virtually resistant to the process of mass formation in the disease e.g., Alzheimer's disease (Riascos et al., 2011). It is also interesting that the expression patterns of calbindin may be different depending on the host age and the types of neurodegenerative diseases (Riascos et al., 2011). Considering this, calbindin expression is significantly lost during normal aging in the BFCN, which are susceptible to AD (Riascos et al., 2011). Besides this, there was more selective co-expression of ChAT and calretinin-ir neurons, primarily in the VDB, SIB, and CPu. These neurons are presumably involved in the cholinergic innervation of the hippocampus and olfactory structures. These ChAT-ir neurons that are co-expressed with calretinin, might be involved in rhythmic oscillatory activity as well as synaptic modulation (Qi et al., 2022a). These might support the concept for functionally specialized cholinergic subtypes. In reference to the pathophysiology of ion instability, it is believed that the pathogenesis of AD is linked to the homeostasis of transition metal ions (Li et al., 2017). For example, the high exposure to manganese (Mn, a metal necessary for basic physiological functions) may leads to accumulation of the metal in a number of organs including the brain (Bakulski et al., 2020). Mn poisoning, which is primarily caused by Mn buildup in the brain, is regarded as a kind of PD. Additionally, Mn is a risk factor for many neurological diseases, including AD, which is caused by oxidative stress, free radical production, and apoptosis (Ijomone et al., 2019). It is intriguing to note that there is Ca^{2+} modulation of neurodegenerative processes and interference of calcium signals may be one of the factors involved in Mn neurotoxicity (Chen et al., 2022). Consequently, it is believed that intracellular calcium homeostasis is critical for the normal maintenance of cell structure and cell functions. Calcium homeostasis has entailed precise conservation of cytosolic Ca^{2+} levels in spite of extracellular influx through numerous synaptic Ca^{2+} channels as well as intracellular release via organelles such as the endoplasmic reticulum, lysosomes, and mitochondria. Moreover, the functions of these organelles are dependent on controlled handling of Ca^{2+} between these different organelles. As a result of this, abnormal signaling may lead to oxidative stress, protein mishandling, synaptic dysfunction, and defective bioenergetics, among other outcomes consistent with AD (McDaid et al., 2020). Therefore, the loss of calbindin or calretinin co-expressive ChAT neurons might have indirect implications for neurodegenerative diseases. For example, the AD or PD were frequently occurred where specific subtypes of cholinergic neurons could be differentially affected. Though, earlier research revealed the parvalbumin-expressing

GABAergic interneurons have a crucial role in maintaining the excitation-inhibition balance in the cortex and contribute to the development of psychiatric disorders like autism, schizophrenia, or AD (Palicz et al., 2024). Interestingly, despite being present in the forebrain region, parvalbumin-ir neurons never contained ChAT immunoreactivity in BFCN and CPu. This molecular segregation of parvalbumin and ChAT reflects a fundamental distinction in their functional and distributional identities.

The present study examined the immunohistochemical relationship between HAP1 and neuropeptide as well as CBP expressing neurons in the BFCN and caudate putamen (CPu) of the adult mouse brain. The expression of HAP1 was almost strong in the BFCN and mild in the CPu. However, the co-expression of HAP1 with CGRP, CCK, calbindin, and calretinin was generally mild to moderate, and with parvalbumin it was absent. Some of the marker showed no co-expression in several nuclei, such as the SIB and CPu. These findings suggest that even though HAP1 is found in the BFCN and striatum, it is expressed in neuronal populations largely different from the prominent characteristics of neuropeptides or CBP markers.

The moderate co-expression of HAP1 with CGRP and CCK observed in the MS and VDB suggests that HAP1 is moderately associated with peptidergic signaling within this area of basal forebrain. CGRP and CCK are indeed known to have actions in the regulation of cholinergic systems, but the interaction is complicated and usually requires the location and receptor subtype (Grabauskas and Owyang, 2017; Hökfelt et al., 2025; Kajekar and Myers, 2008). Excitability and synaptic transmission in different areas of the nervous system such as the brain or peripheral ganglia may be affected by this modulation (Grabauskas and Owyang, 2017; Hökfelt et al., 2025; Kajekar and Myers, 2008). Similarly, the mild co-localization of HAP1 with calbindin and calretinin, and its total absence with parvalbumin suggests that HAP1 in the BFCN and CPu might not fully bear the characteristic feature of CBPs interneuron classes. Rather, HAP1 is capable of contributing to intracellular homeostasis in a way that is independent of classical calcium binding proteins, which is also in line with its known roles in vesicular trafficking, endosomal transport and organelle-associated calcium regulation (Chen et al., 2023; Gunawardena et al., 2014). Its ability to be transported by endosomes and its control of the calcium in the organelles are also supported by its interactions with other trafficking-related proteins at the time, including huntingtin (HTT) and Rab proteins, which additionally connects it to cellular equilibrium and functionality (Chen et al., 2023; Gunawardena et al., 2014; Islam et al., 2025; Li et al., 2000; Rong et al., 2006).

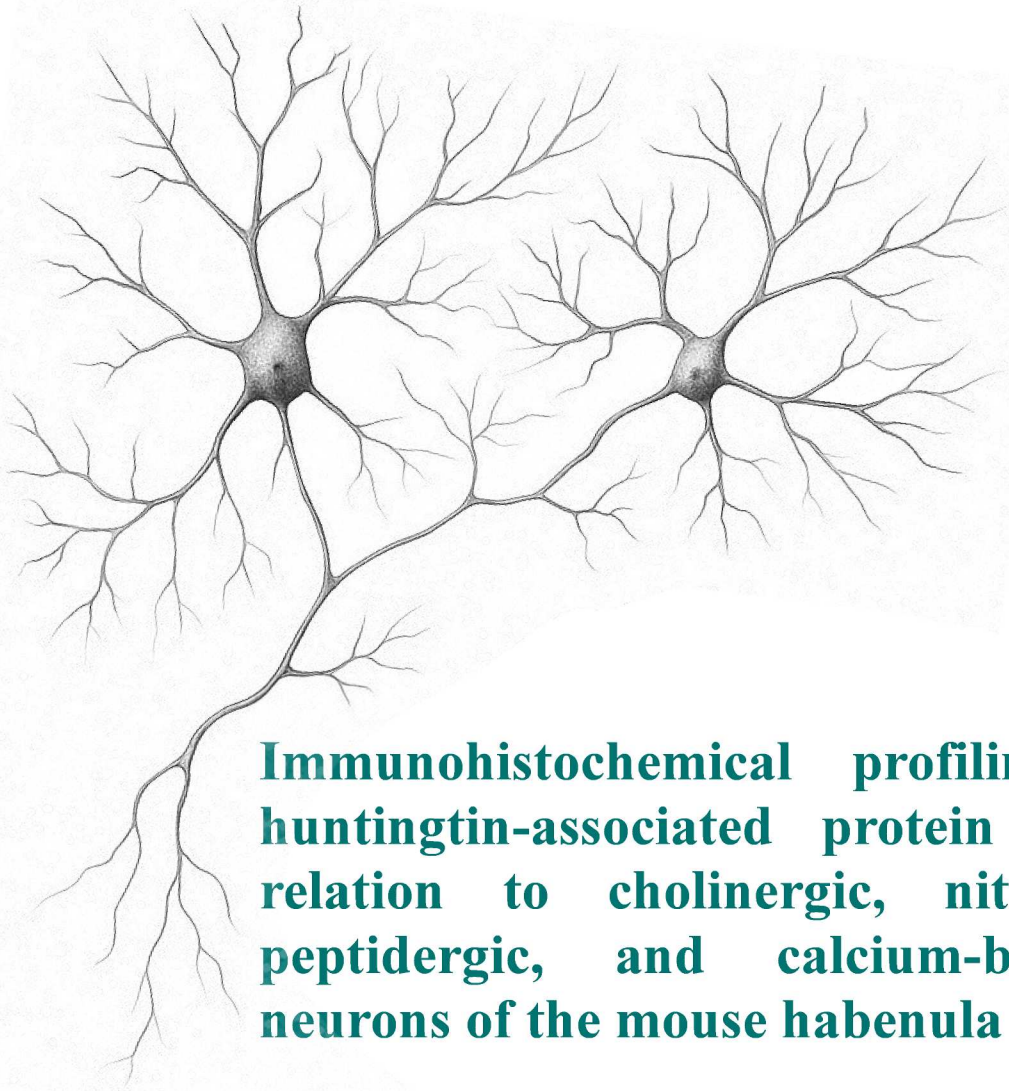
Interestingly, a much wider HAP1 association pattern has been reported in other studies in the enteric nervous system. The authors (Tarif et al., 2021) demonstrated that numerous calretinin, calbindin, NOS, VIP, ChAT, and somatostatin-positive neurons in the myenteric plexus co-expressed HAP1, while CGRP neurons were largely devoid of it. Likewise, another study (Tarif et al., 2023), showed that nearly all cholinergic, secretomotor, and vasodilator neurons expressing ChAT, CGRP, somatostatin, or calretinin in the submucosal plexus were HAP1-positive.

3.5. Conclusion

The neurochemical heterogeneity of ChAT-ir and HAP1-ir neurons throughout the BFCN and the neostriatum (CPu) in adult mice was thoroughly studied in our current investigation. Our findings demonstrated that neuropeptides like CGRP and CCK are commonly co-expressed by cholinergic neurons. We also noticed distinct CBP expression patterns. Together, our data reveal that ChAT-ir neurons in the neostriatum and BFCN are not uniform. Instead, they represent neurochemically distinct subpopulations which indicated by their co-expression with neuropeptides (CGRP and CCK) and CBPs. Understanding this variation could reveal vital insights into the structure and regulation of cholinergic systems. The interpretation of cholinergic activity in motor control and selective vulnerability to neurodegenerative illnesses may also be linked by these studies. In this sense, high-resolution in situ hybridization techniques, like RNAscope, could be employed to spatially map the gene expression to these nuclei which will provide a more detailed image of their molecular makeup and the risk factors of neurodegenerative illnesses. In addition to characterizing cholinergic subtypes, the selective distribution suggests that HAP1 is expressed in neuronal populations that are different from the major peptidergic or calcium-buffering subtypes in the BFCN and CPu. This indicates that the function of HAP1 might be highly region and cell-type specific. Collectively these results suggest that neuroprotective or modulatory actions of HAP1 in the basal forebrain which are probably independent of classical neuropeptide or CBP-expressing neuronal systems. Combined with the cholinergic heterogeneity, these results give a wider perspective of the molecular selection of the BFCN and neostriatum. Moreover, these findings give valuable hints to define their complex structure and differential vulnerability for neurodegenerative diseases. Future studies on marker-specific RNA expression patterns in HAP1-positive neurons will help to further explain these findings as well as give functional implications.



Chapter 4



**Immunohistochemical profiling of
huntingtin-associated protein 1 in
relation to cholinergic, nitrergic,
peptidergic, and calcium-binding
neurons of the mouse habenula**



Abstract

The habenula is a tiny epithalamic region of the brain, which is well known to be a key regulator of a variety of brain functions, including aversion, reward, stress responses, anxiety and mood. Huntingtin-associated protein 1 (HAP1) is a neuroprotective interactor with the causative agents of many neurodegenerative diseases whilst ChAT is a defining marker for cholinergic neurons which are important for modulating synaptic transmission and behavioural regulation. Despite HAP1's important roles in intracellular trafficking and in neuronal function, the distribution and characterization of HAP1 in the mouse habenula have not been systematically explored. Moreover, potential neurochemical relationship of ChAT with other neuronal markers of the mouse habenula is largely unexplored. Hence, this study applied western blotting and immunohistochemistry to neurochemically characterize the HAP1 and ChAT immunoreactivity in the mouse habenula. This study revealed that HAP1-immunoreactive (ir) neurons specifically localize to the lateral habenula (LHb), while medial habenula (MHb) completely devoid of HAP1 expression. Within the LHb, HAP1-ir neurons are highest densities in LHbMC and LHbMPc. Double-label immunofluorescence revealed limited proportion of HAP-1 has the co-expression with ChAT neurons ($8.63 \pm 1.00\%$) and nitrergic (nNOS) neurons ($21.76 \pm 2.14\%$). Moderate proportion of HAP1 co-expression was observed with calbindin ($51.79 \pm 5.25\%$) and calretinin ($58.61 \pm 5.48\%$) neurons, while highest was recorded with CGRP ($83.76 \pm 3.73\%$) and CCK ($74.24 \pm 4.83\%$). Surprisingly, HAP1-ir expression were absent in Substance p (SP) and PV-ir expressing neurons. On the other hand, the ChAT-ir neurons show the selective distribution, where only in the MHb. Moreover, the complementary immunohistochemical analysis of ChAT relationships with markers of neuropeptides further revealed that approximately $38.71 \pm 1.74\%$ of ChAT-positive neurons co-expressed with CCK and $30.14 \pm 2.10\%$ co-expressed with CGRP. These results suggest that there is some selective neurochemical overlap between cholinergic and peptidergic neurons in the MHb. To the sum, these findings show that both HAP1 and ChAT characterize discrete but partially inclusive neuronal subsets in the habenula. Perhaps, HAP1-ir neurons exhibit preferential association with peptidergic and calcium-binding protein expressing neurons. The HAP1 marks a distinct LHb neuronal cohort enriched in CGRP-ir and CCK-ir expressing peptidergic neurons, and also associated with specific calcium-binding phenotypes as well as largely segregated from parvalbumin, SP, nNOS, and cholinergic neuronal populations in habenula. In the MHb, ChAT has selective co-localization with CCK and CGRP-ir neurons. Therefore, the functional specialization of HAP1 and ChAT may potentially contribute to the neurochemical modulation of motivational and affective processes.

Keywords: *HAP1, Habenula, Calcium-binding proteins, Neuropeptides, neuropsychiatric disorders*

4.1. Introduction

Huntingtin-associated protein-1 (HAP1) is a protein that is gaining an increase of neurological importance due to its simultaneous involvement in intracellular trafficking as well as neuroprotection. HAP1 was first revealed as a polyglutamine (polyQ)-length-dependent interactor in huntingtin (HTT), the protein expressed by the gene that causes Huntington's disease (HD) (Gutekunst et al., 1998; Li et al., 1995). HAP1 has been demonstrated to reduce HTT-induced apoptosis by binding to the polyQ HTT (Gutekunst et al., 1998; Li et al., 2003, 1995). HAP1 inhibit apoptosis by binding with the polyQ-expanded receptor in spinal and bulbar muscular atrophy (SBMA) (Takeshita et al., 2006). Besides, HAP1 can cooperate with the contributory agents of nearly polyQ diseases, for example TATA binding protein in spinocerebellar ataxia type 17 (Prigge and Schmidt, 2007), Abelson helper integration site 1 in Joubert syndrome (Sheng et al., 2008), and ataxin 3 in Machado-Joseph disease (Takeshita et al., 2011).

The dot like intracytoplasmic structures of HAP1 is well known as the stigmoid body (STB), one of the promising characteristics of HAP1, that was discovered by immunohistochemistry of HAP1 (Chen et al., 2020; Fujinaga et al., 2007, 2004; Gutekunst et al., 1998; Islam et al., 2022, 2017, 2012; Xiang et al., 2014; Yanai et al., 2020). STB were initially characterized in the previous study as ovoid, non-membranous inclusion bodies with moderate electron density (Shinoda et al., 1993, 1992). The STB could be an important site for the protein aggregation as well as the dynamic sequestration. On the molecular basis, the HAP1 has two isoform (HAP1A and HAP1B) which have different C-terminal amino acid sequences (Fujinaga et al., 2007; Li et al., 1998).

Moreover, HAP1 has emerged as a cargo adaptor that moderates intracellular trafficking by binding with the cytoskeletal motor proteins as well as transport machinery (Chen et al., 2023). A considerable number of NDs, including HD, SBMA, Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and spinocerebellar ataxias (SCAs), are thought to be caused by trafficking defects (Chen et al., 2023). Though the brain regions (amygdala, preoptic area, and hypothalamus) with high STB/HAP1 expression are less susceptible to neuronal loss, but brain areas with little to no HAP1 expression (cerebellum, thalamus, and neocortex) are susceptible to NDs during neurodegenerative stress (Chen et al., 2020; Fujinaga et al., 2007, 2004; Gutekunst et al., 1998; Islam et al., 2022, 2020a, 2017, 2012; Yanai et al., 2020). However, the HAP1 protective role in some specific physiological and neuronal circuit-level are still unidentified. Therefore, it is essential to determine the distribution, localization, and neurochemical characterization of HAP1-expressing neurons in particular brain areas, such as the habenula. Specifically, these are important to understand that how HAP1 interacts with the larger neuronal circuitry, implication with other

neurotransmitters and disease susceptibility.

