

学位論文（博士）

**Immunohistochemical Relationship of
Huntingtin-Associated Protein 1 with Serotonin in
Mouse Brainstem Raphe Nuclei and its Protective Role
in Serotonergic System**

(マウス縫線核における HAP1 の発現解析およびセロト
ニン神経系における保護的役割についての検討)

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ACADEMIC DISSERTATION

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

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Submitted to the
**DIVISION OF NEUROANATOMY
DEPARTMENT OF NEUROSCIENCE
YAMAGUCHI UNIVERSITY GRADUATE SCHOOL OF MEDICINE, JAPAN**

MARCH, 2026

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List of Glossary

10N	: Dorsal motor nucleus of vagus
12N	: Nucleus of the hypoglossal nerve
2Cb	: Second cerebellar lobule
3N	: Oculomotor nucleus
4N	: Trochlear nucleus
4V	: 4th ventricle
5-HT	: 5-hydroxytryptamine (serotonin)
5N	: Trigeminal motor nucleus
6N	: Abducens nucleus
7n	: Facial nerve
7N	: Facial nucleus
AD	: Alzheimer's disease
Akt	: Phosphoinositide 3 kinase (PI3K)
Amb	: Nucleus ambiguus
AP	: Area postrema
Aq	: Aqueduct of Sylvius
ATg	: Anterior tegmental
A β	: Amyloid β
Bcr	: Breakpoint cluster region protein
BDNF	: Brain derived neurotrophic factor
cc	: Central canal
cMnR	: Caudal part of median raphe nucleus
CLi	: Caudal linear nucleus of raphe
CLiw	: Caudal linear nucleus of raphe wing part
DR	: Dorsal raphe nucleus
DRC	: Dorsal raphe nucleus caudal part
DRD/DRd	: Dorsal raphe nucleus dorsal part
DRI/DRi	: Dorsal raphe nucleus interfascicular part
DRL	: Dorsal raphe nucleus lateral part
DRV/DRv	: Dorsal raphe nucleus ventral part
ERK	: Extracellular signal regulated kinase
EW	: Edinger-Westphal nucleus
GFAP	: Glial fibrillary acidic protein

GiA	: Gigantocellular reticular nucleus
GR	: Glucocorticoid receptor
HAP1	: Huntingtin-associated protein 1
HAP1 ^{-/-}	: HAP1 homozygous knockout
HAP1 ^{+/-}	: HAP1 heterozygous knockout
HAP1 ^{+/+}	: HAP1 wild type
HD	: Huntington's disease
HPA	: Hypothalamic–pituitary–adrenal
HTT	: Huntingtin
Iba1	: Ionized calcium binding adaptor molecule 1
IGL	: Intergeniculate leaflet
IP	: Interpeduncular nucleus
IPC	: Interpeduncular nucleus caudal part
IPR	: Interpeduncular nucleus rostral part
ir	: Immunoreactive
isDRd	: Isthmus dorsal raphe nucleus dorsal part
isDRl	: Isthmus dorsal raphe nucleus lateral part
isDRw	: Isthmus dorsal raphe nucleus wing part
isDRv	: Isthmus dorsal raphe nucleus ventral part
KO	: Knockout
mDR	: Mid dorsal raphe
mHTT	: Mutant huntingtin
ml	: Medial lemniscus
mlf	: Medial longitudinal fasciculus
MnR	: Median raphe nucleus
mTORC1	: Mammalian target of rapamycin complex 1
NDs	: Neurodegenerative disorders
NeuN	: Neuronal nuclei
P0	: Postnatal day 0
PAG	: Periaqueductal gray
PD	: Parkinson's disease
PDR	: Posterodorsal raphe
PMnR	: Paramedian raphe nucleus
PnC	: Pontine reticular nucleus caudal part
PnO	: Pontine reticular nucleus oral part

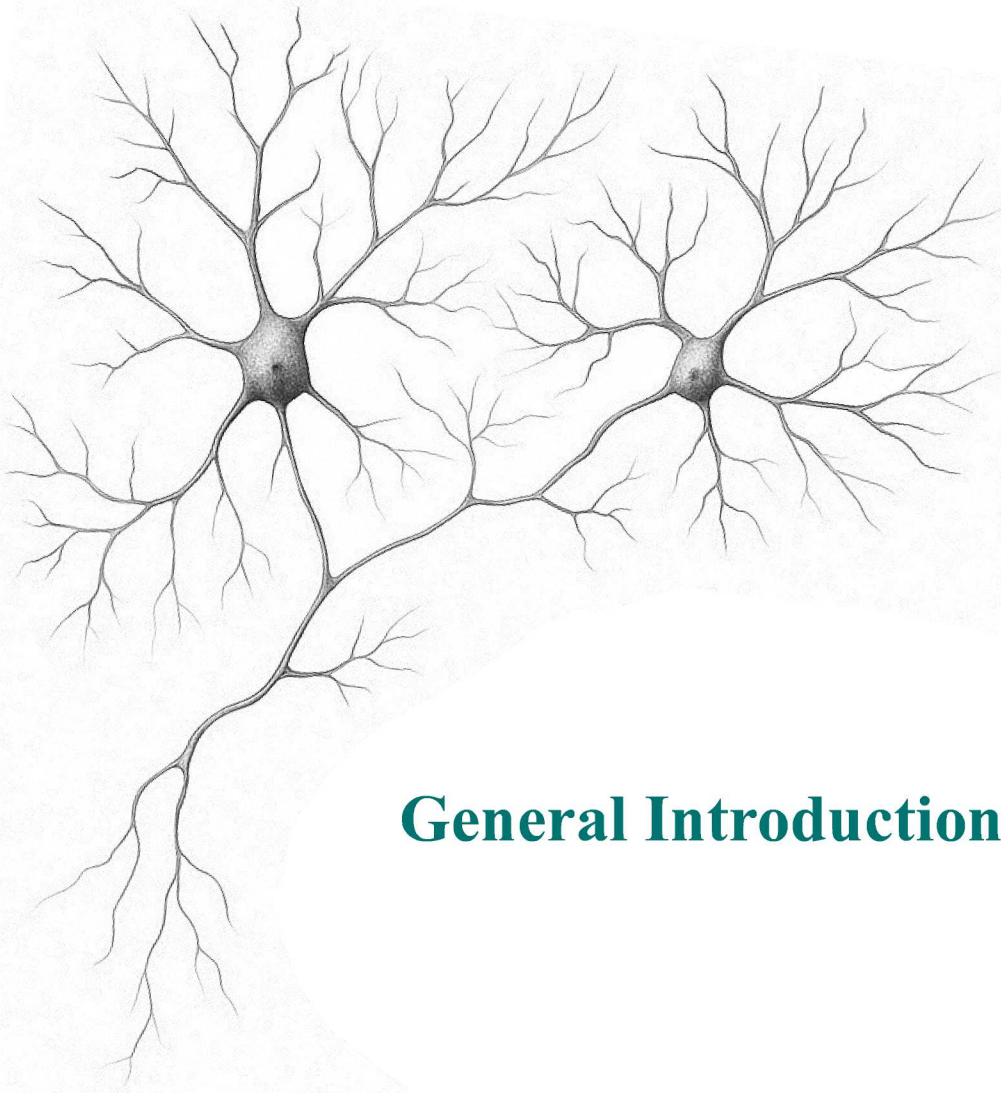
PnR	: Pontine raphe nucleus
PPnR	: Prepontine raphe nucleus
PPy	: Parapyramidal nucleus
py	: Parapyramidal tract
R	: Red nucleus
r1	: Rhombomere 1
r2	: Rhombomere 2
r3	: Pontine rhombomere 3
Rbd	: Rhabdoid nu
RIP	: Raphe interpositus nucleus
rMnR	: Rostral part of median raphe nucleus
RMg	: Raphe magnus nucleus
RMgD	: Raphe magnus nucleus dorsal part
RMgV	: Raphe magnus nucleus ventral part
ROb	: Raphe obscurus nucleus
RPa	: Raphe pallidus nucleus
RRF	: Retrorubral field
RRID	: Research resource identifier
scp	: Superior cerebellar peduncle
SDS–PAGE	: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SIDS	: Sudden Infant Death Syndrome
sp5	: Spinal trigeminal tract
STB	: Stigmoid body
SuL	: Supralemniscal raphe nucleus
TBST	: Tris:buffered saline with 0.1% Tween 20
TPH2	: Tryptophan hydroxylase 2
TrkA	: Tropomyosin related kinase A receptor tyrosine kinase
TrkB	: Tropomyosin receptor kinase B
ts	: Tectospinal tract
TSC1	: Tuberous sclerosis 1
VSRt	: Ventrolateral superficial reticular area
xpy	: Pyramidal decussation
xscp	: Decussation of the superior cerebellar peduncle

Abstract

Huntingtin-associated protein 1 (HAP1) is a component of stigmoid body and is well known as the protective interactor of proteins implicated in multiple neurodegenerative disorders (NDs). Brain areas enriched with HAP1 tend to be relatively resistant to neurodegenerative processes whereas areas with little or no HAP1 expression tend to be preferentially vulnerable. This "HAP1 neuroprotectivity" has been proposed to be a major determinant of regional susceptibility of multiple NDs. Additionally, there is mounting evidence that some NDs and neuropsychiatric diseases are caused by malfunctioning of serotonergic (5-HT) neurons. The raphe nuclei, which are the main source of central 5-HT neurons, have not been fully discussed in the context of the HAP1 protection hypothesis. To address this gap, we showed that HAP1 is abundantly expressed in the entire raphe system, whereby nearly 99% of 5-HT neurons in adult mice show strong HAP1 immunoreactivity in the caudal linear raphe, median raphe, dorsal raphe, suprallemniscal raphe, caudal part of the dorsal raphe, pre-pontine raphe, pontine raphe, raphe magnus, raphe obscurus, raphe pallidus, and parapyramidal raphe nuclei of rostral and caudal clusters. This suggests that there could be a close functional relationship between serotonergic signaling and HAP1. The better comprehension of this association was obtained through the generation of HAP1 homozygous knockout (HAP1^{-/-}) pups by CRISPR/Cas9-mediated genome editing. However, it was challenging to render a comprehensive assessment of their functioning in adulthood as they typically died within 24 hours after birth for inexplicable causes. Therefore, in the present study, postnatal day 0 (P0) HAP1^{+/+} and HAP1^{-/-} pups were used to investigate the neurochemical and morphological interaction between HAP1 and 5-HT neurons at an early developmental stage. Almost 98% of 5-HT neurons co-expressed with HAP1 in both raphe clusters of wild pups. Despite the conserved number of 5-HT neurons in P0 knockout, HAP1^{-/-} pups showed a significant decrease in the somatic volume of 5-HT neurons, and profoundly reduced serotonergic fiber varicosities, throughout the raphe nuclei and their major forebrain targets, including the cortex, hippocampus, hypothalamus, and amygdala. Even HAP1 heterozygous pups (HAP1^{+/-}) showed an intermediate loss of varicosities, indicating the possibility of a gene-dependent effect. Accordingly, tryptophan hydroxylase-2, the rate-limiting enzyme for 5-HT synthesis was significantly reduced in HAP1^{-/-} pups. All of these results suggest that HAP1 is a crucial regulator of 5-HT synthesis and neuronal morphology. Therefore, regulation of intracellular trafficking, synaptic varicosity development, and neurochemical stability may be carried out by HAP1 to preserve 5-HT neuronal integrity and raise the threshold of susceptibility to neurodegeneration.



Chapter 1



General Introduction



1. General Introduction

Huntingtin-associated protein 1 (HAP1) is a neuron-enriched cytoplasmic protein best known as a core component of the stigmoid body (STB), a non-membranous inclusion that serves as a defining morphological marker of HAP1 localization (Shinoda et al., 1992, 1993; Gutekunst et al., 1998; Li et al., 1998b; Fujinaga et al., 2007, 2009; Islam et al., 2012, 2017; Meher et al., 2025b). Since being first identified as a polyglutamine-length-dependent interactor of huntingtin (Li et al., 1995a), its neuroprotective potential in a range of neurodegenerative disorders (NDs) has garnered substantial attention. Numerous studies have shown that HAP1 binds to pathological polyglutamine-expanded proteins and counteracts their apoptotic toxic factors (Fujinaga et al., 2011), increasing the neuronal threshold for degeneration of neurons. Accordingly, the "HAP1 protection hypothesis" suggests that areas of the brain that have abundant HAP1 expression are relatively more resistant to neurodegeneration, while areas of the brain that do not express HAP1, such as the striatum, thalamus, cerebellum, and brainstem motor nuclei, are highly vulnerable to neurodegeneration (Fujinaga et al., 2004, 2009; Islam et al., 2017, 2022, 2025; Wroblewski et al., 2018; Tarif et al., 2023; Meher et al., 2025b). These protective mechanisms may contribute to modulation of neuronal survival in not only HD but also the spinocerebellar ataxias, the spinal and bulbar muscular atrophies, and the Joubert syndrome (Li et al., 2003; Takeshita et al., 2006, 2011; Metzger et al., 2008; Sheng et al., 2008). In addition to its putative neuroprotective roles, HAP1 is implicated in diverse physiological functions, including stress responses, postnatal development, intracellular trafficking, and the regulation of neuronal plasticity (Lin et al., 2010; Chen et al., 2020, 2023).

Serotonin (5-hydroxytryptamine, 5-HT) is another key regulator of neural homeostasis. Originating primarily from the raphe nuclei, which flank the midline along the rostro-caudal length of the brainstem both in animals (Meessen and Olszewski, 1949; Taber et al., 1960) and human (Olszewski and Baxter, 1954). These neurons play a role in a wide range of physiological functions such as brain development, mood and stress responses, feeding, autonomic regulation, circadian rhythms, and higher order neurocognitive functions (Jacobs and Azmitia, 1992; Hornung, 2003). Disruption of 5-HT signaling is a hallmark of numerous NDs and neuropsychiatric disorders (Xing et al., 2024). According to the results of several scientific studies, the shape and/or density of 5-HT neurons are in fact changed in a variety of mental illnesses, including depression (Rajkowska et al., 2017), ASD (Makkonen et al., 2008), schizophrenia (Hrovatin et al., 2020), traumatic brain injury (Abe et al., 2016) and in cases like prenatal exposure of psychotropic drugs (Xu et al., 2004; Maciag et al., 2006; Weaver et al., 2010; Reinebrant et al., 2020), neonatal hypoxia (Reinebrant et al., 2020), or postnatal social isolation (Kuramochi and Nakamura, 2009; Awasthi et al., 2021). In mice, these raphe 5-HT neurons are produced at the beginning of embryogenesis (about E10-E12) and start extending long-range axons shortly after; their dorsal and median subgroups (B6-B8) form distinct, but partly