The cholinergic system in the brain regulates sensory, motor, and cognitive activities by altering the functions of neurons. The cholinergic neurons release acetylcholine (ACh) which is a neurotransmitter and neuromodulator. The ACh may improve the detection of salient sensory cues and reinforcement (Granger et al., 2020), modulate sensory perception (Pinto et al., 2013), gate plasticity (Morishita et al., 2010), and enhance learning and memory (Hasselmo, 2006). Choline acetyltransferase (ChAT) is often employed as a marker of cholinergic neurons since it is only one enzyme that produces ACh (Misgeld et al., 2002). The potential cholinergic neurons in the brain were originally discovered using immunolabeling for ChAT (Granger et al., 2020). The losses or degeneration of cholinergic neurons could lead to several ND like as AD, PD, Rett's syndrome, and autism (Pepeu and Grazia Giovannini, 2017; Witzemann, 2007). To understand the details about the functional heterogeneity and specificity, it is essential to investigate the organization of the cholinergic neurons and their axon arborizations throughout the brain.

The habenula is a small epithalamic part of the brain. This brain area has emerged as a critical regulator for aversion, reward, stress responses, and mood of the individual (Browne et al., 2018). Moreover, the function of habenula are mainly associated with the behavioural output in depression, addiction, and anxiety disorders (Yang et al., 2018). For this reason, habenula has the importance to understand and characterize its molecular architecture. Broadly habenula has two subdivisions, the medial habenula (MHb) and the lateral habenula (LHb) (Andres et al., 1999; Geisler et al., 2003; Kowski et al., 2009). Though the MHb anatomically adjacent to LHb, but it is neurochemically distinct. MHb may have the implication in the function of aversion to anxiety- and arousal-related processes. These properties may underscore a broad organizational principle of MHb where discrete habenular microcircuits differentially regulate the brainstem modulatory tone (Ables et al., 2023; Pang et al., 2016). On the other hand, the LHb encodes negative prediction error and aversive motivational value. LHb also involved in the dopaminergic and serotonergic output through its glutamatergic projections to the rostromedial tegmental nucleus and dorsal raphe (Groos and Helmchen, 2024; Mondoloni et al., 2022). Moreover, LHb link the body internal state with either adaptive or maladaptive behavioral choices (e.g., passive coping, anhedonia, behavioral inhibition) (Groos and Helmchen, 2024; Mondoloni et al., 2022). The LHb neuronal clusters are topographically distinct (Lecca et al., 2020). Notably, recent evidence highlights its multilevel heterogeneity (functional, anatomical, and molecular) which challenges the earlier view of the LHb as a homogeneous nucleus (Lecca et al., 2020). Understanding of the LHb diverse neuronal population is essential. Because LHb may act as a key regulator for the behavioral outcome such as motivation,

emotion, behavioral outcomes, and decision-making (Lecca et al., 2020).

The functional heterogeneity in MHb and LHb are reflected in the selective expression of neurochemical molecular markers. Among the different molecular markers that determine neuronal subtypes, nitric oxide synthase (nNOS) and choline acetyltransferase (ChAT) represent gaseous and cholinergic modulators respectively. Each of these two markers usually contribute to distinct modes of neurotransmission. Likewise, neuropeptides including calcitonin gene-related peptide (CGRP), implicated in pain processing and vasodilation (Iyengar et al., 2017). The other neuropeptide, cholecystokinin (CCK) might be involved in feeding, anxiety, and learning (Ballaz and Bourin, 2021). The existence of these two neuropeptides properties in the neurons of habenula may reveal the neuromodulatory diversity of habenular neurons. Another peptidergic neuronal marker, Substance P (SP) could play a critical role in nociception and stress regulation through its involvement with neurokinin receptors (Humes et al., 2024). In parallel, calcium-binding proteins (CBPs) such as calbindin, calretinin, and parvalbumin may serve as molecular markers of neuronal identity in the habenula. These CBPs have the established roles in the intracellular calcium regulation and distinguishing interneuron subtypes across several brain regions (Medalla et al., 2023; Pace et al., 2020; Parent et al., 1996). Calbindin is widely expressed in excitatory and inhibitory neurons, where it buffers calcium transients and protects neurons (particularly in the circuits that vulnerable to neurodegeneration) against excitotoxicity, (Bezprozvanny and Mattson, 2008). Calretinin may be often enriched in specific interneuron populations. Calretinin also has been implicated on maintaining the firing patterns as well as calretinin enhance the temporal precision of neuronal signaling (Qi et al., 2022b). Parvalbumin (PV) is strongly associated with the fast-spiking GABAergic interneurons. In GABAergic interneurons, the rapid calcium buffering properties of PV might be responsible for high-frequency firing and synchrony of neurons. These functions are essential for maintaining the balance between excitation and inhibition as well as also important in generating oscillatory rhythms in cortical and subcortical circuits (Udakis et al., 2020; Wagatsuma et al., 2023).

Since HAP1 has been shown to be a neuroprotective factor in a number of neurodegenerative conditions, its selective enrichment in certain neuronal populations has garnered a lot of interest. The cellular identity and neurotransmitter phenotype of HAP1-expressing neurons in the habenula are still poorly known. Prior research has shown that HAP1 expression is specific to distinct neuronal populations in various brainstem and forebrain areas. The distribution might be associated with both the capacity of maintain complex modulatory activities and a relative resistance to neurodegeneration (Fujinaga et al., 2007, 2004; Islam et al., 2025, 2023, 2022, 2017; Tarif et al., 2023; Yanai et al., 2020). Therefore, a thorough study of the HAP1 distribution and its neurochemical relationships in

the habenula is required to elucidate the potential role of trafficking scaffold in the molecular specialization of habenula circuits. On the other hand, cholinergic neurons are identified in the brain and spinal cord (Witzemann, 2007), but the neurochemical heterogeneity of ChAT in the habenula is poorly understood. To ascertain the expression, comparative distribution, and neurochemical properties of HAP1 and ChAT in the mouse habenula; we performed the immunohistochemical study with different neuronal marker such as nNOS, SP, CCK, CGRP, calbindin, calretinin, and parvalbumin.

4.2. Materials and methods

4.2.1. Animals

Adult male mice (C57BL/6J) aged eight weeks were acquired from Japan SLC Inc. located in Shizuoka, Japan. Although there is dispute on when adulthood begins, 8 to 12 weeks is the typical cutoff point for classifying mice as adults. Perhaps, mice may acquire sexual maturity and the capacity to reproduce between the ages of 6 and 8 weeks (Jackson et al., 2017). The mice were housed in cages that were maintained at a consistent temperature between 22°C and 24°C. During the 12-h light-dark cycle (8:00 AM to 8:00 PM), the mice were fed a typical pellet meal and water. The experimental procedures used in this study were authorized by Yamaguchi University School of Medicine's Committee on the Ethics of Animal Experimentation and carried out in compliance with Japanese laws governing animal research (Law No. 105, Notification No. 6). A total of thirty-three mice were sacrificed during this investigation. Thirty mice were used for immunohistochemistry, while three mice were used for Western blotting. Every attempt was made to reduce the number of mice slaughtered and their suffering. A combination of butorphanol tartrate (Vetorphale, Meiji Seika Pharma, Tokyo, Japan; 5.0 mg/kg), midazolam chloride (Midazolam, Sandoz, Basel, Switzerland; 4.0 mg/kg), and medetomidine chloride (Domitor, Orion Pharma, Espoo, Finland; 0.3 mg/kg) was used to thoroughly anesthetize the mice. Intraperitoneal injections of 0.25–0.30 ml of the anesthetic mixture were given to each animal. The mice were sacrificed by transcardial perfusion with either normal saline for Western blotting or 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for immunohistochemistry once a deep level of surgical anesthesia was reached, as evidenced by the loss of corneal reflexes and pedal withdrawal. A peristaltic pump (AP-3300, AdVANTEC, Tokyo, Japan) was used for the perfusion. The whole brain was then extracted from the mice after they had been decapitated.

4.2.2. Sample preparation for Western blotting

Basal forebrain and habenula segments were homogenized using 5 µl/ml of protease inhibitor (P8340; Sigma-Aldrich) and T-PER tissue protein extraction reagent (78510; Thermo Scientific, Rockford, IL, USA). Using a Portable Power Homogenizer (S-203, AS ONE Corporation, Osaka, Japan), the homogenization procedure was carried out on ice. Centrifugation of the homogenates was performed for 30 mins at 4 °C and 17,000 rpm. The protein content of each lysate was ascertained by collecting the supernatant and using the BCA Protein Assay Kit (Pierce™, Thermo Scientific, Waltham, MA, USA).

4.2.3. Tissue preparation for immunohistochemistry

The brains were stored in a 30% sucrose solution in 0.1 M PB for five to seven days after being collected, and they were submerged in the same fixative agent for the whole night. The brain was then frozen in dry ice powder and put in a cryostat (Leica CM1900, Germany) for coronal slices that were 30 µm thick.

4.2.4. Antibodies

Table 1 lists the details of primary antibodies with their working dilutions, whereas Table 2 provides specifics on the secondary antibodies.

4.2.5. Western blotting

Following our earlier research, the Western blotting was carried out (Afrin et al., 2025; Islam et al., 2017; Meher et al., 2025b). To summarize, 7.5% SDS-polyacrylamide gel electrophoresis was used to load and separate 80 µg of each protein. It was then moved onto a membrane made of polyvinylidene difluoride. The membrane was then cleaned in Tris-buffered saline with 0.1% Tween-20 (TBST) and blocked for 1 h at 20°C using 5% skim milk. The samples were treated with either mouse monoclonal anti-α tubulin (1:200,000) or goat polyclonal anti-HAP1 R19 (1:20,000) primary antibody for overnight at 4°C (Table 1). The membrane was cleaned with TBST and then incubated with the horseradish peroxidase-linked anti-goat (1:10,000) or anti-mouse IgG (1:20,000) secondary antibody for 2h at 20°C (Table 2). Protein bands were analyzed using enhanced chemiluminescence reagents (ECL Select, GE Healthcare) after three rounds of TBST washing, and pictures were taken with an Amersham Imager 600 (GE Healthcare). The HAP1/α-tubulin and ChAT/α-tubulin immunoreactive bands' relative optical density ratios were calculated using ImageJ software (NIH, Bethesda, MD, USA).

Table 1. Detailed information on the primary antibodies used in the present study

Antibody	Immunogen	Host/ Clonality	Code	Source	Dilution	Reference
Anti-HAPI (R19)	Rat HAPI C-terminus	Goat polyclonal	Cat# sc-8770, RRID: AB_647322	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:20,000	(Afrin et al., 2025)
Anti- α tubulin	Microtubule derived from chicken embryonic brain	Mouse monoclonal	Cat# T6199, RRID: AB_477583	Sigma-Aldrich, St. Louis, MO, USA	1:200,000	(Afrin et al., 2025)
Anti-GFAP	Purified human brain GFAP	Rabbit polyclonal	Cat# G9269, RRID: AB_477035	Sigma-Aldrich, St. Louis, MO, USA	1:1,000	(Afrin et al., 2025)
Anti-Iba1	Synthetic peptide corresponding to the C terminus of Iba1	Rabbit polyclonal	Cat# 019-19741, RRID: AB_839504	Wako, Osaka, Japan	1:1,000	(Islam et al., 2022)
Anti-NeuN	Synthetic peptide of Human NeuN aa 1–100	Rabbit Polyclonal	Cat# ab177487, RRID: AB_2532109	Abcam, Cambridge, UK	1: 5,000	(Islam et al., 2022)
Anti-ChAT	Synthetic peptide within Pig Choline Acetyltransferase aa 150–250	Rabbit monoclonal	Cat# ab178850, RRID: AB_2721842	Abcam, Cambridge, UK	1: 5,000	(Meher et al., 2025b)
nNOS	C-terminal synthetic peptide sequence corresponding to amino acids (1419–1433) of human nNos coupled to KLH	Rabbit polyclonal	Cat# 24287, RRID: AB_572256	Immunostar, Hudson, WI, USA	1:2000	(Tarif et al., 2023)
SP	Entire peptide sequence of substance P	Rabbit polyclonal	Cat# AB1566, RRID: AB_2271494	Millipore, Billerica, MA	1:500	(Islam et al., 2017)
CCK	Synthetic sulfated CCK-8 bound to KLH	Rabbit polyclonal	Cat# C2581, RRID: AB_258806	Sigma-Aldrich, St. Louis, MO	1:1,000	Islam et al., 2012
CGRP	Synthetic CGRP (rat) conjugated to KLH	Rabbit polyclonal	Cat# C8198, RRID: AB_259091	Sigma-Aldrich, St. Louis, MO	1:1,000	Tarif et al., 2023
Calbindin	Recombinant rat calbindin D-28 K (CB)	Rabbit polyclonal	Cat# CB-38a, RRID: AB_2721225	Swant, Marly, Switzerland	1:25,000	Tarif et al., 2023
Calretinin	Recombinant human calretinin a 6-his tag at the N-terminal	Rabbit polyclonal	Cat# CR7697, RRID: AB_2619710	Swant, Marly, Switzerland	1:2000	Tarif et al., 2023
Parvalbumin	Purified carp. muscle	Mouse monoclonal	Cat # 235; RRID: AB_10000343	Swant, Marly, Switzerland	1:1,000	(Magno et al., 2021)

Table 2. Detailed information on the secondary antibodies and other reagents used in the present study

Antibody	Immunogen	Host	Code/Source	Application	Dilution	References
Biotinylated goat anti-rabbit	IgG, isolated from rabbit serum	Goat	Cat# AP180B, RRID: AB_92569 Millipore, Darmstadt, Germany.	Immunoperoxidase staining	1:1,000	(Afrin et al., 2025)
Biotinylated polyclonal goat anti-mouse	Purified mouse IgG	Goat	Cat# E0433, RRID: AB_2687905, Dako, Denmark	Immunoperoxidase staining	1:1,000	(Köhler et al., 2025)
Biotinylated donkey anti-goat	IgG, isolated from goat serum	Donkey	Cat# AP180B, RRID: AB_92569 Millipore, Darmstadt, Germany	Immunoperoxidase staining	1:1,000	(Islam et al., 2022)
Alexa Fluor 488 donkey anti-goat	IgG (H + L) goat	Donkey	Cat# A11055, RRID: AB_2534102 Invitrogen, Eugene, OR, USA	Immunofluorescence staining	1:1,000	(Yanai et al., 2020)
Alexa Fluor 594 donkey anti-rabbit	IgG (H + L) goat	Donkey	Cat# A21207, RRID: AB_141637 Invitrogen, Eugene, OR, USA	Immunofluorescence staining	1:1,000	(Yanai et al., 2020)
Alexa Fluor 488 donkey anti-mouse	IgG (H+L) highly cross-adsorbed	Donkey	Cat# A21202, RRID: AB_141633 Invitrogen, Eugene, OR, USA	Immunofluorescence staining	1:1000	(Zhang et al., 2022)
HRP-conjugated streptavidin	N/A	N/A	Cat# P0397, Agilent Dako, Glostrup, Denmark	Immunoperoxidase staining	1:1,000	(Meher et al., 2025a)

4.2.6. Immunoperoxidase staining

The immunohistochemistry methodology for single immunoperoxidase evolved from our earlier research (Islam et al., 2023, 2022, 2021, 2020a, 2020b, 2017, 2012; Jahan et al., 2015; Tarif et al., 2021; Wroblewski et al., 2018; Yanai et al., 2020). In summary, 0.3% Triton X-100 containing 10% normal donkey serum or 10% normal goat serum was used to block free-floating brain sections. Next, goat anti-HAP1 R19 (1:20000), rabbit anti-ChAT (1:5000), rabbit anti-nNOS (1:2000), rabbit anti-SP (1:500), rabbit anti-CCK (1:1000), rabbit anti-CGRP (1:1000), rabbit anti-calbindin (1:25000), rabbit anti-calretinin (1:2000), and mouse anti-parvalbumin (1:1000) primary antibodies (Table 1) were added in the successive brain sections respectively, which were then incubated at 20°C for 5 days (Islam et al., 2022, 2017; Meher et al., 2025a, 2025b; Tarif et al., 2023). To perform the HAP1 pre-adsorption test, a particular blocking peptide (sc-8770P, Santa Cruz Biotechnology, Santa Cruz, CA, USA) against the HAP1 R19 antibody was added to the diluted primary antibody and left at 4°C overnight. The sections were then treated with either biotinylated donkey anti-goat or goat anti-rabbit or goat anti-mouse secondary antibody for 3h at 20°C (Table 2). The brain sections were then treated with peroxidase-conjugated streptavidin (P0397, Dako, Glostrup, Denmark) for 2h at 20°C. The sections were stained for 10 to 20 min at 4°C using a nickel-enhanced diaminobenzidine (DAB; Wako Pure Chemical Industries, Ltd., Osaka, Japan) reaction. After mounting the sections on glass slides, they were dehydrated using increasing the concentration of xylene and alcohol solutions, left to air dry for 30 min, and then embedded with Entellan Neu (Merck KGaA, Darmstadt, Germany).