overlapping patterns of innervation in their targets in the forebrain (Alonso et al., 2013; Awasthi et al., 2021). Although their cytoarchitectonic boundaries are subtle, these nuclei are unified by a shared enrichment of serotonergic neurons, as first revealed by classical histofluorescence techniques (Dahlström and Fuxe, 1964). The immunohistochemical detection by tryptophan hydroxylase (TPH), 5-HT, and serotonin transporter (SERT) then strengthened our knowledge of cellular composition and topographic projection patterns (Joh et al., 1975; Steinbusch, 1981; Ovalle et al., 1995; Sur et al., 1996; Zhou et al., 2017). A conserved organizational plan was revealed across mammals including humans by these earlier studies, in spite of the species-specific differences (Hubbard and Carlo, 1974; Schofield and Everitt, 1981; Schofield and Dixon, 1982; Azmitia and Gannon, 1983; Felten and Sladek, 1983; Takahashi et al., 1986; Hornung and Fritschy, 1988; Nishi et al., 2000). Notably, the development and terminal differentiation of these monoaminergic axons are possibly influenced by intracellular trafficking, cytoskeletal regulation, and neurotrophins signaling (Ren and Feng, 2007; Speranza et al., 2015; Aiken and Holzbaur, 2021). However, molecular signals which dictate these raphe axons targeting, local arbor complexity, and synaptic bouton (varicosities) assembly are incompletely defined. On the other hand, serotonergic neurons exhibit marked susceptibility to environmental and internal stressors, especially within distinct subregions of the raphe nuclei that possess unique electrophysiological and receptor profiles. Beyond its classical neurotransmitter role, 5-HT also acts as a trophic molecule during embryogenesis and contributes to structural remodeling of the developing and adult brain. Thus, the understanding of those factors involved in homeostatic maintenance of the integrity of serotonergic neurons is of high neurobiological relevance.

One critical requirement for serotonergic function is the regulation of 5-HT synthesis, which depends on the brain-specific isoform TPH2. Perturbations in TPH2 expression or serotonergic axon structure during early development can alter emotional behavior, stress reactivity, cognitive functions and increase the vulnerability to neurodevelopmental or psychiatric disorders (Walther and Bader, 2003). Given both the HAP1 and 5-HT systems are critically involved in neuronal stability, regulation of stress and resistance to degeneration, a possible functional interplay between both systems has been an emerging topic of interest. Evidence from hypothalamic studies suggests that HAP1 can modulate glucocorticoid receptor (GR) signaling (Chen et al., 2020), thereby influencing stress-related pathways that may also engage serotonergic circuits. Because dysfunction of the 5-HT system is strongly implicated in the progression of several NDs (Mahmood and Silverstone, 2001; Huot et al., 2011; Viswanath et al., 2013; Politis and Niccolini, 2015), the presence of HAP1 within serotonergic neurons may represent an intrinsic protective mechanism that enhances neuronal resilience against chronic stress or degenerative insults.

In light of the context, we initially hypothesized that HAP1 had a neuroprotective function within the raphe serotonergic system, as it may be expressed in the brainstem raphe nuclei and have specific immunohistochemical interactions with serotonin neurons. The distinct distribution of HAP1 in the raphe nuclei is believed to correlate with specific neurochemical effects on the serotonergic system, highlighting its importance in cellular specialization. However, the neuroanatomical relationship between HAP1 and serotonergic neurons across the raphe nuclei has not been thoroughly studied, despite the physiological and clinical significance of both systems. Therefore, it is crucial to clarify the expression, distribution, and immunohistochemical interaction of HAP1 with 5-HT in raphe nuclei in order to better understand the potential role of HAP1 in the maintenance and protection of serotonergic neurons. Importantly, the well-established roles of HAP1 in trafficking and receptor stabilization raise the question of whether HAP1 also contributes to the development or maintenance of 5-HT circuits. Moreover, the developmental guidance cues that direct raphe neurons to their appropriate targets remain incompletely defined. Therefore, the mechanisms that stabilize early 5-HT projections and ensure their precise arborization across brainstem and forebrain areas are only beginning to be explored by our findings. Using the HAP1-genotype mouse, we further attempted to provide a more comprehensive map of the serotonergic neurons in the raphe nuclei. These analyses provide an important fundamental framework into how HAP1 contributes to serotonergic system development and highlight the potential functional significance in HAP1-deficient mice, which may influence neurodevelopmental and neuropsychiatric vulnerability. Consequently, the current study was designed with the following objectives in view of the aforementioned hypothesis:

- **Objective 1:** Neuroanatomical mapping of huntingtin-associated protein 1 across the rostral and caudal clusters of mouse raphe nuclei and its immunohistochemical relationships with serotonin.
- **Objective 2:** Huntingtin-associated protein 1 deficiency in mouse brainstem raphe nuclei impairs serotonin synthesis and leads to disrupted neuronal fiber-arborization in neonatal mice.



Chapter 2



Review of Literatures



2.1.1. Anatomical organization of serotonergic neurons in the raphe nuclei

The serotonergic neuron clusters may be allocated, on the basis of their distribution and main projections, into two clusters: the rostral cluster, which is confined to the mesencephalon and rostral pons and has major projections to the forebrain, and the caudal cluster, which extends from the caudal pons to the caudal portion of the medulla oblongata and has major projections to the caudal brainstem and to the spinal cord (Alonso et al., 2013).

2.1.1.1. The rostral cluster of brainstem raphe nuclei

The raphe nuclei comprise this rostral cluster, accounting for 85% of all serotonergic neurons in the brain. The majority of rostral serotonergic neurons in the brainstem are found in the caudal linear nucleus (CLi) (Halliday and Törk, 1986). The CLi is densely packed and lies dorsally and caudally to the interpeduncular nucleus (IP). 5HT neurons in the CLi were mainly confined at the caudal level of the mid-brainstem. Neurons were encircled and resided caudally in the CLi at the midline in the decussation of the dentothalamic axons of the superior cerebellar peduncle (scp) and stretched rostrally and laterally towards the red nuclei (R). Small and pyriform 5-HT neurons in CLi were oriented to the median plane, giving them a distinctive and compact morphological appearance.

The dorsal raphe nucleus (DR) expands to the mid-pons from the level of the oculomotor nucleus. The rostral half of DR contains the majority of its serotonergic neurons, which are dispersed across the nucleus's dorsal (DRD), ventral (DRV), interfascicular (DRI), and ventro-lateral (DRVl) subdivisions (Baker and Lindley, 1992). The neurons also extend laterally while remaining confined to the midline. The caudal subdivision of the dorsal raphe nucleus (DRC) extends to the level of the abducens nucleus. Laterally, neurons of the DRVl are adjoining with the serotonergic neurons of the median raphe nucleus (MnR) of the lateral reticular formation.

DR and CLi were separated from MnR by the decussation of the superior cerebellar peduncle (xscp). 5-HT neurons were sparsely scattered in MnR. The MnR reaches the position of the trigeminal motor nucleus (5N) from the caudal limit of the xscp. At the paramedian space, which is inhabited by the anterior tegmental nucleus (ATg) and rhabdoid nucleus (Rbd), the rostral part of MnR is bordered by these nuclei. Ventrally, the MnR is also marked by the region resided by the rostral interpeduncular (IPR) nucleus. It is divided into a median region (MnR proper) complemented in the caudal half by a paramedian region and the lateral extensions in the reticular formation: the supramedian nucleus (SuL) (Baker et al., 1991). Primates have a highly developed population of serotonergic neurons in raphe pontis oralis (PnO) (Hubbard and Carlo, 1974;

Schofield and Everitt, 1981; Schofield and Dixon, 1982; Felten and Sladek, 1983; Azmitia and Gannon, 1986; Hornung and Fritschy, 1988). More than 80% of MnR neurons produce serotonin in the median area; this neuronal percentage sharply decreases in the lateral divisions of the nucleus (Baker et al., 1991).

The rostral pons of the raphe nuclei includes the prepontine raphe nucleus (PPnR) and the pontine raphe nucleus (PnR). The PPnR, located in the caudal region of the MnR, is a distinct population of 5-HT cells. PPnR followed by PnR. 5-HT cells were disseminated to a very limited extent in PnR.

The afferent projections to DR and MnR arise mainly from forebrain structures that are a part of the limbic system. The ventral tegmental area, the laterodorsal tegmentum nuclei, the cingulate cortex, the interpeduncular nucleus, several hypothalamic nuclei, and the lateral habenula are the sources of glutamatergic projections (Behzadi et al., 1990). GABAergic innervation is abundant in DR neurons (Qing-Ping et al., 1992), some of which originate in the hypothalamus (Peyron et al., 1997). In contrast, the RMg and RPa nuclei as well as the nucleus prepositus hypoglossi were the origin of ascending projections to MnR (Behzadi et al., 1990).

The efferent projections of the rostral group ascend by two pathways, which are parallel. The dorsal raphe projection runs in a dorsal tract parallel to the medial longitudinal fasciculus (Azmitia and Gannon, 1983, 1986) while the MnR and caudal DR projection is in a ventral tract that is parallel to the paramedian division of MnR and enters the ventral tegmental area at the dorsal margin of the interpeduncular nucleus (Azmitia and Segal, 1978; de Olmos and Heimer, 1980; Jacobs and Azmitia, 1992).

As the dorsal raphe projections, which innervate the cerebral cortex, are higher in non-human primates, the dorsal route is larger and has comparatively more myelinated serotonergic fibers (Azmitia and Gannon, 1983). At the caudal diencephalon, the dorsal and ventral pathways merge and split into two: (a) a projection that travels through the internal capsule and ends in the lateral cerebral cortex (Van Der Kooy and Kuypers, 1979; Steinbusch, 1981; Porrino and Goldman - Rakic, 1982; Tigges et al., 1982; Waterhouse et al., 1986) and (b) a projection that passes through the medial forebrain bundle, innervating the amygdala, basal forebrain, and hypothalamus before continuing across the cingulum and ending in the medial cerebral cortex and an area of the hippocampus (Azmitia and Segal, 1978; Steinbusch, 1981; Imai et al., 1986; Vertes and Martin, 1988; Datiche et al., 1995). The DR and MnR innervation to the forebrain primarily overlaps with specific cellular

targets and regional dissemination. All regions and layers of the cerebral cortex have tiny, widely spaced varicosities on axons that originate in DR; however, these varicosities are more prevalent in layer IV of the cerebral cortex and primary sensory areas (Morrison et al., 1982; Halliday and Törk, 1986; Campbell et al., 1987; Berger et al., 1988; Hornung et al., 1990).

At the ultrastructural level, it has shown that the varicosities of DR neurons contribute to the volume transmission system rather than forming real chemical synapses. These axons constitute the majority of the serotonergic cortical innervation (Beaudet and Descarries, 1978; de Lima et al., 1988; Dori et al., 1996; Lambe et al., 2000; Pepeu and Grazia Giovannini, 2017). MnR serotonergic neurons, on the other hand, have axons with large, closely spaced varicosities, are more prevalent in the frontal and hippocampal regions, and have terminals that establish real chemical synapses at the ultrastructural level (Halasy et al., 1992; Acsády et al., 1993; Lambe et al., 2000). Furthermore, it has been demonstrated that large varicose serotonergic axons terminate in dense clusters, referred to as "baskets," with varicosities encircling particular populations of interneurons in the hippocampus of rodents (Freund et al., 1990), the primary auditory cortex of cats (Mulligan and Törk, 1988), and the frontal, cingular, and hippocampal cortex of non-human primates (Hornung et al., 1990; Hornung and Celio, 1992) and of man (Kosofsky and Molliver, 1987; Trottier et al., 1996). Serotonergic axons were visualized using either 5-HT or TPH antibodies. In the cerebral cortex of primates, the 5-HT_{1A}, 5-HT_{2A}, and 5-HT₃ receptors are among the most thoroughly studied serotonin receptors. They are widely distributed and expressed by major neurons as well as local circuit neurons. As a result, serotonin may play a number of different regulatory functions in cortical cell activity (Lidow et al., 1989; Ropert and Guy, 1991; Jacobs and Azmitia, 1992; Azmitia et al., 1996).

In addition to its primary ascending projection, the rostral group also includes projections to the cerebellum (Torigoe et al., 1986), the superior colliculus (Villar et al., 1988), many brainstem nuclei (Thompson et al., 1995), and the spinal cord (Kazakov et al., 1993).

2.1.1.2. The caudal cluster of brainstem raphe nuclei

A maximum of 15% of all serotonergic neurons are raphe nuclei, which make up the caudal group of the raphe system. A gap in the middle of the pons that is devoid of serotonin-containing neurons divides these caudal neurons from the rostral cluster.

The raphe magnus nucleus (RMg) neurons are situated next to the midline. With 30,000 neurons, RMg is the biggest caudal group serotonergic nucleus (Halliday et al., 1988; Rikard-Bell et al., 1990; Wu et al., 1993). The raphe obscurus nucleus (ROb) neurons are found in the dorsal

half of the medulla over its entire length, close to the midline. With just about 1000 neurons, the serotonergic neurons of the raphe pallidus nucleus (RPa) make up the smallest group in the raphe system (Hornung, 2003). Between the pyramids and the medial lemniscus that covers them, they are situated close to the midline. Serotonergic neurons appear to be limited to the rostral half of this nucleus, while its rostro-caudal extension cytoarchitectonically coincides with that of the inferior olive.

The caudal group's efferent projections descend via two parallel routes in the spinal cord after terminating in the brainstem's lateral reticular formation as well as visceral and somatic motor nuclei (Li et al., 1993; Manaker and Fogarty, 1995). The ventro-medial pathway starts partially in the ROb and RPa and ends in the intermediate and ventral horn in addition to the dorsal horn, whereas the dorso-lateral pathway starts mostly in the RMg and ends in the dorsal horn (Holtman et al., 1990; Yang et al., 1994; Arita et al., 1995; Hosogai et al., 1998). According to previous studies, the dorsal pathway produces axonal arbors with tiny varicose fibers, whereas the ventro-medial pathway produces massive varicose fibers that establish conventional synapses with spinal neurons (Ridet et al., 1993, 1994).