4.2.7. Double-label immunofluorescence staining

The immunofluorescence immunohistochemistry method was acclimatised from previously published works (Afrin et al., 2025; Islam et al., 2017; Jahan et al., 2015; Meher et al., 2025a, 2025b; Tarif et al., 2023, 2021; Yanai et al., 2020). In summary, 10% NDS containing 0.3% Triton X-100 was added to the free-floating brain sections for 2 to 3 h at 20°C for blocking. Next, the sections were incubated in goat anti-HAP1 R19 (1:20,000) or rabbit anti-ChAT (1: 5000) with either a combination of rabbit anti-nNOS (1:2000) or rabbit anti-SP (1:500) or rabbit anti-CCK (1:1000) or rabbit anti-CGRP (1:1000) or rabbit anti-calbindin (1:25000) or rabbit anti-calretinin (1:2000) or mouse anti-parvalbumin (1:1000) primary antibodies (Table 1). Additionally for neuronal characterization sections were incubated goat anti-HAP1 R19 antibody (1:20000) in combination with either rabbit anti-GFAP (1:1000) or rabbit anti-Iba1 (1:1000) or rabbit anti-neuronal nuclei (NeuN; 1:5000) primary antibodies (Table 1). All sections were incubated at 20°C for 5 days, diluted with 1% NDS in PBS and 0.3% Triton X-100 (Table 1). After being cleaned, sections of brain were incubated with the appropriate species-specific secondary antibodies for 3h at 20°C [donkey anti-goat conjugated to Alexa 594 (A11058) or donkey anti-rabbit conjugated to Alexa 488 (A32790) or donkey anti-mouse

conjugated to Alexa 488 (A21202)] ([Table 2](#)). The secondary antibodies were diluted with PBS containing 1% NDS. The sections were covered with Fluoromount/Plus (K048, Diagnostic Biosystems, Pleasanton, CA, USA) after being cleaned with PBS and allowed to air dry for 30min on glass slides.

4.2.8. Photomicrographs

Photomicrographs for immunoperoxidase staining photos were captured using an Eclipse E80i photomicroscope (Nikon) attached to a colour digital USB 2.0 camera (Lumenera Corporation, Ottawa, Canada). A confocal microscope (Leica Stellaris-8 CRS, Wetzlar, Germany) with 20x and 40x objectives was used to take immunofluorescence photomicrographs. Adobe Photoshop Elements 2021 (Adobe Systems, Inc., San Jose, CA, USA) was then used to import the photos.

4.2.9. Tissue analyses and cell counting

Nissl (cresyl violet) staining technique was taken from earlier studies ([Bjorkli et al., 2022](#)). Nissl stain was applied on adjacent section; the exact locations of each nucleus incorporated within habenula were identified ([Fig. 1a-b](#)). Additionally, the distribution of HAP1, ChAT, nNOS, SP, CCK, CGRP, or CBPs-ir cells in the mouse habenula was ascertained by employing immunoperoxidase staining of the neighbouring sections. The intensity of expression from these immune-positive neurons in the habenula was ascertained. Using a x20 objective, immunofluorescence images were captured and imported into the ImageJ program (NIH, Bethesda, MD, USA). A total of five mice were used for cell counting. The cells were counted according to the method outlined in previous work ([Islam et al., 2025, 2022, 2017; Jahan et al., 2015](#)). The actual number of HAP1, ChAT, nNOS, SP, CCK, CGRP, or CBPs-ir cells were determined. The co-expression proportions of HAP1 and ChAT with different neuronal markers in habenula were determined. In each immunofluorescent staining, a minimum of four brain sections from a single animal were used for each nucleus, with a maximum distance of 60 μ m between the sections.

4.2.10. Terminology

The terms utilized in this study were acquired from a mouse brain atlas ([Franklin and Paxinos, 2008](#)).

4.3. Result

4.3.1. The general expression of HAP1 in the habenula of mouse brain

The present study revealed the detail characterization of HAP1 expression within the habenula, with particular emphasis on HAP1 localization and cellular identity in the habenula of mouse brain. In order to define the habenular subregions in mice, we used the anatomical subdivision which was initially suggested in rats by the author (Andres et al., 1999) and further confirmed and implemented by the immunohistochemical and electrophysiological data (Aizawa et al., 2012; Geisler et al., 2003; Kowski et al., 2009). In accordance with Andres et al. (1999) proposal, the medial habenula (MHb; pink in **Figure 1A**) is divided into superior (MHbS), inferior (MHbI), central (MHbC), lateral (MHbL), and commissural parts, while the lateral habenula (LHb) comprises medial (LHbM; light green in **Figure 1A**) and lateral divisions (LHbL; dark green in **Figure 1A**). The LHbM which is further subdivided into superior part (LHbMS) parvocellular part (LHbMPc), central part (LHbMC), and magnocellular part (LHbMMg' **Figure 1A**). Moreover, the LHbL were subdivided into parvocellular part (LHbLPc), magnocellular part (LHbLMc), oval part (LHbLO) of habenula (**Figure 1A**). This framework was used in the present study to localize HAP1 expression within the habenula of adult mice. The Nissl stain was able to distinguish between the medial and lateral subdivisions of the habenula of a mouse with ease thus serving as a good cytoarchitectonic reference point to further examination of HAP1 expression (**Figure 1B**). The expression of HAP1 immunoreactivity in the habenula of mouse was determined by Western blotting and immunohistochemistry. Western blot analysis confirmed the expression of HAP1 in the habenula, with immunoreactive bands as around 85 kDa to 90 kDa, in the mouse brain (**Figure 1C**). By performing the immunohistochemistry, HAP1 immunoreactivity was also evident in the lateral area of the habenula (**Figure 1D**), suggesting a region-specific role for this protein. At the cellular level, HAP1-ir cells typically exhibited a unique staining pattern, characterized by the presence of distinct cytoplasmic inclusions bodies which is STB. This STB is embedded within the diffusely immunoreactive cytoplasm (**Figure 1E**).

Interestingly, a subpopulation of HAP1-expressing cells did not have any discernible STB but only diffuse cytoplasmic labeling (**Figure 1E**), indicating that there was heterogeneity in the subcellular HAP1 expression. The specificity of this immunolabeling was confirmed using pre-adsorption controls with the help of a particular blocking peptide against the primary antibody anti-HAP1, which significantly suppressed the immunoreactivity of HAP1 (**Figure 1F**). To determine the cellular phenotype of the HAP1-expressing neurons in the LHb, we applied a double-label immunofluorescence to the markers of glial and neuronal populations. HAP1 immunoreactivity did not colocalize with GFAP (Glial fibrillary acidic protein) positive astrocytes (**Figure 1G-I**) or Iba1 (Ionized calcium-binding adaptor) positive microglia (**Figure 1J-L**). In contrast, a substantial

proportion of HAP1 positive cells were co-expressed with NeuN (Neuronal nuclei), thereby establishing the neuronal identity of HAP1 expressing cells within the habenula but were devoid of any glial cell characteristics. Taken together, these findings suggest that the expression of HAP1 within the habenula is neuron-specific with some showing distinct STB-like features. These results point at a possible structural and functional specialization of HAP1 in habenula of mouse brain.

4.3.2. Distribution of HAP1-ir neurons in the mouse habenula

Systematic analysis of regional distribution of HAP1-immunoreactive (HAP1-ir) neurons in the rostrocaudal length of the habenula was examined in adult mice. Serial coronal sections were analysed and mapped, to identify the localization of HAP1-ir cells (**Figure 2A-L; Figure 3A-L**). The HAP1 immunoreactivity was uniformly detected in the LHb across all the rostrocaudal levels with the MHb having no HAP1-ir neurons. Immunoreactive neurons were distributed to a restricted group of subnucleus between rostral (Bregma -0.82 mm) and caudal levels (Bregma -2.08 mm). These findings indicate that HAP1 expression in the habenula is selective to the LHb and absent in the MHb. At a glance these findings confirm a subregion-specific role of HAP1 in habenular function.

4.3.3. Comparative expression pattern of HAP1-ir cells with ChAT, nNOS, SP, CCK, CGRP, and CBPs in the mouse habenula

The comparative neurochemical distribution of HAP1-ir neurons (**Figure 4A**) was performed by comparing with the cholinergic (ChAT, **Figure 4B**), gaseous (nNOS, **Figure 4C**), neuropeptidergic (SP, **Figure 4D**; CCK, **Figure 4E**; and CGRP, **Figure 4F**), and CBP (calbindin, **Figure 4G**; calretinin, **Figure 4H**; and parvalbumin, **Figure 4I**) neuronal markers across the habenula of the adult mouse (**Table 3**).

In consistent with our mapping, HAP1-ir cells were confined to the LHb, with strong immunoreactive expression in the LHbMC and LHbMPc (**Table 3; Figure 1D; Figure 4A**), while moderate to strong HAP1-ir expression was also observed in the LHbMS and LHbMMg (**Table 3; Figure 1D; Figure 4A**). Though the LHbLMc exhibited weak to moderate HAP1 immunoreactivity, while LHbLO and LHbLPc showed weak immunoreactive expression of HAP1. Notably, the MHb subdivisions (MHbS, MHbL, MHbC, MHbI) were utterly devoid of HAP1 immunoreactive expression (**Table 3; Figure 1D; Figure 4A**).

When we compared HAP1-ir expression with other markers, cholinergic immunoreactive neurons (ChAT, **Figure 4B**) were exclusively localized to the MHb, but were absent in the LHb (**Table 3; Figure 4B**). Strong ChAT-ir expression was found in specific MHb subdivisions (MHbL, MHbC,

and MHbI). Interestingly, MHbS and a part of LHb (LHbMPc) showed weak ChAT-ir expression. Though, the LHb was largely ChAT-negative, apart from a trace signal in the LHbMPc. Thus, HAP1-ir neurons had the LHb selective distribution that is largely complementary to ChAT-ir neurons in the MHb enriched pattern (**Table 3**).

The gaseous neuronal marker, nNOS showed a weak or absent immunoreactivity which distributed sporadically across the LHb subdivisions (**Table 3, Figure 4C**). Within the LHb, weak nNOS immunoreactivity was disseminated in LHbMC, LHbMPc, LHbLMc, and LHbLPc; while LHbMS, LHbMMg, and LHbLO had no immunoreactivity (**Table 3, Figure 4C**). All MHb subdivisions were nNOS-negative. Though its distribution was more diffuse and did not resemble the dense clustering of HAP1-ir neurons, few nNOS-ir neurons showed little overlap with the HAP1 dense subnuclei (**Table 3, Figure 4C**).

Among the neuropeptides (**Figure 4D–F**), SP (**Figure 4D**) was highly selective for MHbS (Showed strong SP immunoreactivity) and absent elsewhere (in all LHb and remaining MHb subdivisions). The SP-ir fibers were observed in the LHb particularly in the LHbMPc. Another neuropeptide, CCK-ir neurons (**Figure 4E**) were broadly represented, with strong immunoreactive expression in LHbMC, LHbMPc, LHbLMc, and most of the MHb subdivisions except MHbS where weak to moderate CCK immunoreactivity was observed (**Table 3, Figure 4E**). Moderate to strong CCK expression was seen in LHbMS, LHbMMg, and LHbLO, while weak to moderate level of expression was observed in LHbLPc (**Table 3, Figure 4E**). Additionally, the peptidergic neuronal marker, CGRP (**Figure 4F**) was one of the most pervasive neuropeptide markers that exhibiting strong immunoreactivity across the MHb and nearly all LHb subnuclei (LHbMS, LHbMC, LHbMMg, LHbLMc, LHbLO) except LHbMPc and LHbLPc where moderate to strong CGRP expression was observed (**Table 3, Figure 4F**). Notably, the CGRP enriched LHb substantially coincide with those harboring the highest HAP1-ir cells (LHbMC or LHbMPc), whereas SP is segregated to MHbS (**Table 3; Figure 4A, D–F**).

CBP expression further showed heterogeneous patterns across the habenular subdomains (**Figure 4G–I**). Calbindin-ir cells (**Figure 4G**) were strongly expressed in LHbMS, LHbMC, and LHbMPc. Weak to moderate calbindin-ir expression was observed in LHbMMg and LHbLPc (**Table 3, Figure 4G**). A subdivision of LHb (LHbLMc) and specific areas of MHb (MHbS, MHbL, MHbI) had weak calbindin immunoreactivity. Interestingly, there were no calbindin immunoreactivity in the LHbLO and MHbC (**Table 3, Figure 4G**). The another CBP, calretinin (**Figure 4H**) had strong immunoreactive expression in LHbMS, LHbMC, LHbMPc, LHbMMg, LHbLMc, and across all

MHb subnuclei (MHbS, MHbL, MHbC, and MHbI). Weak to moderate and weak calretinin-ir expression were observed in LHbLPc and LHbLO respectively (Table 3, Figure 4H). The PV-ir cells (Figure 4I) showed a strikingly restricted distribution pattern, where PV was absent from most LHb subnuclei except for a weak to moderate expression in LHbLMc and LHbLO (Table 3, Figure 4I). Surprisingly, no PV immunoreactivity was observed in the MHb (Table 3, Figure 4I).

Table 3. Comparative expression pattern of cholinergic, gaseous, neuropeptide, and calcium-binding protein neuronal markers in the habenula of adult mouse

Adult mice Habenula		Different neuronal markers intensity of immunoreactivity								
		Neurotransmitter Type								
		Cholinergic		Gaseous	Neuropeptide			Calcium-binding protein		
		HAP 1	ChAT	nNOS	SP	CCK	CGRP	Calbindin	Calretinin	PV
Lateral Habenula (LHb)	LHbMS	++	-	-	-	++	+++	+++	+++	-
	LHbMC	+++	-	+/-	-	+++	+++	+++	+++	-
	LHbMPc	+++	+/-	+/-	-	+++	++	+++	+++	-
	LHbMMg	++	-	-	-	++	+++	+	+++	-
	LHbLMc	+	-	+/-	-	+++	+++	+/-	+++	+
	LHbLO	+/-	-	-	-	++	+++	-	+/-	+
Medial Habenula (MHb)	LHbLPc	+/-	-	+/-	-	+	++	+	+	-
	MHbS	-	+/-	-	+++	+	+++	+/-	+++	-
	MHbL	-	+++	-	-	+++	+++	+/-	+++	-
	MHbC	-	+++	-	-	+++	+++	-	+++	-
	MHbI	-	+++	-	-	+++	+++	+/-	+++	-

(- indicates no expression, +/- indicates weak, + indicates weak to moderate, ++ indicates moderate to strong, and +++ indicates strong expression). For detail on abbreviations, see Abbreviations.

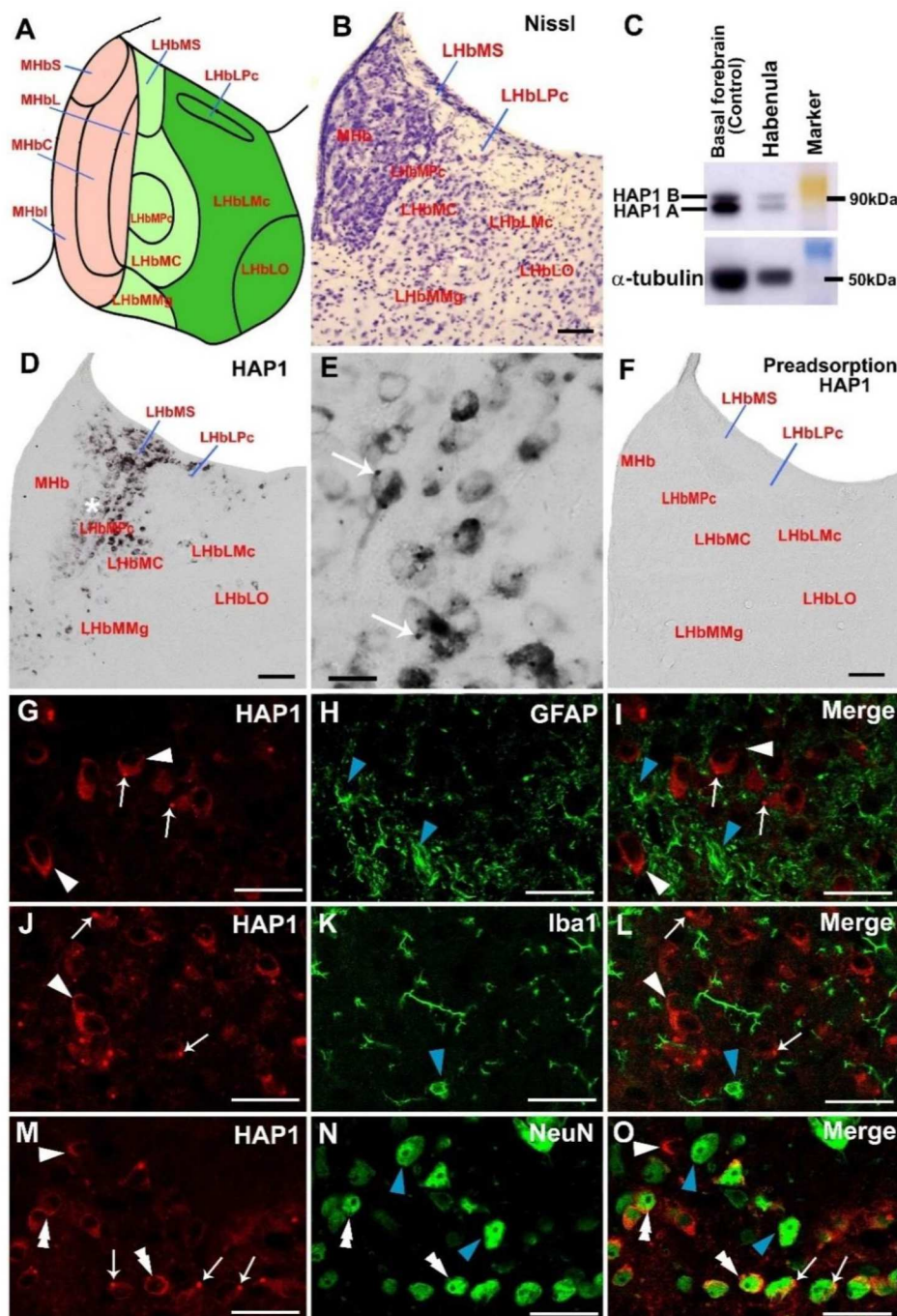


Figure 4.1. Western blotting, Nissl (cresyl violet) and double-label immunofluorescence immunohistochemistry for HAP1 with Glial or Neuronal markers in the habenula of adult mouse. (A) Subdivision of the MHb (pink), medial division of the LHb (light green), and lateral division of the LHb (dark green), according to previous studies (Andres et al., 1999). (B) Representative forebrain section was examined employing Nissl staining for delineating habenula nuclei. (C) Western blot analysis showing the HAP1 immunoreactive (ir) bands and α -tubulin was used for loading control. (D) Immunoperoxidase staining showing the presence of HAP1 immunoreactivity in the LHb. Figure “E” is the enlargement of * in Figure “D”, indicating the HAP1-ir stigmoid body (STB). (F) Preincubation with a blocking peptide against the anti-HAP1 antibody eliminated the HAP1 immunoreactivity. Photomicrograph showing double-label immunofluorescence staining of HAP1 and GFAP (G-I), Iba1 (J-L), or NeuN (M-O) in the habenula. Double arrowheads indicate the cells positive for both HAP1 and NeuN. Single white arrowheads indicate cells single-positive for HAP1. Cyan arrowheads indicate cells single-positive for GFAP or Iba1 or NeuN. White arrows indicate HAP1-immunoreactive (ir) STB. Scale bar = 50 μ m in “B, D, and F” and “G-O”; scale bar = 20 μ m in “E”. For details on abbreviations, see Abbreviations.