RMg and rostral RPa receive afferent projections from a number of hypothalamic nuclei, the dorsolateral periaqueductal gray, the central nucleus of the amygdala, the bed nucleus of the stria terminalis, and the medullary reticular formation (Zagon, 1993; Hermann et al., 1997). Moreover, rostral RPa neurons and spinally projecting RMg neurons receive direct catecholaminergic synaptic inputs (Tanaka et al., 1994). The RMg also receives convergent inputs from the retro facial nucleus and the pneumotaxic region in the medial parabrachial area (Gaspar and Lillesaar, 2012). Additionally, RMg, ROb, and RPa midline raphe neurons receive convergent inputs from ventrolateral periaqueductal gray matter neurons and visceral sensory afferents (Snowball et al., 1997).

2.2.1. Functional implications of serotonergic circuitry in health and neurological disease

Serotonin neurons are regarded as being one of the most extensively projecting neuromodulatory networks in the mammalian brain. Distinct subpopulations exhibit heterogeneous afferent and efferent connections, receptor profiles, developmental trajectories, and physiological activities. The early maturation of serotonergic neurons and the significant establishment of long-range cellular connections during embryonic and early postnatal development render them particularly susceptible to disruptions in cytoskeletal dynamics, vesicular trafficking, axonal transport mechanisms, and trophic signaling. Growing evidence indicates that neurodegeneration

results from mutations in proteins within the microtubule-dependent intracellular transport system (Gunawardena et al., 2014; Vicario-Orrí et al., 2014; Reinebrant et al., 2020). Therefore, dysfunctions in circuits originating from these raphe nuclei have been associated with significant neurological and psychiatric problems. Functionally, serotonin is a neuroregulatory component that influences/regulates a wide spectrum of brain activities, including motor control, circadian rhythm modulation, sleep-wake cycling, stress and emotional management, and sensory and autonomic processing (Brummelte et al., 2017).

2.2.1.1. Motor function and movement regulation

The importance of serotonin in controlling motor activity is exemplified by the motor syndrome generated by serotoninomimetic drugs, which is characterized by head movements, tremors, and stiffness patterns (Jacobs and Azmitia, 1992). During the waking period, the baseline activity of raphe neurons increases in the caudal division in tandem with swallowing (Ridet et al., 1993) or hyperpnea brought on by carbon dioxide exposure (Veasey et al., 1995; Ribeiro-do-Valle, 1997) or in the rostral division in tandem with complex orofacial movements (chewing, licking, or grooming) (Fornal et al., 1996). Recently, a patient who had a vascular stroke that only affected a portion of the caudal brainstem, including the RMg and rostral ROb, was reported to have the "Fourire prodromique," an uncontrollable motor pattern of laughing (ASSAL, 2000). Regulation of visceral motor response is also influenced by serotonin, as evidenced by the RPa projection to the dorsal motor nucleus of the vagus involved in the neural regulation of the stomach mucosa (Kaneko et al., 1998).

2.2.1.2. Serotonergic control of sleep and circadian rhythms

Early research revealed a correlation between electrical activity in the serotonin system and the sleep-waking cycle (Jouvet, 1969). More recently, this correlation was further defined by the contribution of the DR projection onto the cholinergic latero-dorsal tegmental nucleus (Steininger et al., 1997). According to McGinty and Harper (1976), DR activity is lowest during paradoxical sleep (REM-sleep) and highest during waking hours (McGinty and Harper, 1976). As a result, the cholinergic mesopontine neurons that generate REM sleep are no longer inhibited (Luebke et al., 1992; Thakkar et al., 1998). For axial and proximal limb muscles, serotonergic neurons fire in tandem with the central rhythm pattern generators and motor tone (Jacobs and Azmitia, 1992).

The suprachiasmatic nucleus of the hypothalamus (SCh) is considered the primary circadian pacemaker and is regulated by a variety of photic and non-photic therapies (Hastings, 1997). A phase shift in SCh is caused by electrical stimulation of DR and MnR, sources of the hypothalamic serotonin innervation (Leander et al., 1998). Serotonin applications on suprachiasmatic neurons in

vitro might replicate this (Prosser et al., 1993). Sleep difficulties have thus contributed to a prevalent non-motor sign of PD. Consequently, PD is associated with sleep disruption due to decreased serotonergic activity in the midbrain raphe, basal ganglia, and hypothalamus (Wilson et al., 2018). Increasing serotonin levels in the brain may be a potential way to treat sleep disruption in PD and may also be relevant to other NDs.

2.2.1.3. Serotonin in mood regulation and affective disorders

The symptoms of anxiety and depression are believed to be influenced by abnormal neuronal morphological characteristics, such as spine density and axonal or dendritic branching (Shimada et al., 2024). Serotonin neurotransmission is implicated in several aspects of mood disorders and drug addiction, according to pharmacological, genetic, or functional imaging investigations (Meltzer et al., 1998; Harrison, 1999; Aghajanian and Marek, 2000). Experimental research on animals with pharmacologically or genetically altered neurotransmission further supports this. Numerous diseases have been linked to changes in serotonin receptors or transporters. There have been reports of both genetic variation for the serotonin transporter and increased serotonin levels in autistic individuals (Cook and Leventhal, 1996; Klauck, 1997). Some of the effects of antipsychotic medications and hallucinogens are mediated via the 5-HT_{2A} receptor, while major depressive disorder patients' brains express less of this receptor (Biver et al., 1997; Scruggs et al., 2000). However, the dorsal raphe nucleus has more serotonergic neurons in suicide victims (Underwood et al., 1999), which might reflect a predisposition with a developmental origin. Additionally, individuals with significant depression or suicidal conduct have altered levels of serotonin transporter and 5HT_{1A} receptor expression, which lowers serotonin activity overall (Stockmeier et al., 1998; Arango et al., 2001). The information now available additionally confirms that serotonin is directly connected to the regulation of aggressive behavior, though the mechanisms of action are yet unknown (Gingrich and Hen, 2000). These are most likely many and may include developmental pathways. Even serotonin could regulate the reward system together with other neurotransmitters (Koob, 1992).

2.2.1.4. Serotonergic modulation of pain pathways

The RMg and the ventro-lateral periaqueductal gray (part of DR) are two structures in the raphe system that are involved in the central regulation of noxious stimuli (Fields et al., 1991; Wang and Wang, 1994). These structures are linked by a descending projection. However, the synaptic relay of pain afferents in the spinal trigeminal nucleus and dorsal horn of the spinal cord is one of the earliest sites for pain modulation. This circuit incorporates the descending projection of a mixed population of serotonergic and non-serotonergic neurons of the rostro-medial medulla, which includes the RMg (Skagerberg and Björklund, 1985). During stressful circumstances, tonic noxious stimuli can activate

serotonergic neurons (Milne and Gamble, 1990; Mitchell et al., 1998).

2.2.1.5. Developmental roles of serotonin and neurodevelopmental vulnerability

Remarkably, the idea that serotonin has a modulatory role in brain development has been prompted by the early synthesis of serotonin and the early axon growth in developing raphe neurons, when cell division, migration, and differentiation are still prominent in their target areas (Lauder, 1993; Turlejski, 1996). Meanwhile, serotonin depletion modifies the cell division of many populations of neural precursors in mouse embryos (Lauder and Krebs, 1978). Defective neuronal circuit formation in the cortex, subcortical structures, brainstem, and cerebellum of autistic individuals, as well as defects of neurogenesis, neuronal migration, neuronal growth, differentiation, and arborization, are among the cascades of abnormalities caused by developmental defects in raphe nuclei during the early stages of fetal life (Wegiel et al., 2024). Dysfunction of the serotonin transporter (SERT) also affects the uptake of extrasynaptic serotonin during perinatal CNS development, and the regulation of extracellular serotonin concentration (Lamiay et al., 2006; Chen et al., 2015). These could be involved in the origin of sensory and cognitive deficits associated with neurodevelopmental disorders (Chen et al., 2015). Additionally, the MAO-A deficient mouse exhibits aberrant dendritic growth in the phrenic motoneurons, which is correlated with a delayed maturation of the respiratory pattern (Bou-Flores et al., 2000). This characteristic is similar to that of sudden infant death syndrome victims, who have higher levels of serotonin metabolites in the cerebrospinal fluid (Cann-Moisan et al., 1999).

2.2.1.6. Serotonergic system disruption in neurodegenerative disorders

In Alzheimer's disease (AD) and PD, a loss of serotonergic neurons in the rostral nuclear complex of the raphe has been observed (Halliday et al., 1992; Frechilla et al., 2001). This loss is thought to be secondary to pathological changes that occur in the target areas of these projections (Rüb et al., 2000). Hence, 5-HT terminals are among the earliest degenerating structures in PD, leading to both motor symptoms and non-motor characteristics including mood disorders and sleep difficulties (Halliday et al., 1990; Politis and Niccolini, 2015). Serotonergic vulnerability is particularly pertinent since the raphe-forebrain 5-HT system is degenerated early in AD. AD brains exhibit a reduced 5-HT neuron number, a decreased level of 5-HT and 5-HIAA, a reduced receptor binding, and a marked loss in serotonergic innervation in cortex and hippocampus. These serotonergic deficits contribute not only to depressive and anxiety symptoms but also to impaired neurogenesis, mitochondrial dysfunction, circadian rhythm disruption, and reduced synaptic plasticity, all of which accelerate AD progression. In addition, serotonin receptors (e.g. 5-HT1A, 5-HT2A, 5-HT4, 5-HT6 and 5-HT7) modulate neuroplasticity, A β metabolism and inflammatory

signaling; therefore, reduced serotonergic innervation could further dysregulate such receptor-mediated protective pathways.

2.3.1 Neuroprotective mechanisms of HAP1 and regional vulnerability in neurological disorders

2.3.1.1. Structure, localization, and cellular functions of HAP1

HAP1 interacts with multiple cargo and motor proteins and is involved in the maintenance of neuronal integrity through supporting axonal transport, and stabilizing trophic and stress-related signaling pathways (Lin et al., 2010; Chen et al., 2020, 2023). Consistent with these functions, HAP1-deficient mice exhibit severe postnatal neurodegeneration, highlighting its requirement for neuronal survival and circuit maturation (Chan, 2002; Sheng et al., 2006). The expression of HAP1 inhibits neuronal apoptosis resulting from proteotoxic or metabolic stress, while HAP1 deficiency affects vesicular transport, induces receptor instability, and disrupts cytoskeletal dynamics (Li et al., 1995a; Colomer et al., 1997; Gutekunst et al., 1998; Fujinaga et al., 2009). Recent research has revealed a significant amount of HAP1 expression in the nuclei of the mouse brainstem (Fujinaga et al., 2004; Islam et al., 2022), indicating a functional relationship between HAP1-dependent trafficking mechanisms and the stability of serotonergic neurons in the raphe nuclei. Parallel to the growing concept of HAP1-dependent neuroprotection, disruption of the serotonergic system in HAP1-deficient mice could play a major role in numerous neurodegenerative or neurodevelopmental disorders.

2.3.1.2. HAP1-dependent intracellular trafficking mechanisms

HAP1 appears as a significant intracellular trafficking element within the neuroprotective context, perhaps influencing the stability of the serotonergic system. Neuronal survival, synaptic plasticity, and long-range neurotransmission all depend on this intracellular trafficking (Aiken and Holzbaur, 2021). In order to coordinate both anterograde and retrograde transport inside neurons, HAP1 functions as a molecular scaffold that combines intracellular cargo to microtubule-based motor proteins to facilitate both anterograde and retrograde transport within neurons (Chen et al., 2023; Wu et al., 2024). According to biochemical and proteomic research, HAP1 directly supports retrograde axonal transport by interacting with the dynein complex (dynactin p150 subunit), whereas anterograde trafficking along microtubules is facilitated by its association with kinesin light chain, specifically KLC2 (Chen et al., 2023). Crucially, phosphorylation controls HAP1's affinity for these motor complexes, allowing for activity-dependent adjustment of cargo trafficking efficiency. Importantly, phosphorylation regulates HAP1 affinity for these motor complexes, allowing for activity-dependent modification of cargo trafficking efficiency. In

addition, HAP1 interacts with proteins such as Abelson helper integration site-1 (AHI1), breakpoint cluster region protein, synaptophysin, pericentromeric protein 1, Duo. These are together corroborate in intracellular transport, endocytosis, and cytokinesis, which regulate the cytoskeleton, synaptic signaling and free radical production (Colomer et al., 1997; Sheng et al., 2008; Engel, 2015; Mackenzie et al., 2016; Huang et al., 2019).

HAP1's trafficking partners encompass a range of neurobiologically critical cargos. These include neurotrophic factors such as BDNF and proBDNF, membrane receptors such as GABA_AR, tyrosine kinase receptor B (TrkB), epithelial growth factor receptor (EGFR), and tyrosine kinase receptor A (TrkA) (Klein et al., 1993; Kittler et al., 2004), and synaptic vesicle associated proteins. These interactions with HAP1 enhance the stability of internalized receptors, facilitates their recycling and return to the plasma membrane, prevents premature lysosomal degradation, and preserving synaptic responsiveness (Chen et al., 2023). Similarly, HAP1 interacts with GR and stabilizes GR in the cytoplasm (Chen et al., 2020). Loss of HAP1 disrupts this protective stabilization, leading to reduced surface expression of critical receptors and attenuation of downstream trophic signaling. These mechanisms are particularly relevant in neurodegenerative conditions such as HD, AD, and PD, where defects in vesicle trafficking, receptor recycling, and axonal transport contribute to early neuronal vulnerability (Wu et al., 2024).

Beyond receptor and vesicle trafficking, HAP1 also interfaces with endocytic and secretory pathways. HAP1 deficiency impairs clathrin-mediated internalization in neurons and adrenal chromaffin cells through clathrin light chain B and sec23A, suggesting a broader role in maintaining endocytic homeostasis. Through this interaction, HAP1 modulates intracellular Ca²⁺ release, linking organellar dynamics and synaptic activity to intracellular transport mechanisms (Mackenzie et al., 2017).

Collectively, understanding HAP1's trafficking functions thus provides an essential foundation for elucidating how HAP1 deficiency destabilizes serotonin neuronal circuits and contributes to the pathophysiology of neurodevelopmental and neurodegenerative disorders.