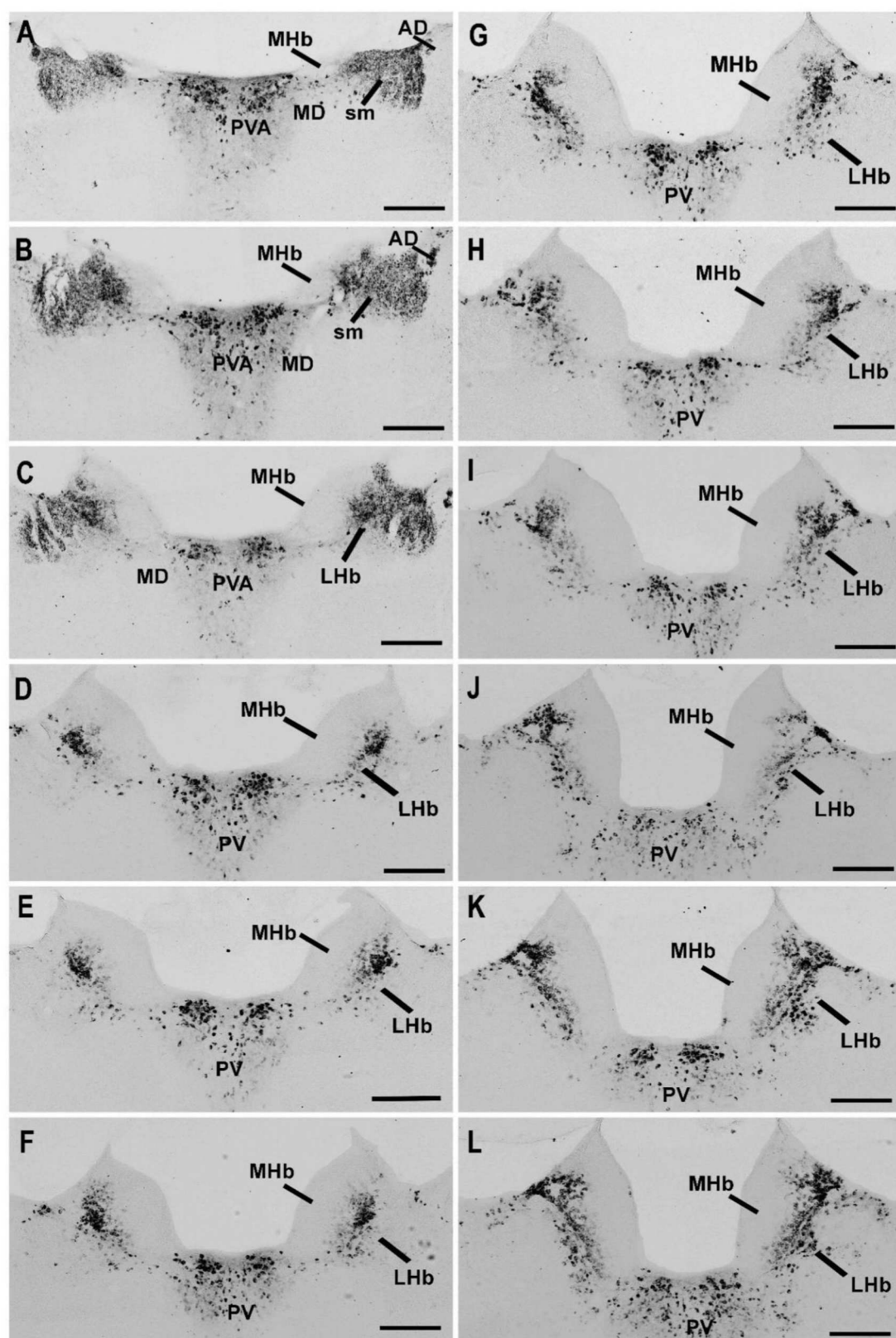


Figure 4.2. HAP1 immunoreactivity in the habenula of the mouse brain from rostral to caudal (Bregma -0.82mm to -1.34mm). Photomicrographs showing the HAP1-ir cells are distributed throughout the lateral habenula and completely devoid in the medial habenula from rostral to caudal. Scale bar = 500µm. MHb, medial habenular nucleus; LHb, lateral habenular nucleus; Sm, stria medullaris; AD, anterodorsal thalamic nucleus; MD, mediodorsal thalamic nucleus; PV, paraventricular thalamic nucleus; PVA, paraventricular thalamic nucleus, anterior part

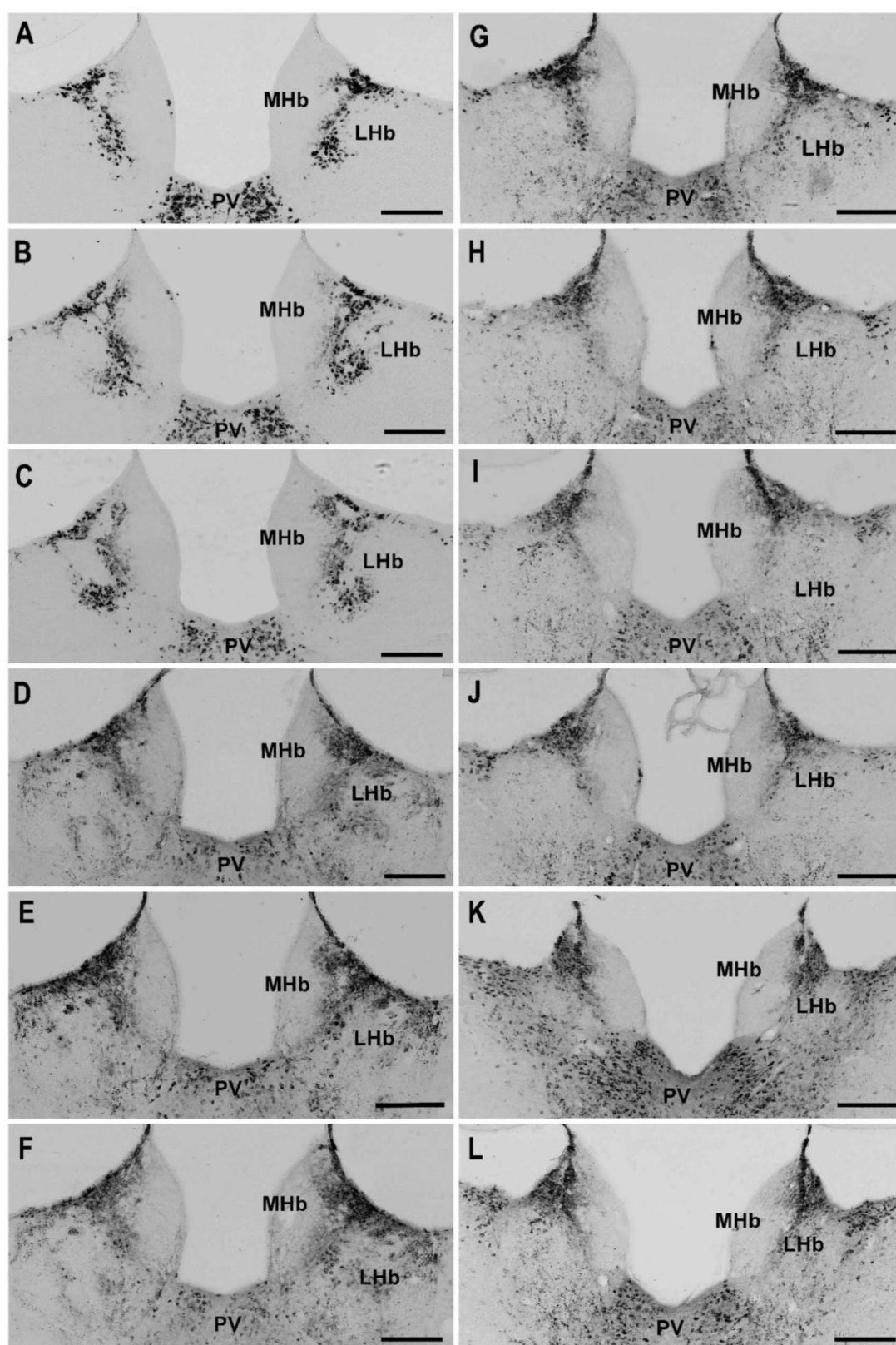


Figure 4.3. HAP1 immunoreactivity in the habenula of the mouse brain from rostral to caudal (Bregma -1.34mm to -2.086mm). Photomicrographs showing the HAP1-ir cells are distributed throughout the lateral habenula and completely devoid in the medial habenula from rostral to caudal. Scale bar = 500µm. MHb, medial habenular nucleus; LHb, lateral habenular nucleus; Sm, stria medullaris; AD, anterodorsal thalamic nucleus; MD, mediodorsal thalamic nucleus; PV, paraventricular thalamic nucleus; PVA, paraventricular thalamic nucleus, anterior part

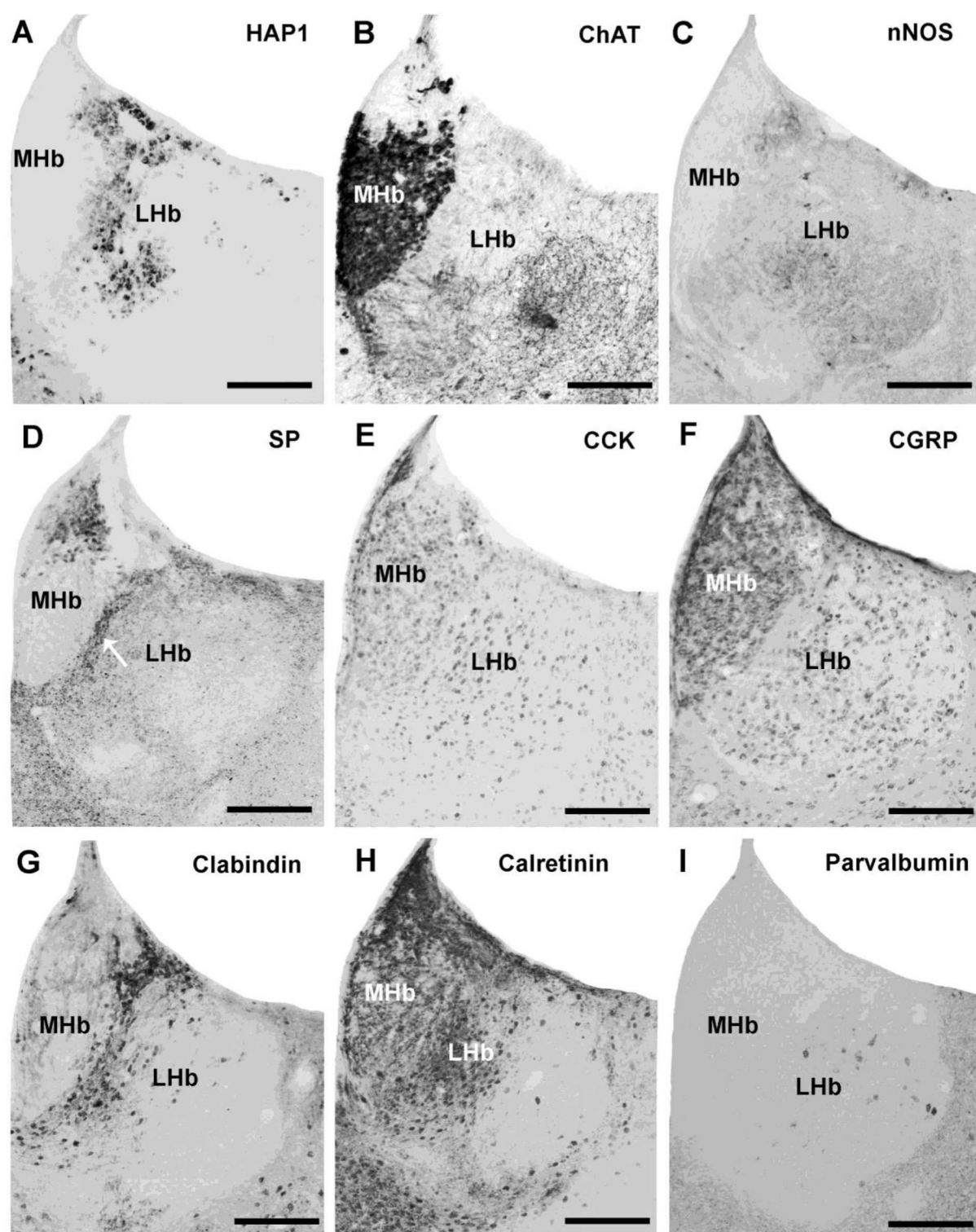


Figure 4.4. Immunoperoxidase immunohistochemistry for HAP1, ChAT, nNOS, neuropeptide, and calcium-binding protein expressing cell markers. (A-I) distribution of HAP1, ChAT, nNOS, SP, CCK, CGRP, calbindin, calretinin, and parvalbumin immunoreactivity across the habenula in the adult mouse forebrain. White arrows indicate SP-ir fibers that are stored in dense-core vesicles (D). Scale bar = 500µm. ChAT, Choline acetyltransferase; CGRP, Calcitonin gene-related peptide; CCK, Cholecystokinin; nNOS, Neuronal nitric oxide synthase, SP, Substance P, MHb, medial habenular nucleus; LHb, lateral habenular nucleus.

4.3.4. Immunohistochemical relationship of HAP1 with cholinergic-expressing neurons

Double-label immunofluorescence revealed a limited proportion of co-localization between HAP1 and ChAT-ir neurons in the LHb (LHbMPc; **Figure 5A-C**). Quantitative analysis showed that $18.27 \pm 3.44\%$ of ChAT-ir positive neurons expressed HAP1, while $8.63 \pm 1.00\%$ of HAP1-ir positive cells showed ChAT immunoreactivity (**Table 4, Figure 5A-C**). Morphologically, a large number of HAP1-ir positive somata and STB were found in close apposition to ChAT-ir neurons, but only a small proportion of the neurons were co-expressed. These data show that most of the HAP1 neurons in the lateral habenula are independent of the cholinergic neuronal group and this is in line with the relative paucity of ChAT immunoreactivity in this area.

4.3.5. Immunohistochemical relationship of HAP1 with gaseous neuromodulator-expressing neurons

Double-label immunofluorescence revealed a clear, though partial, co-localization of HAP1 with nNOS-ir expressing neurons in the LHb (LHbMC; **Figure 5D-F**). Quantitative analysis demonstrated that $28.04 \pm 5.38\%$ of nNOS-positive cells had immunoreactivity with HAP1, whereas $21.76 \pm 2.14\%$ of HAP1-ir positive neurons contained nNOS immunoreactivity (**Table 4, Figure 5D-F**). Moreover, a number of HAP1-ir neurons were localized to regions neighboring nitrergic populations. Although some of the nitric oxide-producing neurons provide a subgroup of HAP1-ir positive cells, most HAP1-ir neurons in the LHb were non-nitrergic neuronal phenotypes.