2.3.1.3. Possible neuroprotective role of HAP1 in neurodegenerative disorders

The development of NDs, such as AD, HD, PD, amyotrophic lateral sclerosis (ALS), spinal and bulbar muscular atrophy (SBMA), and spinocerebellar ataxia (SCA), is recognized to be significantly influenced by intracellular transport failure. Thus, protein mislocalization and the

buildup of undegraded proteins in neurons are common outcomes of intracellular trafficking abnormalities (Chen et al., 2023). Many proteins implicated in NDs, including TATA binding protein (TBP), ataxin-3, and AR, interact with HAP1. The intracellular transport of certain molecules could be impacted by the aberrant interactions of these mutant proteins with HAP1, which plays a significant role in the pathophysiology of NDs (Takeshita et al., 2006, 2011; Prigge and Schmidt, 2007). In particular, the pathophysiology of neurodegeneration in HD is significantly influenced by the abnormal interaction of HAP1 with mutant HTT (Chen et al., 2023). HAP1 may also control the subcellular movement of the amyloid precursor protein (APP) in AD. HAP1 influences the kinesin-dependent APP axonal transport in neurons, and the transport of APP vesicles from the cell body to neurites is reduced in the absence of HAP1 (McGuire et al., 2006). In general, HAP1 lowers A β levels in neurons and controls APP trafficking to the non-amyloidogenic pathway (Yang et al., 2012). This suggests that HAP1 deficiency could exacerbate AD pathology by impairing APP processing and promoting toxic amyloid accumulation. In PD and other neurodegenerative contexts, disrupted intracellular transport is a well-known pathogenic mechanism, and the role of HAP1 as a trafficking scaffold positions it as a potential modulator of neuron survival and homeostasis (Chen et al., 2023).

2.3.1.4. HAP1 in pain pathways

HAP1 is highly expressed in sensory neurons, including dorsal root ganglion (DRG) neurons and dorsal horn of the spinal cord (Islam et al., 2017, 2020a). In neuropathic pain models, HAP1 levels increase in these regions; its deficiency reduces the excitability of nociceptive neurons, suppresses pain behaviors (allodynia, hyperalgesia), and lowers the membrane expression of Cav1.2 channels in sensory neurons (Pan et al., 2023). Through this mechanism, HAP1 controls Ca²⁺ influx, BDNF synthesis, and BDNF release in sensory neurons, all of which are critical for pain sensitization (Yang et al., 2011; Zhou et al., 2021; Pan et al., 2023). Loss of HAP1 also appears to dampen neuroinflammatory activation (astrocytes, microglia) in nerve injury models, suggesting that HAP1 contributes to the central sensitization component of chronic pain (Pan et al., 2023).

2.3.1.5. HAP1 in depression and stress pathology

HAP1 contributes to stress-related signaling through its stabilization of the GR in the cytoplasm of hypothalamic neurons (Chen et al., 2020). Reduced HAP1 impairs GR expression and signaling, which is critical for the stress response and neurogenesis. In addition, AHI1, which belongs to a class of proteins that interact with HAP1 for mutual stabilization (Sheng et al., 2008), interacts with GRs so as to stabilize each other in the cytoplasm (Wang et al., 2021; Wei et al., 2024). Significantly, AHI1 deficiency promotes the degradation of GRs in the cytoplasm and reduces the

nuclear translocation of GRs in response to stress (Wang et al., 2021). The absence of AHI1 in neurons leads to a depressive-like phenotype and decreased sensitivity to antidepressants (Xu et al., 2004; Wang et al., 2021), while increased levels of the mitochondrial AHI1/GR complex improve depressive behaviors (Wang et al., 2023). Moreover, HAP1 influences postnatal hippocampal neurogenesis by regulating BDNF/TrkB receptor trafficking, pathways that are strongly implicated in mood regulation and resilience (Peng et al., 2015; Xiang et al., 2015). Through these mechanisms, HAP1 dysregulation may contribute to depressive-like behaviors by disrupting neurotrophic support and stress-hormone signaling.

The comprehensive localization of HAP1 in the brainstem (Fujinaga et al., 2004; Islam et al., 2022) suggests that it may play a part in maintaining the integrity of serotonergic neurons by regulating trophic receptor trafficking, stabilizing stress-related proteins (GR), and safeguarding metabolic processes that sustain 5-HT synthesis. Furthermore, HAP1 could be implicated in cytoplasmic alterations of GR and AR nuclear translocation in a ubiquitin–proteasome system (Fujinaga et al., 2011). In mice, HAP1 and Ahi1 commonly interact to control the development of the brainstem and cerebellum. HAP1 was similarly reduced when Ahi1 was suppressed, and neurite outgrowth in neuronal culture was impeded by shortened Ahi1, which is associated with the mutations in Joubert syndrome (Sheng et al., 2008). It has been shown that Ahi1 controls the GR nuclear translocation during stress. The decrease in Ahi1 expression lowers cytoplasmic GR levels, encourages ligand-dependent GR nuclear translocation, and reduces ER β protein production in the nucleus. This GR can bind to the ER β promoter to suppress the ER β expression, though E2 may raise ER β at protein levels. However, TPH2 protein expression in the brainstem and hippocampus of male Ahi1 KO mice was considerably lower than that of control animals, while Ahi1 KO had no effect on SERT expression. Intriguingly, the lower E2 content brought on by Ahi1 KO in male mice could not compensate for the decrease in ER β expression. As a result, male Ahi1 KO mice exhibited decreased TPH2 and 5-HT levels and depressive behavior as a result of their reduced E2 content. Therefore, through the Ahi1/GR/ER β /TPH2 pathway in the brain, Ahi1 controls the synthesis of serotonin and may also be involved in the control of sex differences in depressive behaviors (Wang et al., 2022). Given that, TPH2, the rate-limiting enzyme in central serotonin synthesis, is sensitive to stress pathways (Chen and Miller, 2013), it's possible that HAP1's regulatory influence on Ahi1 and GR signaling has an indirect effect on serotonergic function.