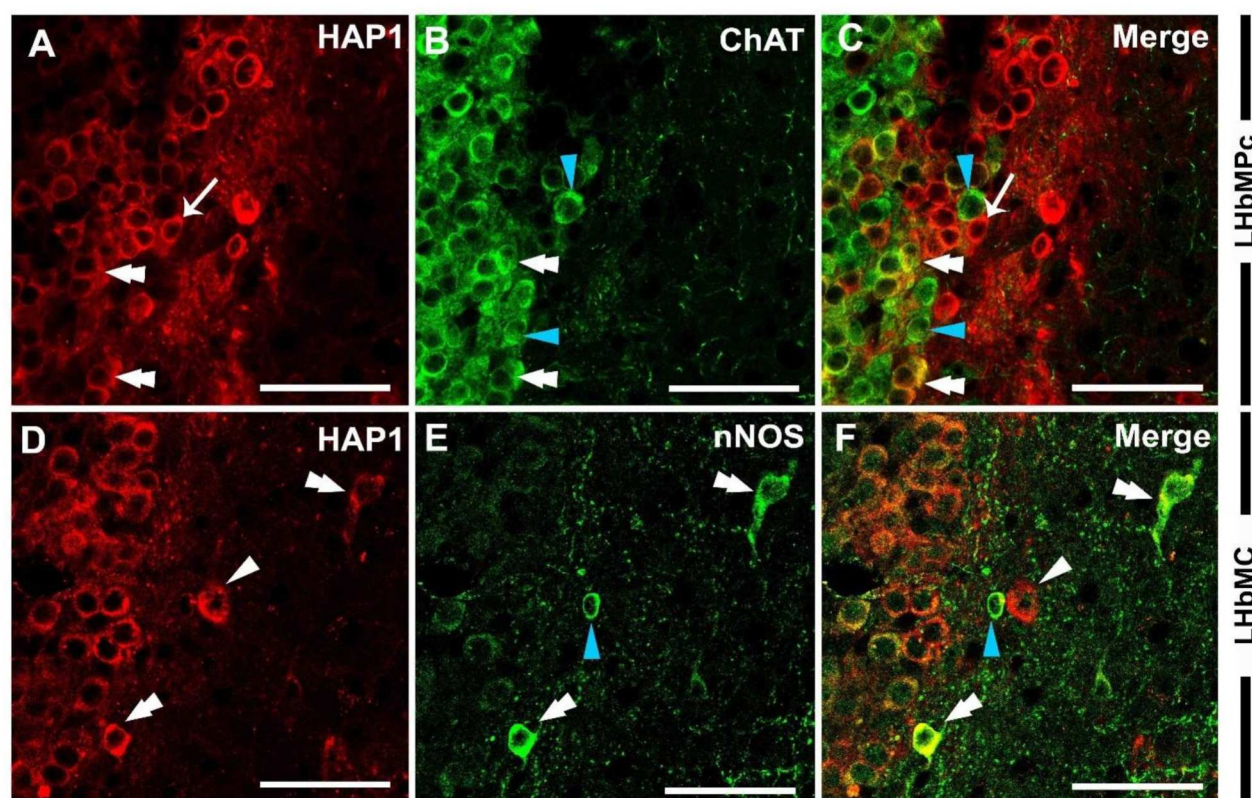


Figure 4.5. Double-label immunofluorescence immunohistochemistry for HAP1 with ChAT or nNOS in lateral habenula of adult mouse. Photomicrographs showing immunolabeling of HAP1 with ChAT (A-C) or nNOS (D-F) in the lateral habenula of adult mouse. Single white arrowheads indicate the cells positive only for HAP1, cyan arrowheads indicate cell positive for ChAT or nNOS, white arrows indicate HAP1-immunoreactive (ir) STB, and double white arrowheads indicate the cells positive for both HAP1 and ChAT or nNOS. Scale bar = 50 μ m in “A-F”. ChAT, Choline acetyltransferase; nNOS, Neuronal nitric oxide synthase, LHbMPc, parvocellular part of the medial division of lateral habenular nucleus, LHbMC, central part of the medial division of lateral habenular nucleus.

4.3.6. Immunohistochemical relationship of HAP1 with neuropeptide-expressing neurons

Double-label immunofluorescence analyses revealed distinct patterns of co-expression between HAP1 and peptidergic markers in the LHb (**Figure 6A-I**). The SP-ir fibers were observed in LHb. SP-ir neuronal fibers showed no detectable HAP1 immunoreactivity. This finding is due to SP-ir cells preferentially localize to the MHb (particularly MHbS) rather than the LHb (**Table 4; Figure 6A-C**). In contrast, a notable degree of co-localization of HAP1 and CCK-ir neurons was observed. Quantitative analysis demonstrated that $27.11 \pm 1.66\%$ of CCK had the HAP1 immunoreactivity, while the majority of HAP1-ir positive neurons ($74.24 \pm 4.83\%$) had CCK immunoreactivity (**Table 4; Figure 6D-F**). These findings indicate that CCK-ir neurons form a substantial subpopulation within the HAP1-expressing cohort of the LHb. The most striking association of HAP1 was observed with CGRP. Approximately $25.22 \pm 1.41\%$ of CGRP-ir positive neurons had HAP1 immunoreactivity, and most of the HAP1-ir positive neurons ($83.76 \pm 3.73\%$) expressed CGRP (**Table 4; Figure 6G-I**). The extensive co-expression highlights CGRP-ir positive neurons as the predominant peptidergic phenotype of HAP1-ir expressing cells in the LHb. Together, these results indicate that HAP1-ir neurons were not found in SP-ir positive neurons in LHb. However, it is highly concentrated in other peptidergic neurons, including CCK and especially CGRP-expressing neuronal subtypes. These findings suggest that HAP1 might be preferential markers in peptidergic circuits regulated by the habenular signaling.

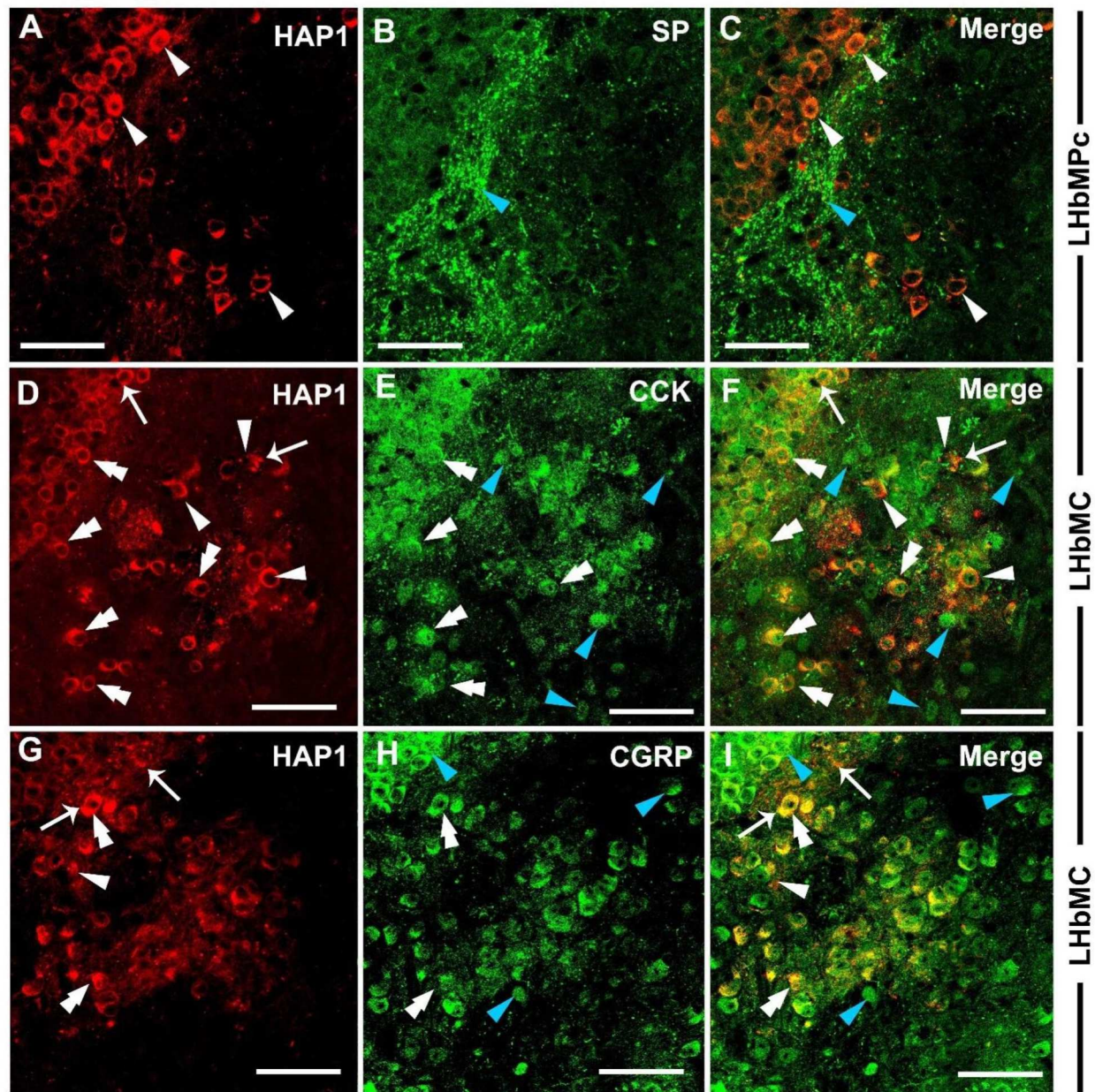


Figure 4.6. Double-label immunofluorescence immunohistochemistry for HAP1 with peptidergic neuronal marker (SP, CCK, and CGRP) in lateral habenula of adult mouse. Photomicrographs showing immunolabeling of HAP1 with SP (A-C) or CCK (D-F) or CGRP (G-I) in the lateral habenula of adult mouse. Single white arrowheads indicate the cells positive only for HAP1, cyan arrowheads indicate cell positive for CCK or CGRP or SP-ir fibers that are stored in dense-core vesicles (B-C), white arrows indicate HAP1-ir STB, and double white arrowheads indicate the cells positive for both HAP1 and SP or CCK or CGRP. Scale bar = 50µm in “A-I”. SP, Substance P; CCK, Cholecystokinin; CGRP, Calcitonin gene-related peptide, LHbMPc, parvocellular part of the medial division of lateral habenular nucleus, LHbMC, central part of the medial division of lateral habenular nucleus.

4.3.7. Immunohistochemical relationship of HAP1 with CBP expressing neurons

Double-label immunofluorescence revealed a substantial proportion of co-expression between HAP1 and CBP expressing neurons within the LHb (**Figure 7A-I**). Quantitative analysis showed that $41.62 \pm 3.61\%$ of calbindin positive neurons contained HAP1, while more than half of HAP1-ir positive neurons ($51.79 \pm 5.25\%$) had calbindin immunoreactivity (**Table 4, Figure 7A-C**). An even greater co-expression of HAP1 was observed with calretinin, where $40.45 \pm 4.27\%$ of calretinin -ir cells were HAP1 positive, and $58.61 \pm 5.48\%$ of HAP1-ir cells had the calretinin immunoreactivity (**Table 4, Figure 7D-F**). In contrast, parvalbumin-ir positive neurons showed no detectable overlap with HAP1-ir cells (**Figure 7G-I**) which might be due to the scarcity of parvalbumin expression in the LHb (**Table 3, Figure 7G-I**). Morphologically, co-expression was clearly identified between HAP1 and calbindin or calretinin neuron (**Figure 7A-F**). Perhaps, HAP1-ir neurons in the LHb are preferentially associated with calretinin and calbindin expressing neuronal populations, but are excluded from the parvalbuminergic phenotype.

Table 4. Co-expression ratios of HAP1 and different neuronal markers in the lateral Habenula in adult mice.

Relationship of HAP1 with neuronal markers in adult mice Habenula			Ratio (%)
Neurotransmitter Type	Marker	Interrelations	Mean $\pm$ SD
Cholinergic Neurons	ChAT	HAP1/ChAT	18.27 ± 3.44
		ChAT/HAP1	8.63 ± 1.00
Gaseous Neuromodulator	nNOS	HAP1/nNOS	28.04 ± 5.38
		nNOS/HAP	21.76 ± 2.14
	SP	HAP1/SP	-
		SP/HAP1	-
Peptidergic Neurons	CCK	HAP1/CCK	27.11 ± 1.66
		CCK/HAP1	74.24 ± 4.83
	CGRP	HAP1/CGRP	25.22 ± 1.41
		CGRP/HAP1	83.76 ± 3.73
	Calbindin	HAP1/Calbindin	41.62 ± 3.61
		Calbindin/HAP1	51.79 ± 5.25
Calcium-binding protein	Calretinin	HAP1/Calretinin	40.45 ± 4.27
		Calretinin/HAP1	58.61 ± 5.48
	Parvalbumin	HAP1/Parvalbumin	-
		Parvalbumin/HAP1	-

Values represent the mean $\pm$ SD of co-expression ratios (%) in adult mice (n = 5). HAP1, Huntingtin protein 1; SD, standard deviation; SP, Substance P; CCK, Cholecystokinin; CGRP, Calcitonin gene-related peptide; ChAT, Choline acetyltransferase; nNOS, Neuronal nitric oxide synthase.

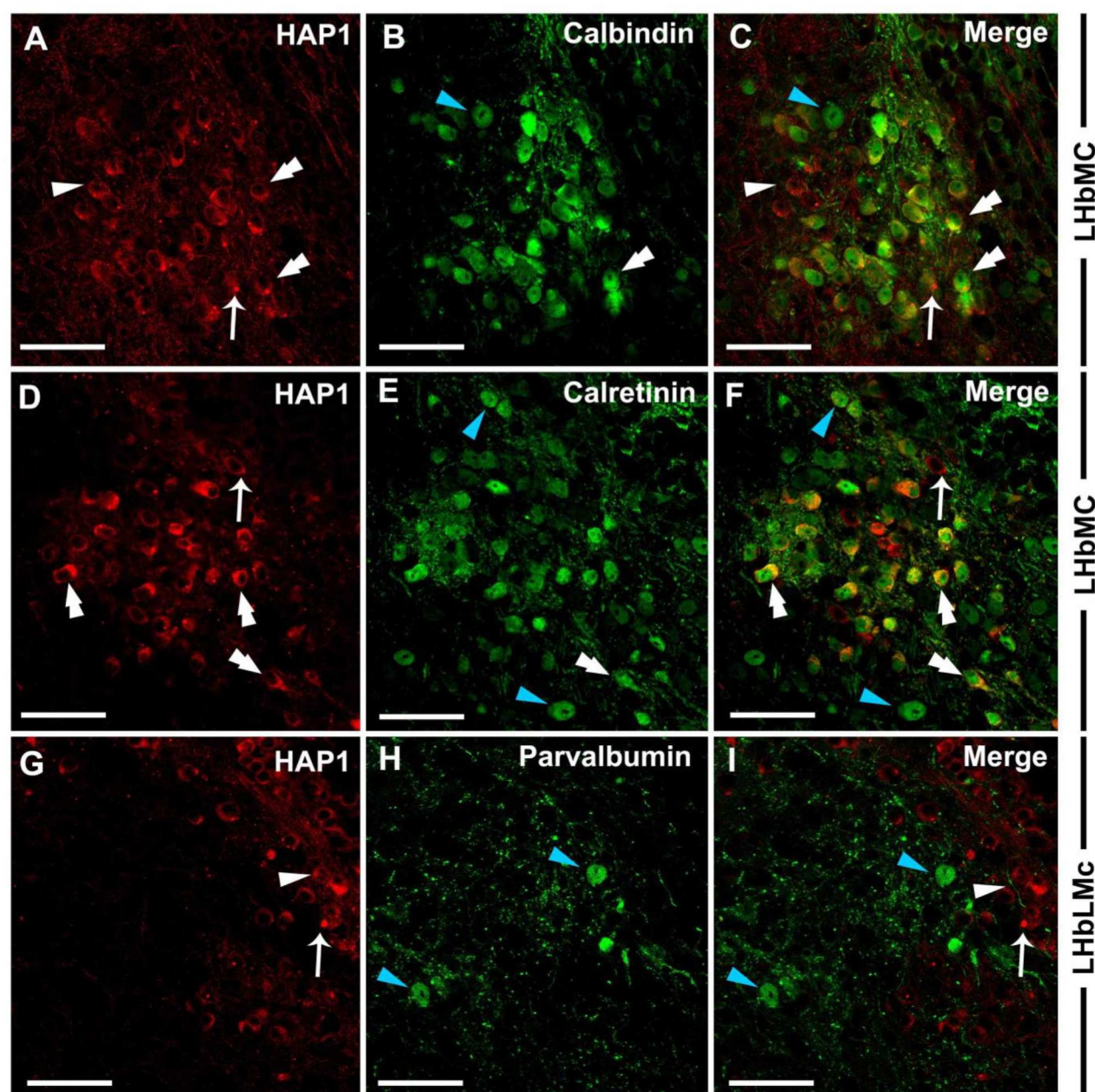


Figure 4.7. Double-label immunofluorescence immunohistochemistry for HAP1 with Calcium binding proteins (Calbindin, Calretinin, and Parvalbumin) in lateral habenula of adult mouse. Photomicrographs showing immunolabeling of HAP1 with calbindin (A-C) or calretinin (D-F) or parvalbumin (G-I) in the lateral habenula of adult mouse. Single white arrowheads indicate the cells positive only for HAP1, cyan arrowheads indicate cell positive for calbindin or calretinin or parvalbumin, white arrows indicate HAP1-ir STB, and double white arrowheads indicate the cells positive for both HAP1 and calbindin or calretinin or parvalbumin. Scale bar = 50µm in “A-I”. LHbMC, central part of the medial division of lateral habenular nucleus.

4.3.8. Immunohistochemical relationship of ChAT with neuropeptide or CBP expressing neurons

The immunohistochemical analysis revealed that there were neurochemical relationships between ChAT-ir neurons and certain peptidergic markers (Table 5). The CCK and CGRP-ir neurons showed that a considerable proportion of co-expression with ChAT. Approximately $38.71 \pm 1.74\%$ of ChAT-ir neurons co-expressed with CCK positive neurons, while $30.14 \pm 2.10\%$ of ChAT-ir neurons co-expressed CGRP positive neurons. Conversely, $18.99 \pm 0.67\%$ of CCK-ir neurons and $22.66 \pm 1.78\%$ of CGRP-ir neurons had the ChAT positive expression. These findings suggest the partial but meaningful co-expression of cholinergic neurons with the peptidergic neuronal populations (CCK and CGRP). However, there were no significant co-localization of ChAT with SP or CBP (calbindin, calretinin, and parvalbumin) was observed in the habenula.

Table 5. Co-expression ratios of ChAT and different neuronal markers in the lateral Habenula in adult mice.

Relationship of ChAT with neuronal markers in adult mice Habenula			Ratio (%)
Neurotransmitter Type	Marker	Interrelations	Mean $\pm$ SD
Peptidergic Neurons	SP	ChAT/SP	-
		SP/ChAT	-
	CCK	ChAT/CCK	38.71 ± 1.74
		CCK/ChAT	18.99 ± 0.67
	CGRP	ChAT/CGRP	30.14 ± 2.10
		CGRP/CHAT	22.66 ± 1.78
Calcium-binding protein	Calbindin	ChAT/Calbindin	-
		Calbindin/ChAT	-
	Calretinin	ChAT/Calretinin	-
		Calretinin/ChAT	-
	Parvalbumin	ChAT/Parvalbumin	-
		Parvalbumin/ChAT	-

Values represent the mean $\pm$ SD of co-expression ratios (%) in adult mice (n = 5). CHAT, Huntingtin protein 1; SD, standard deviation; SP, Substance P; CCK, Cholecystokinin; CGRP, Calcitonin gene-related peptide; ChAT, Choline acetyltransferase; nNOS, Neuronal nitric oxide synthase

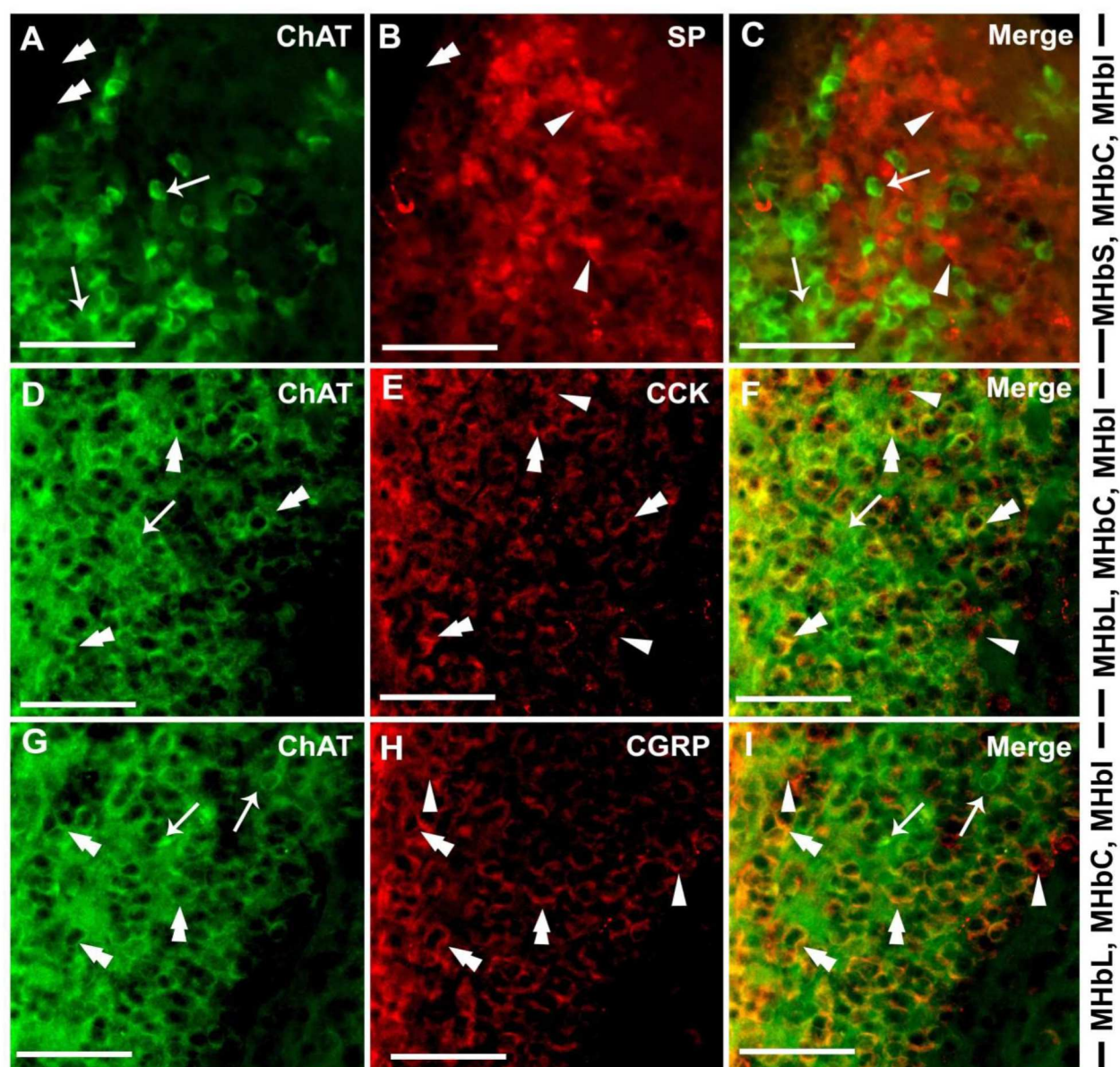


Figure 4.8. Double-label immunofluorescence immunohistochemistry for ChAT with peptidergic neuronal marker (SP, CCK, and CGRP) in medial habenula of adult mouse. Photomicrographs showing immunolabeling of HAP1 with SP (A-C) or CCK (D-F) or CGRP (G-I) in the medial habenula of adult mouse. Single white arrows indicate the cells positive only for ChAT, white arrowheads indicate cell positive for SP or CCK or CGRP, and double white arrowheads indicate the cells positive for both ChAT and SP or CCK or CGRP. Scale bar = 50 μ m in “A-I”. SP, Substance P; CCK, Cholecystokinin; CGRP, Calcitonin gene-related peptide, MHb, Medial Habenula; MHbC, central part of MHb; MHbI, inferior part of MHb; MHbL, lateral part of MHb; MHbS, superior part of MHb.

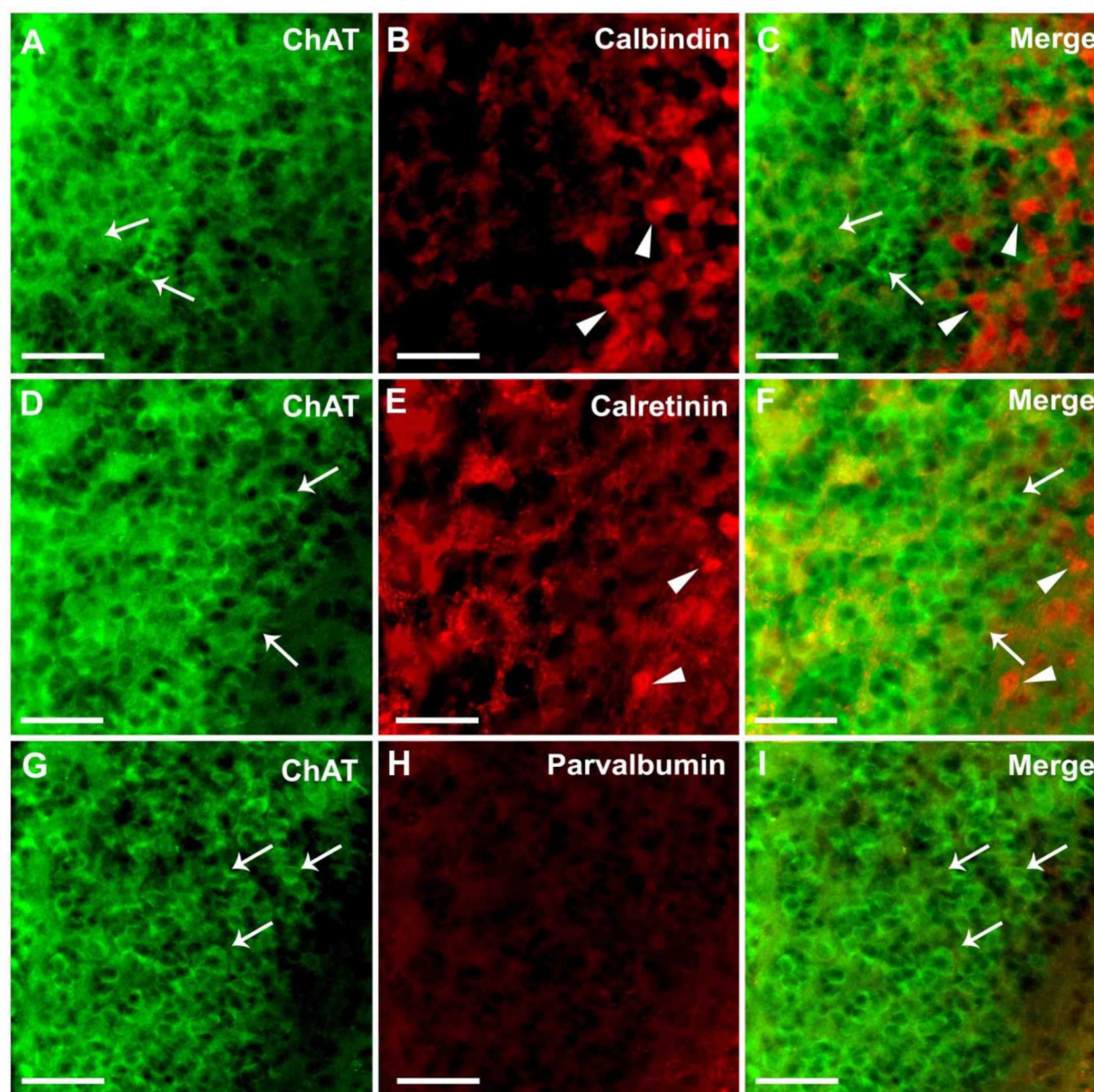


Figure 4.9. Double-label immunofluorescence immunohistochemistry for ChAT with Calcium binding proteins (Calbindin, Calretinin, and Parvalbumin) marker in medial habenula of adult mouse. Photomicrographs showing immunolabeling of HAP1 with calbindin (A-C) or calretinin (D-F) or parvalbumin (G-I) in the medial habenula of adult mouse. Single white arrows indicate the cells positive only for ChAT, white arrowheads indicate cell positive for calbindin or calretinin or parvalbumin, and double white arrowheads indicate the cells positive for both ChAT and calbindin or calretinin or parvalbumin. Scale bar = 50µm in “A-I”. MHb, Medial Habenula; MHbC, central part of MHb; MHbI, inferior part of MHb; MHbL, lateral part of MHb; MHbS, superior part of MHb.

4.4. Discussion

To the best of our observation, the present study is the first to identify the expression, comparative distribution, and neurochemical characterization of HAP1 with cholinergic (ChAT), gaseous neuromodulator (nNOS), neuropeptide (SP, CCK, and CGRP), and CBP (calbindin, calretinin, and parvalbumin) neuronal marker in the habenula nuclei of adult mice. The possible outcome for the neurochemical heterogeneity of HAP1 on neuroprotection in the mouse habenula is presented in the Figure 8. The habenula is a highly conserved epithalamic structure that acts as a relay between forebrain limbic circuits and midbrain monoaminergic nuclei. Although HAP1 expression has been investigated using in situ hybridization (Fujinaga et al., 2009, 2004), Western blotting (Li et al., 1998), RT-PCR (Li et al., 2000), and immunohistochemistry (Niu et al., 2011; Sheng et al., 2008) across various regions of the rodent brain, its cellular localization, comparative immunohistochemical distribution, and neurochemical characterization in the habenula have not been explored in the mouse brain. In the present study, we confirm the existence of two isoforms of HAP1 (HAP1A and HAP1B) in habenula of the mouse brain by Western blotting. These results coincide with earlier research (Fujinaga et al., 2009; Gutekunst et al., 1998; Islam et al., 2022, 2017; Li et al., 1998) where HAP1 exists in two isoforms in the rodent brain, spinal cord, or enteric nervous system. It is well known that HAP1A induces cytoplasmic STB, whereas HAP1B gives rise to diffuse cytoplasmic expression (Fujinaga et al., 2007; Li et al., 1998).

According to our current research, HAP1 is selectively expressed in LHb neurons with most HAP1-ir cells exhibit characteristic STB (smaller or larger) in their diffusely immunoreactive cytoplasm, expanding the known neuroanatomical distribution of this HAP1 and providing new insight into possible mechanisms by which habenular neurons regulate their connectivity and plasticity. However, there are some strongly stained HAP1-ir cells where the STB are challenging to identify under light microscopy despite their existence (Afrin et al., 2025; Islam et al., 2022; Meher et al., 2025b). HAP1 has been widely examined in the other parts of the brain, especially the hypothalamus, the amygdala, and limbic circuits, where it is over-expressed in neurons that regulate homeostasis, stress-related responses, and neuroendocrine processes (Fujinaga et al., 2007, 2004; Islam et al., 2025; Li et al., 1996; Meher et al., 2025b; Seo et al., 2019; Sheng et al., 2008). In hypothalamus, HAP1 plays a vital role in intracellular transport of growth factor receptor like TrkA and TrkB to promote the neuronal responsiveness to neurotrophic support (Rong et al., 2006; Sheng et al., 2008). Likewise, HAP1 relates with GABA receptor subunits and AMPA receptor complexes that determine the synaptic stability and plasticity (Li et al., 2002). This receptor-associated function aligns with our observation that HAP1 immunoreactivity in the habenula is confined to neurons and absent from glial populations. These fundings suggest that HAP1's fundamental role across brain

regions is the regulation of neuronal connectivity and survival through receptor trafficking and intracellular transport. The previous studies showed that HAP1 immunoreactivity is present in neurons rather than glial cells in the adult mouse forebrain, brainstem, spinal cord, or enteric nervous system (Afrin et al., 2025; Islam et al., 2025, 2023, 2022, 2017; Meher et al., 2025b; Tarif et al., 2023; Yanai et al., 2020).

The expression of HAP1 in the habenula is very selective as it is only localized to the LHb and not in the MHb. Such a specific distribution pattern indicates a sub regions functional specialization of HAP1 in habenular circuitry. It has been known that the LHb and MHb differ significantly in terms of their interrelations, their composition of neurotransmitters, and their functions concerning behavior. The MHb is most closely related to the interpeduncular nucleus and it has been implicated in nicotine dependence and cholinergic signaling (Ables et al., 2023). On the other hand, the LHb combines the inputs of the limbic and basal ganglia and it is a key regulator of aversion, negative reward prediction and mood-related behaviors (Browne et al., 2018). Therefore, the selective expression of HAP1 to LHb neurons suggests that this protein can selectively be involved in the regulation of stress, motivation, and affective regulation circuits without implication to the cholinergic and interpeduncular pathways of the MHb. HAP1 confined to LHb neurons emphasizes the concept that HAP1 is not a ubiquitous neuronal protein but is concentrated in populations with metabolic activity and high requirements in synaptic plasticity as well as intracellular trafficking (Li et al., 2000). Moreover, HAP1 has been demonstrated to engage in interaction with intra cellular trafficking machinery and neurotrophic signaling proteins, comprising even with the hepatocyte growth factor-regulated tyrosine kinase substrate (Chen et al., 2023; Rong et al., 2006; Yang et al., 2011). Moreover, its implication to BDNF receptors, thus affecting receptor recycling, intracellular transport, and neuronal survival (Chen et al., 2023; Rong et al., 2006; Yang et al., 2011). Such functions of HAP1 are important in maintaining receptor trafficking and synaptic output to promote aversion-related signaling in the LHb. These results indicate that LHb might have the strong inhibitory effects on midbrain dopaminergic and serotonergic neurons (Groos and Helmchen, 2024; Mondoloni et al., 2022). Nevertheless, the current distribution pattern of HAP1 expression is consistent with the complex neuromodulatory structure of the LHb that obtains separate enkephalinergic projections of the lateral hypothalamus and the septoperiventricular areas (Shinoda and Tohyama, 1987). These observations indicate that the HAP1-expressing neurons are able to coordinate the simultaneous presence of various neuropeptidergic signals in the circuit.

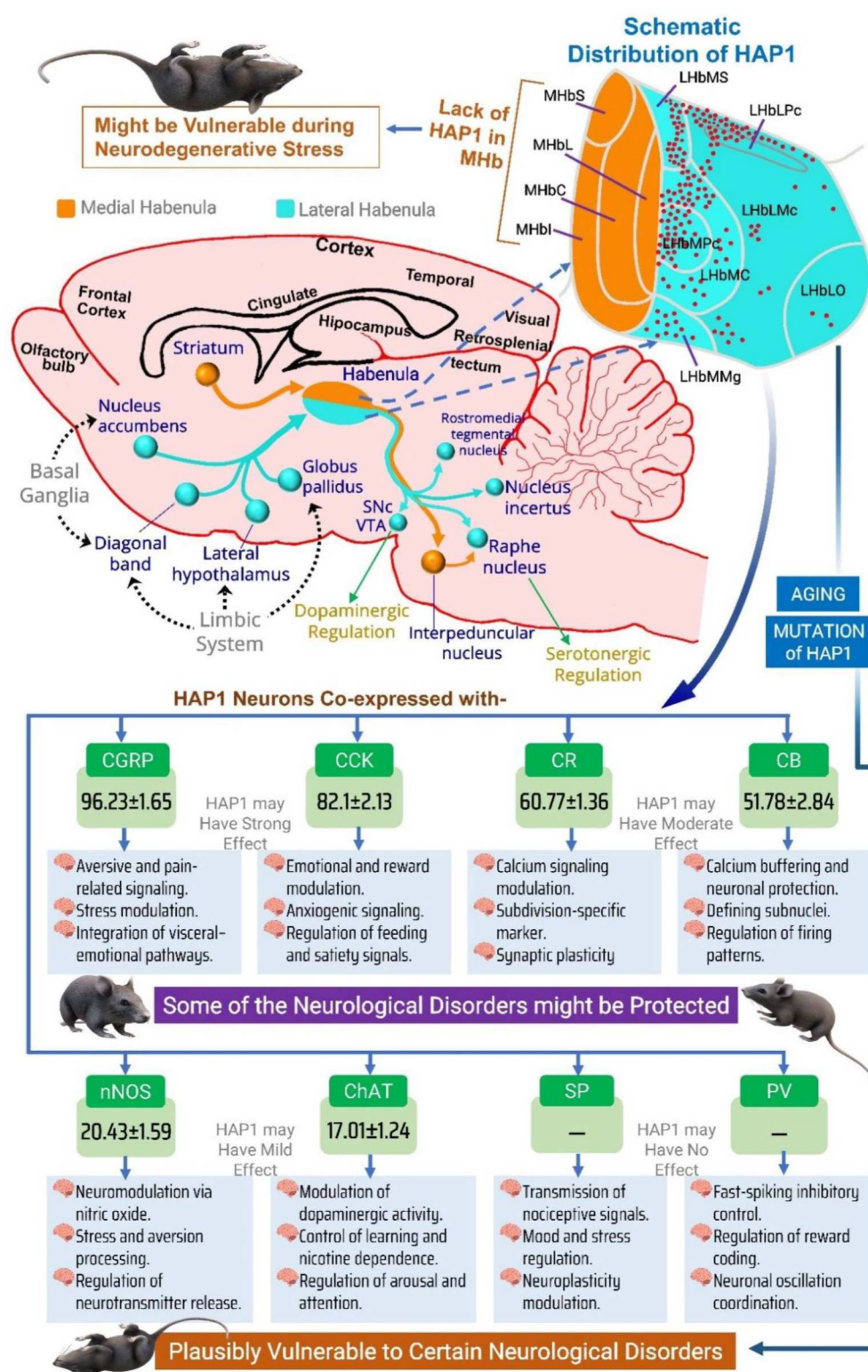


Figure 4.10. HAP1 co-expression with neuronal markers reveals potential mechanisms of neuroprotection in the mouse habenula. The afferent and efferent neuronal distribution of the mouse habenula, adapted and modified from Aizawa (2022). HAP1 shows high co-expression with CGRP and CCK, moderate co-expression with CR and CB, low co-expression with nNOS and ChAT, and is absent in Substance P (SP) and parvalbumin (PV) expressing neurons. This pattern suggests that the brain functions that regulated by the neuronal populations with high HAP1 co-expression are likely to be protected under neurodegenerative stress, while moderately expressing neurons may be partially protected, and neurons lacking HAP1 may be highly vulnerable. Loss of HAP1 due to aging or mutation could render all habenular neurons susceptible to neurodegeneration. For details on abbreviations, see Abbreviations.

The interactive distribution of HAP1 and cholinergic neurons is especially significant to comprehend the neuronal segregation in the adult mouse habenula. HAP1 expression is restricted to the LHb, while ChAT are localized to the MHb (Aizawa et al., 2012). These findings indicate that neurochemical segregation of habenular subcircuits. This distinction can be attributed to the specific mechanisms of the two habenular pathways, such as the MHb-IPN circuit (mediated by acetylcholine and is associated with aversion and nicotine-related behavior) and the LHb-midbrain circuit (responsible for the brain function to learn negative results and anticipate unpleasant events) (Antolin-Fontes et al., 2015; Sosa et al., 2021). Moreover, the partial co-expression of HAP1-ir with cholinergic (ChAT-ir expressing) neurons suggests that HAP1 may not a major component of classical cholinergic signaling in the LHb. Nevertheless, the presence of HAP1-immunoreactive somata and terminal-like structures in adjacent apposition to ChAT-ir neurons that involved in potential synaptic or modulatory connections. These pattern of distribution may predict that the HAP1-positive neurons may have the indirect influence to cholinergic signaling in the habenula (Chung et al., 2023; Meher et al., 2025b; Pang et al., 2016). For instance, HAP1 may have effect with the modulatory role of acetylcholine in habenula that are connected to nicotine addiction and mood regulation (Chung et al., 2023; Meher et al., 2025b; Pang et al., 2016). In hypothalamic feeding circuits, HAP1 is not always expressed in orexigenic neurons but assists in the stability and trafficking of receptors crucial for energy balance (Timper and Brüning, 2017).

Regarding gaseous neurotransmitter systems, nNOS-ir neurons were also found in the lateral habenula, which suggests that nitric oxide signaling may be involved in the epithalamic circuit of brain. Nitric oxide is a neuromodulator with a diffusion effect on the plasticity of synapses, excitability, and circuitry of stress (Hardingham et al., 2013). The expression of nNOS in the LHb may suggest that nitric oxide could also play a role in the formation of the habenular output, especially in the pathways associated with aversive processing and mood control. Based on the co-expression of HAP1 and nNOS, a portion of HAP1 co-expression were found with nNOS, indicating that nitric oxide signaling can be one of the functional partners of HAP1 in the LHb. The selective enrichment of HAP1 in a subpopulation of nitrergic neurons makes it a potential that HAP1 can be the trafficking factor of the nNOS-based signaling complex, and can regulate the excitatory drive of LHb neurons output. This is in line with the previous study that has attributed HAP1 with the roles of stabilizing and trafficking of membrane receptors (including TrkB and GABA receptors) which play crucial roles in neuronal excitability and survival (Chen et al., 2023; Rong et al., 2006; Sheng et al., 2008).

The functional heterogeneity of the habenular subdivision is highlighted by the unique distribution patterns of neuropeptides in the habenula. The SP-ir cells were highly selective to the MHbS (Aizawa et al., 2012). The SP immunoreactivity in the dorsal part of habenula indicating its well established role in the medial habenula–interpeduncular nucleus (MHb-IPN) pathway (Burgunder and Young, 1989). Specifically, SP-ir neuronal fibers are found to have their origin in the MHb and extend to the LHb and terminate in the IPN where fibers are contained in dense-core vesicles and mediate cholinergic signaling and other neuromodulatory functions (Burgunder and Young, 1989). In the initial immunohistochemical investigations showed that SP-ir fibers in LHb are generated by the main sources of the rostral entopeduncular nucleus and medial habenula, which create specific medial and lateral subdivisions with intensive peptidergic innervation (Shinoda et al., 1984). This distribution indicates that SP might be selectively involved in aversive behaviors and rewards that mediated by MHbS-IPN circuit without involving the LHb. However, the interpeduncular nucleus is the primary efferent projection of habenular neurons, which has a complex subnuclear structure with separate and partially co-expression with enkephalinergic and SP neurons (Shinoda et al., 1988). This neuronal distribution and connection may provide a neuromodulatory transmission framework of the habenula-interpeduncular axis. Furthermore, this neuronal framework could be influenced by HAP1-positive neurons. Such preferential distribution of SP in the MHb could be responsible for the absenteeism of HAP1 and SP co-expression. Because the SP mainly projects to the interpeduncular nucleus and is less directly involved in the aversion-related output of the LHb (Contestabile et al., 1987). The expression of CCK-ir neurons in LHbMC, LHbMPc, and LHbLMc suggest involvement of CCK in the modulation of LHb output. Particularly CCK might interfere in the affective and motivational circuits through local microcircuitry or may have projections to midbrain targets. The selective expression of CCK, across the LHb subnuclei (moderate to strong in LHbMS, LHbMMg, LHbLO, and from weak to moderate in LHbLPc) highlights the possibility of region-specific neuromodulatory roles of CCK within the LHb. The HAP1-ir enrichment with CCK-ir expressing neurons is an indication to a specific functional specialization by subpopulation. It is also well known that CCK-ir is a neuromodulator of emotional and motivational states of consciousness and it has been established that CCK-ir signaling has considerable links to anxiety, stress responsiveness, and reward processing (Ballaz and Bourin, 2021; Rotzinger and Vaccarino, 2003). The considerable proportion of co-expression between HAP1-ir and CCK-ir neurons in the LHb suggests that HAP1 might be fundamental to the intracellular trafficking and stability of CCK-ir mediated modulation in the habenular output to midbrain dopaminergic and serotonergic functions (Sobrido-Cameán et al., 2020). The other peptidergic marker, CGRP showed strong immunoreactivity across nearly all the LHb and MHb subregions of habenula. The highest co-expression of CGRP with HAP1 particularly in the LHb territories (LHbMC and LHbMPc) raises the

interesting prospect of an interaction between CGRP signaling and HAP1-mediated intracellular trafficking. Perhaps, CGRP is an effective neuropeptide which responsible for the nociception, stress responses, and aversion-related circuits (Iyengar et al., 2017). The co-expression of CGRP-ir with HAP1-ir neurons strongly suggests that HAP1 might be used as a molecular signature of this peptidergic phenotype in the LHb. The HAP1 enrichment in CGRP neurons may facilitate the stability of CGRP. These stable CGRP is crucial for long-range signaling to the brainstem and midbrain that are involved in pain and stress integration (Edvinsson and Ho, 2010). Furthermore, the existence of HAP1-ir in CGRP neurons may also provide protection against excitotoxic stress. Because the HAP1 has been proposed to have neuroprotective effects through its interactions with intracellular transport machinery (Chen et al., 2023; Gauthier et al., 2004; Li et al., 1995, 1996).

The heterogeneity in CBP expression observed in subregions of the habenula that underscores the diversity of the molecular and functional aspects within this epithalamic structure. CBP-ir neurons were predominantly localized in the medial portions of the lateral habenula (LHbMS, LHbMC, and LHbMPc) which indicates that these sub regions may be involved in specialized functions of calcium-dependent signaling and neuronal excitability. Contrary, the weaker calbindin-ir expression in LHbMMg, LHbLPc, and LHbLMc, and its absence in LHbLO along with the almost no expression in the MHb suggest that calbindin-dependent buffering may be selectively important to particular populations of LHb neurons. These results might be potentially reflecting the differences in CBP's synaptic inputs or firing patterns (Bezprozvanny and Mattson, 2008; Chard et al., 1993; Medalla et al., 2023). The another CBP, calretinin-ir neurons exhibited a widespread distribution throughout the majority of LHb and MHb subnuclei. The broader distribution of calretinin-ir neuronal expression might suggests a more generalized role of calretinin in regulating intracellular calcium dynamics throughout the habenular circuits. Moreover, this calretinin expression may contribute to the integrative processing of different afferent inputs and also may modulate the outputs to the area of midbrain that involved in reward and aversion (Hua et al., 2018; Medalla et al., 2023; Qi et al., 2022b). Comparatively, the weak expression of calretinin in LHbLPc and LHbLO highlight that the broadly expressed calretinin may have subregional specificity. The preferential co-expression of HAP1 with calbindin-ir and calretinin-ir neurons in the LHb provides valuable hints as to the possible functional identity of CBP. Understanding the Co-expression between HAP1 with calbindin-ir and calretinin-ir neuron may has often been used as a marker for specialized functional roles in certain brain regions where neuronal circuits integrate aversive and reward-related signals (Chard et al., 1993; Hua et al., 2018; Medalla et al., 2023). The strong co-expression of HAP1-ir with calretinin-ir and calbindin-ir neurons suggests that HAP1-ir may be preferentially engaged in neurons that are highly functional and has the metabolic importance. Surprisingly, the PV (another CBP) showed a

restricted pattern with only weak to moderate expression in LHbLO and LHbLMc as well as completely absent in the MHb. This highly selective distribution of PV is consistent with the proposed role of PV-ir positive interneurons. PV might control the local inhibitory signals and fast-spiking activity within the LHb circuits, and this LHb circuit may regulate the synchronous activity in cortical and subcortical networks (Druga et al., 2023; Medalla et al., 2023; Nahar et al., 2021). However, this selective distribution of PV might be responsible for the absence of HAP1 in the PV expressing neurons. The absence of PV neurons in the HAP1 indicating that there may have limited role of HAP1 neuron in the presynaptic calcium signals and also in the paired-pulse facilitation (Fairless et al., 2019; Müller et al., 2007). However, the selective association of HAP1 with calbindin and calretinin neurons indicate its possible role in excitatory and modulatory processes that is linked to the fine-tuning of calcium dynamics (Fairless et al., 2019). These observations are in line with previous studies of HAP1, in which HAP1 has been implicated in intracellular trafficking of neurotrophic factors, receptor clustering, and synaptic vesicle transport (Chen et al., 2023; Li et al., 1998, 1995; Rong et al., 2006; Sheng et al., 2008).

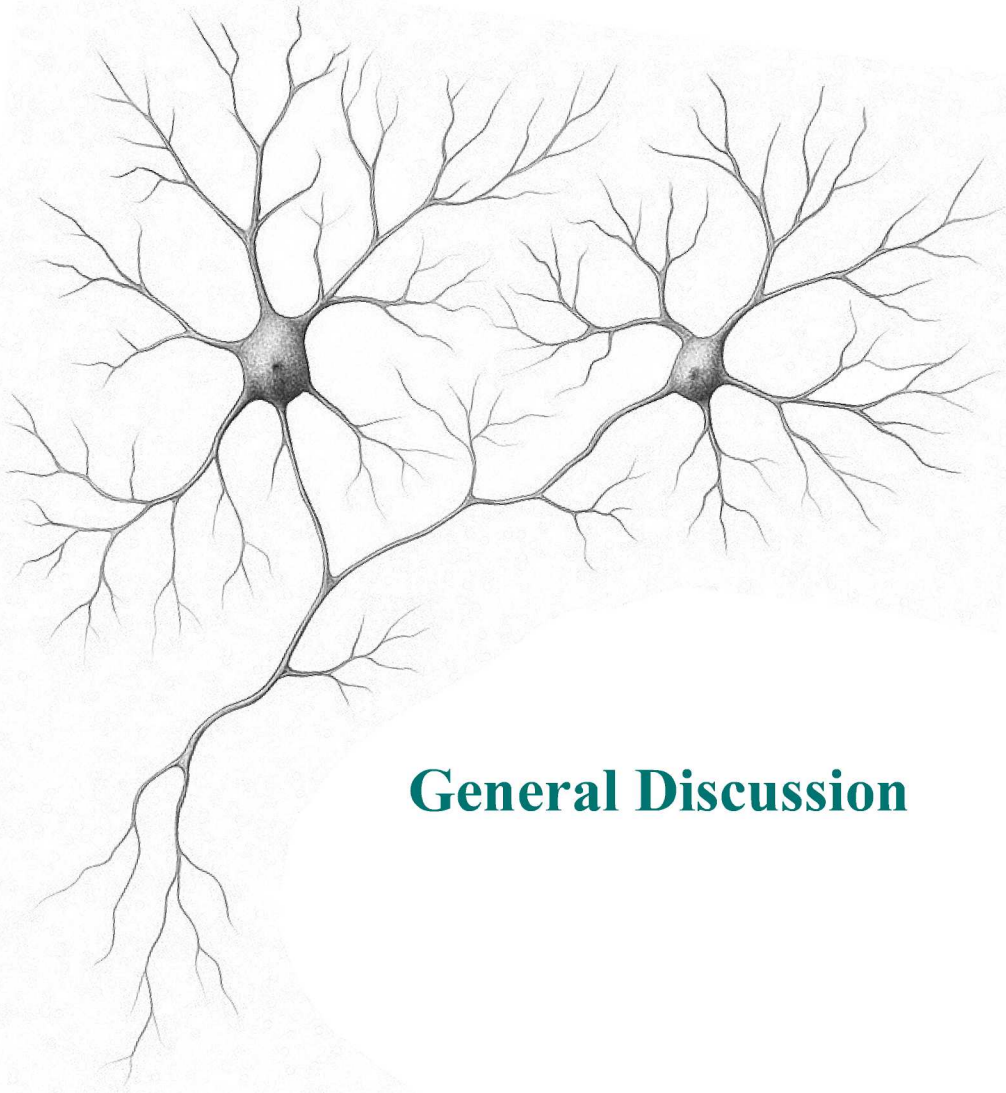
Habenula cholinergic neurons are the source of a major cholinergic pathway in the central nervous system. The Cholinergic neurons in the habenula mainly projected to the IPN neurons through the fasciculus retroflexus (fr) (Renier et al., 2014). The presence of co-expressive ChAT-ir neurons with CCK and CGRP shows that cholinergic transmission may be substantially connected with peptidergic control in these circuits rather than with other neurochemical categories (e.g., SP or CBP expressing interneurons). This result could further be supported by the other study in the basal forebrain where higher proportion of cholinergic neurons were co-expressing with CGRP and CCK immunoreactive neurons (Meher et al., 2025a). However, this co-expression may enhance the dynamic regulation of the MHb cholinergic neurons mediated functions such as the drug addiction (Nicotine addiction and nicotine withdrawal symptoms), anxiety, and depression (Lee et al., 2019).

4.5. Conclusion

The findings of this current research indicated that HAP1 was completely absent in the MHb and specifically localized in the LHb. Conversely, majority of the ChAT-ir neurons located in the MHb. The HAP1-ir neurons had particularly been more prevalent in the LHbMC and LHbMPc which frequently co-localized with calcium-binding proteins (calbindin, calretinin) and peptidergic markers (CGRP, CCK). On the contrary, HAP1 was not expressed in the SP and parvalbumin neuronal subtypes. Rather than being cholinergic or inhibitory, HAP1 is selective to interact with excitatory and modulatory groups of neurons as demonstrated by its molecular specificity. HAP1 distribution with CGRP and CCK-expressing neurons suggests that HAP1 does play a role in the peptidergic regulation of aversion and stress but co-expression with CBP suggests that HAP1 is an active participant in calcium-dependent excitability and plasticity. Besides, HAP1 may provide intracellular maintenance of the elevated synaptic and metabolic requirements of such pathways, based on the known role of the LHb in regulating midbrain monoaminergic mechanisms and negative motivational valence imprinting. Though the ChAT-ir neurons had the CGRP and CCK positive immunoreactivity but the SP and CBP immunoreactivity was completely absent. Lastly, our findings all demonstrate that HAP1 is a molecular marker of LHb-specific neural networks, whereas ChAT is for MHb. Furthermore, habenular dysregulation as well as the ChAT and HAP1 functional abnormalities may have been connected to neuropsychiatric diseases, including addiction, depressive disorder, and neurodegenerative disease etc. Future studies of conditional deletion of HAP1 in clinical models may also reveal it as a therapeutic target and a biomarker in neuropsychiatric and neurodegenerative diseases.



Chapter 5



General Discussion



5.1. General Discussion

I hypothesized that HAP1 would be regionally expressed in selective neuronal populations and have certain immunohistochemical relationships with ChAT-expressing neurons. I further hypothesized that both HAP1 and ChAT neurons would show neurochemical heterogeneity throughout the BFCN, CPu, and habenula (Hb). These hypotheses were based on the known role of HAP1 in intracellular trafficking, vesicle transport, neuronal protection and the known vulnerability of cholinergic neurons in ND. To carry out this study, I used western blotting, Immunoperoxidase and double label immunohistochemistry to examine the distribution, co-expression and neurochemical diversity of HAP1 and ChAT with various neuropeptides (CCK and CGRP) and calcium binding proteins (CBPs) in the adult mouse fore brain.

First, I hypothesized that HAP1 would be expressed in the cholinergic nuclei of the forebrain, and interact with ChAT positive neurons. I found that HAP1 was highly distributed across the BFCN (MS, VDB, HDB, and SIB). I found strong HAP1 and ChAT co-expression in the MS and VDB, and little to no overlap in the striatum, where ChAT neurons were present but had no HAP1 immunoreactivity. This regional variation suggests that HAP1 expression is not constant throughout the cholinergic systems, but HAP1 is determined by functional needs. The MS and VDB cholinergic neurons project densely to hippocampal and cortical areas that brain area is involved in learning, attention, and emotional regulation. The cholinergic neurons have long axons and high metabolic activity, which makes the cholinergic neurons very sensitive to intracellular transport and protein turnover (Wurtman, 1992). The high expression of HAP1 in these cholinergic neurons probably facilitates efficient axonal transport and synaptic homeostasis. Thus plays a role in cholinergic neurons resistance to neurodegeneration. In contrast, striatal cholinergic interneurons are local modulatory elements with specific firing patterns, which do not require long distance transport of neuronal signals (Mallet et al., 2019). The lack of HAP1 in such cells may be the reason for their increased susceptibility during diseases such as Huntington's disease in which vesicular trafficking shortages are prominent (Chen et al., 2023; Gauthier et al., 2004; Li et al., 1995, 1996).

For the second hypothesis, I suggested that ChAT-positive and HAP1-positive neurons in the BFCN and CPu would exhibit different neurochemical heterogeneity that will be indicated by co-expression with neuropeptides and CBPs. I found that ChAT neurons exhibited considerable co-localization with CGRP, and CCK especially in HDB and CPu. I also found calbindin as well as calretinin selectively co-expressed with ChAT in specific nuclei whereas parvalbumin did not overlap ChAT in any region. HAP1-positive neurons also displayed selective and region-dependent co-expression with these markers. HAP1 was moderately co-localized with CGRP and CCK in the MS,

VDB and HDB but not in the SIB. HAP1 was also mildly co-expressed with calbindin and calretinin in forebrain but not in CPu. These results suggest that both ChAT and HAP1 neurons are chemically heterogeneous and that ChAT and HAP1 neurochemical identity is differentially fine-tuned based on regional function.

The patterns of neurochemicals that I found have obvious functional implications. CGRP and CCK are neuropeptides that influence excitability and neurotransmission in limbic and motor circuits. CGRP and CCK co-expression with ChAT indicates that subsets of cholinergic neurons have other modulatory features in addition to acetylcholine release, so that the CGRP and CCK can modulate network activity in a more flexible manner. The selective presence of calbindin and calretinin suggests the differences in the buffering and dynamics of calcium in cholinergic neurons. CBP are important in maintaining calcium homeostasis within cells and are responsible for preventing excitotoxicity, which play important roles in the long-term survival of neurons. The co-expression of HAP1 together with CBP- and neuropeptide-containing neurons indicates that HAP1 may be able to act with these molecules to stabilize calcium-dependent processes and maintain vesicular transport (Chen et al., 2023; Li et al., 1998, 1995; Rong et al., 2006; Sheng et al., 2008). Therefore, the molecular heterogeneity seen among ChAT and HAP1 neurons that are the expression of different physiological functions.

In my third hypothesis I investigated the habenula, where I hypothesized that HAP1 and ChAT would have differential and selective expression patterns. I found that HAP1 was specifically localized to the LHb, particularly dense in the LHbMC and LHbMPc subregions while ChAT expression was limited to the MHb. Within the LHb, HAP1 had a strong co-expression with CGRP and CCK, moderate co-expression with calbindin and calretinin, and a minimal overlap with nNOS. I also found no co-localization of HAP1 to substance P or parvalbumin. In contrast, ChAT neurons in the MHb were selectively co-expressed with CGRP and CCK. These results show that HAP1 and ChAT identify different, but partially overlapping, neuronal populations in the habenula, which presumably represent different aspects of the habenular circuitry.

The habenula is an anatomically well-maintained structure that integrates emotional and motivational information. The LHb, which is abundant in HAP1, is known to modulate aversive and reward prediction pathways through projections to dopaminergic and serotonergic centers (Sobrido-Cameán et al., 2020). The preferential association of HAP1 with CGRP and CCK in this habenula indicates that HAP1-positive neurons are peptidergic modulation and stress-related signaling specialized neurons. Cholinergic ChAT neurons of the MHb have an output to the interpeduncular

nucleus and are considered to be involved in mood and motivational control (Renier et al., 2014). The little overlap between HAP1 and ChAT suggests functional compartmentalization in which HAP1 mediates peptidergic modulation in LHb circuits and ChAT mediates cholinergic modulation in MHb circuits. Functionally and chemically, these markers emphasize the chemical and functional heterogeneity underlying the involvement of the habenula in affective processing (Aizawa et al., 2012).

5.2. Methodological Considerations

The purpose of this well-planned research was to investigate the neurochemical connections between ChAT and HAP1 in several adult mouse brain areas. Adult male C57BL/6J mice were selected because they are often utilized in neuroscience studies and have consistent neuroanatomical patterns. To lessen stress, every animal was kept under carefully regulated lighting and temperature settings. They were given ad libitum food and drink. Ethical approval and proper animal handling were followed according to the official Japanese guidelines, which contributed to ethical and reliable research practices.

The tissue preparation technique has been enhanced for maintaining the intricate structures of the brain. Prior to perfusion, deep sedation was achieved through the use of MMB anesthesia. The brain tissue was kept stable and protected from damage during sectioning by fixation with 4% paraformaldehyde and further in 30% sucrose. For microscopic examination and antibody binding, coronal sections were cut at a thickness of 30 μ m. These procedures ensured that both structural and antigenic properties of the tissue were maintained.

Two primary immunohistochemistry techniques were used in the investigation. The single immunoperoxidase approach was useful in visualizing the distribution of each neurochemical marker. Co-localization between HAP1, ChAT, and other markers such as CGRP, CCK, and calcium-binding proteins may be found using the double-labeling immunofluorescence technique. A comprehensive understanding of neurochemical organization was obtained by combining the two methods. Every antibody was commercially obtainable and its availability has been verified in earlier research. To minimize variability, all parts were kept under the same circumstances. Long incubation periods were used to improve the precision of antibody binding, particularly for proteins like HAP1 that are present in little amounts.

High precision was used in the acquisition and processing of the images. A Nikon brightfield microscope and a Leica Stellaris-8 confocal microscope were utilized to get precise pictures. To

ensure picture integrity, brightness and contrast adjustments were applied consistently. To measure and count co-expressed cells, ImageJ software was used. This helps to preserve integrity in the gathering of data. Nissl staining in nearby sections ensured accurate neuronal distinctions and validated anatomical boundaries.

Finally, the study design emphasized reproducibility through sample replication, with multiple sections analyzed per animal and consistent at equal intervals between sections. As a result, sample bias was decreased and observation reliability was raised. Immunohistochemistry may have some technical drawbacks, such as variations in staining intensity or antibody affinity, but these were reduced by rigorous technical control. In order to comprehend the neurochemical diversity and relationships of HAP1 and ChAT in the adult mouse forebrain, the methodological design as an entirety provided reliable, repeatable, and anatomically precise information.

5.3. Future Directions

The findings from this study present a number of promising avenues for further research. I found that HAP1 is selectively expressed in some cholinergic and peptidergic neurons. However, the underlying sub-molecular mechanism of how HAP1 affects neuronal survival and signaling is not well understood. Future studies should examine the interaction of HAP1 with its known partners such as huntingtin, dynactin and kinesin to regulate axonal transport and synaptic stability. This can be resolved with ultrastructural and live-cell imaging of HAP1-dependent trafficking in identified neuronal populations.

Another important direction is to determine the alteration of the HAP1 expression under pathological conditions. Since HAP1 is linked to Huntington's and other neurodegenerative diseases, experimental models of neuronal injury or neurodegeneration should be used to study whether the HAP1 level is altered by stress. Through transgenic or knockout mice, we can study if the absence or excess of HAP1 protein has any effect on the survival of cholinergic neurons in the basal forebrain or habenula. These experiments would resolve the issue of whether HAP1 is neuroprotective or simply a structural marker of resilient neurons.

It is also important to study the signal pathways associated with the co-expression of HAP1 with neuropeptides and calcium binding proteins. The current results showed that HAP1 is overlapped with CGRP, CCK, calbindin, and calretinin in selective regions. Future studies should investigate if this co-expression is a result of common transcriptional regulation or post-translational regulatory mechanisms. Transcriptomic profiling of HAP1 and ChAT expressing neurons with

single-cell RNA sequencing has the potential to identify unique sets of gene networks that govern their neurochemical identity and functional roles.

Further, the findings in the habenula imply that HAP1 and ChAT define different neuronal circuits that are related to emotion and motivation. It would be interesting to use optogenetic tools to trace the projections of HAP1-positive neurons and ascertain their connectivity with monoaminergic centers. Electrophysiological recordings could then be used to uncover the role of HAP1-expressing neurons in synaptic plasticity, reward and stress.

Another future study would be to examine the developmental pattern of HAP1 expression. It is currently unknown whether HAP1 shows up at an early stage in the neuronal differentiation process or later after the establishment of mature circuitry. Investigating the postnatal and adult stages of the brain may be useful in determining when HAP1 becomes functionally integrated into cholinergic and peptidergic systems. This would also elucidate whether its neuroprotective roles are developmentally programmed or activity dependent.

Finally, therapeutic implications should be explored in future studies. If HAP1 indeed supports neuronal stability and modulates calcium or vesicular dynamics, then pharmacological enhancement of HAP1 function may become a potential strategy for neuroprotection. Investigating small molecule or gene therapy strategies to increase HAP1 expression in vulnerable cholinergic systems might offer an insight into the translational potential of HAP1.

5.4. Final Conclusions

In this study, the neurochemical distribution and relationships of HAP1 and ChAT were investigated in major forebrain nuclei including the BFCN, CPu and habenula of adult mice. HAP1-immunoreactive neurons were moderately to highly distributed in the MS, VDB, SIB, LSI, LSV and LHb but absent in the MHb. In contrast, ChAT-immunoreactive neurons were numerous in the BFCN and MHb, whereas very little HAP1 was expressed in the CPu. This selective distribution may explain the increased vulnerability of the cholinergic neurons of the striatum to neurodegenerative processes.

The co-expression of HAP1 and ChAT was high in the MS, VDB, LSI and LSV suggesting a neuroprotective role of HAP1 within basal forebrain cholinergic systems. These neurons, which project to the hippocampus, amygdala and hypothalamus, may be supported by the HAP1 mediated

mechanisms to maintain the memory, emotional and cognitive functions during the time of stress or degeneration.

Cholinergic neurons in the basal forebrain and neostriatum were also characterized by significant neurochemical heterogeneity. Cholinergic neurons co-expressed with neuropeptides (CGRP, CCK) and other CBP (calbindin, calretinin) suggesting distinct subpopulations with specialized regulatory functions. However, HAP1 demonstrated only limited overlap with these peptidergic and CBP-expressing neurons and therefore, its actions may be occurring in discrete or selective neuronal circuits. This pattern supports the concept that HAP1 is a region- and cell type-specific, classically neuropeptide system-independent, functioning protein.

In the habenula, HAP1 was highly expressed in the LHb, and specifically in the LHbMC and LHbMPc, where it co-expressed with CGRP, CCK and several CBPs (calbindin and calretinin). These results reveal that HAP1 has a preferential interaction with excitatory and modulatory neurons. HAP1 association with CGRP and CCK raises the possibility of a role in peptidergic control of aversion and stress. The co-expression with CBPs raises the possibility of a role in calcium-dependent excitability and synaptic plasticity. The absence of HAP1 in SP and parvalbumin neurons further argues for its selective molecular specificity.

Statement of Published Work Derived from This Dissertation

This dissertation is based on original research conducted by the author during the period of doctoral study at Yamaguchi University Graduate School of Medicine, from 01 April 2023 to 31 March 2026. Portions of the research work and findings presented in this dissertation have been published in peer-reviewed scientific journals. These publications represent integral components of the present dissertation and have contributed substantially to the development of its scientific content and interpretations.

The published papers include the following:

1. **Meher, M. M.**, Afrin, M., Jahan, M. R., Nozaki, K., Masumoto, K., Yanai, A., & Islam, M. N. (2025). Neurochemical heterogeneity of ChAT-immunoreactive neurons in the basal forebrain cholinergic nuclei and striatum in reference to CGRP, CCK, and calcium-binding proteins. *Acta Histochemica*, 127(4), 152291. <https://doi.org/10.1016/j.acthis.2025.152291>
2. **Meher, M. M.**, Islam, M. N., Yanai, A., Afrin, M., Jahan, M. R., Nozaki, K., Masumoto, K., & Shinoda, K. (2025). Immunohistochemical relationships of huntingtin-associated protein 1 with choline acetyltransferase in the forebrain cholinergic nuclei of adult mice. *Cell and Tissue Research*. <https://doi.org/10.1007/s00441-025-03996-w>

In addition to the published works, the author is preparing manuscript for a **third research article** based on the experimental findings presented in **Chapter 4 (Study III)**. This manuscript is intended for submission to a peer-reviewed journal following the completion of the dissertation.

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