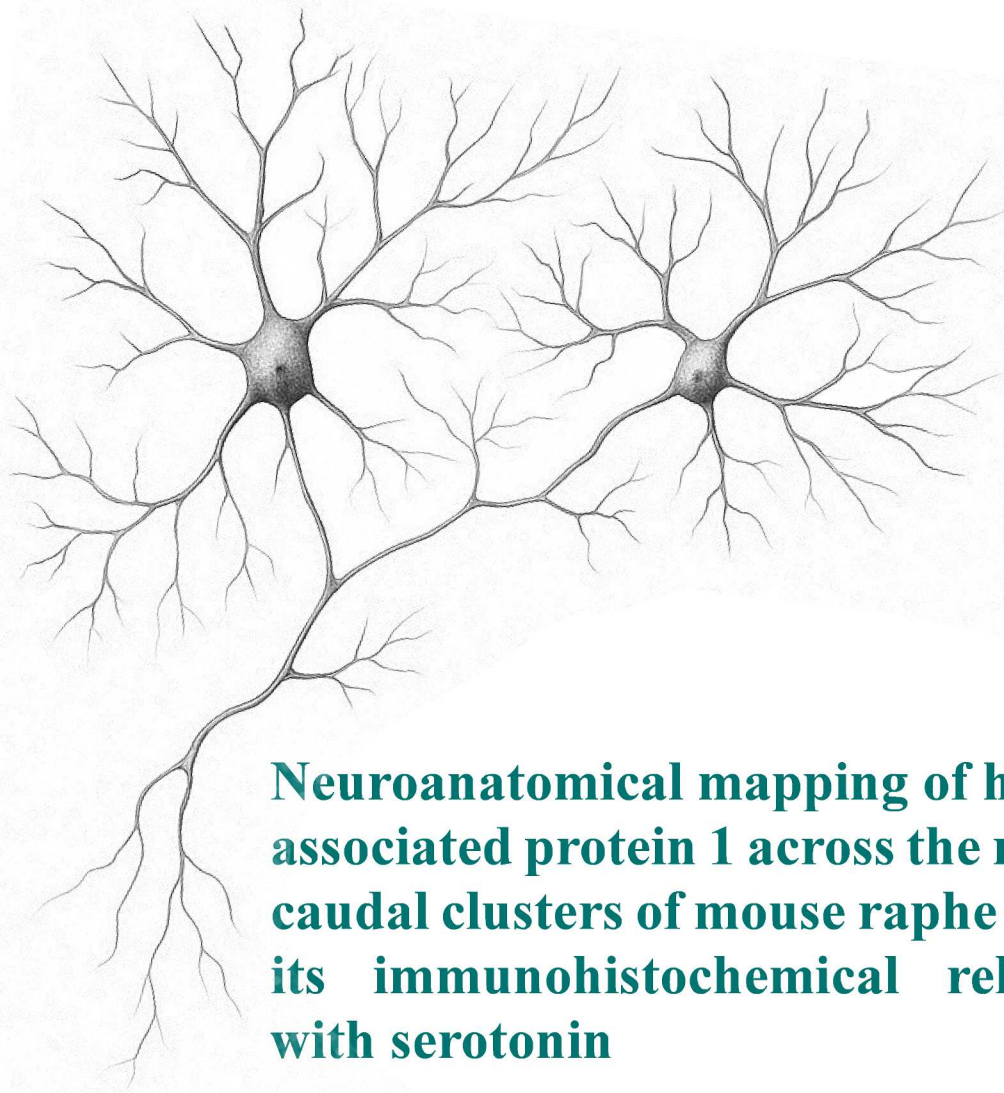
Taken together, HAP1 is necessary for microtubule-based cargo trafficking and the dysfunction of HAP1 may exacerbate the serotonergic susceptibilities to neurological diseases by affecting axonal maintenance, synaptic vesicle trafficking, or receptor trafficking in 5-HT neurons. This raises the possibility that HAP1 dependent maintenance of serotonergic circuitry is a previously underappreciated component of various non-motor symptoms or NDs resilience.

2.2 Summary of literature gaps and rationale for the present study

The 28,000 5-HT-producing neurons (Ishimura et al., 1988) in the mouse brain function as parallel sub-systems with distinct physiological traits, connections, and behavioral activities rather than as a single population (Ren et al., 2018; Okaty et al., 2019). In addition, serotonin neurons target particular areas and cells in certain circuits of the sensory, motor, or limbic systems, although they project densely and divergently across all brain divisions. Numerous neurological and psychiatric diseases have been associated with alterations in serotonergic neurotransmission, affecting either receptors or the transporter, in which HAP1 might have implications. The review of research together suggests that HAP1 may serve as a molecular stabilizer in 5-HT neurons, enhancing degeneration thresholds via regulating cargo transport, stress response, and synaptic structure. Therefore, investigating the developmental and functional consequences of HAP1 deficiency in the serotonergic system is crucial for elucidating how dysfunction in these pathways may contribute to the pathophysiology of neurodegenerative disorders, where early serotonergic susceptibility is increasingly acknowledged. Experimental investigation might have generated transgenic mice (HAP1-knockout model) to study the mechanisms underlying these pathological processes. The crucial role that HAP1 plays as a morphogenetic modulator on 5-HT neurons in both the developing and adult brain might be highlighted by this area of research. Therefore, a detailed analysis of the topographical distribution and cytoarchitecture of these serotonergic neuronal populations may provide an anatomical basis for hypothesizing the pathophysiological role of HAP1 in cognitive behavior as well as for understanding potential alterations in a variety of neurological and psychiatric disorders. Our work may contribute to the future breakthroughs in serotonin research. The rapid growth of genetic, proteomic, and imaging techniques would then enable the development of animal models to replicate the circumstances of certain pathological processes, and clinical neuroscience would become more precise in characterizing these disorders.



Chapter 3



Neuroanatomical mapping of huntingtin-associated protein 1 across the rostral and caudal clusters of mouse raphe nuclei and its immunohistochemical relationships with serotonin



Abstract

Huntingtin-associated protein 1 (HAP1) is a crucial component of the stigmoid body (STB) and is recognized as a neuroprotective interactor with causative proteins for several neurodegenerative disorders (NDs). Due to HAP1 protectivity, brain regions rich in STB/HAP1 are typically shielded from neurodegeneration, whereas areas with little or no STB/HAP1 are often affected in NDs. Mounting evidence suggests that serotonin (5-HT) neuron dysfunction contributes to various NDs. While the raphe nuclei denote the origin of 5-HT neurons, HAP1 protectivity has yet to be determined there. To accomplish this, the present study evaluated the expression and detailed neuroanatomical distribution of HAP1 throughout the rostral and caudal clusters of raphe nuclei in adult mice brains and their morphological relationships with 5-HT by employing Western blotting and immunohistochemistry. Our results indicated that in the rostral cluster, HAP1-ir cells were extensively distributed across the caudal linear raphe, median raphe, dorsal raphe, supramedian raphe, caudal part of the dorsal raphe, pre-pontine and pontine raphe nuclei. In the caudal cluster, HAP1-ir neurons were disseminated throughout the raphe magnus, raphe obscurus, raphe pallidus, parapyramidal, and raphe interpositus nuclei. Our double-immunofluorescence labeling results confirmed that most of the 5-HT neurons contained HAP1 immunoreactivity throughout the rostral and caudal clusters of the raphe nuclei. These suggest that HAP1 is crucial for modulating/protecting serotonergic functions, plausibly by upholding 5-HT neuronal plasticity/integrity by raising the threshold for neurodegeneration. Our current findings might provide a fundamental basis for further research aimed at elucidating the role of STB/HAP1 in the pathophysiology of serotonin neurons.

Keywords: *HAP1, Stigmoid body, Serotonergic neuron, Neurodegenerative disorder, Neuroprotection*

3.1. Introduction

Huntingtin-associated protein 1 (HAP1) is considered a neural interactor of huntingtin, a protein responsible for Huntington's disease (HD) (Li et al., 1995a). HAP1 is principally enriched in the neurons of the brain, spinal cord, and enteric nervous system (Gutekunst et al., 1998; Li et al., 1998b; Fujinaga et al., 2007, 2011; Islam et al., 2012, 2017; Tarif et al., 2021, 2023). HAP1 is most often expressed in the stigmoid body (STB), a physiological entity that is approximately 0.5–3 μm in diameter and considered a spherical to oval-shaped, non-membrane-bound cytoplasmic inclusion (Shinoda et al., 1992, 1993). Therefore, HAP1 has been known as an essential component and the determinant marker of the STB.

The rodent brain contains two spliced HAP1 isoforms, HAP1A and HAP1B, each with a distinct C-terminal sequence ((Li et al., 1998b; Fujinaga et al., 2007). While transfection of HAP1B-cDNA can lead to diffuse cytoplasmic expression, transfection of HAP1A-cDNA can induce the formation of cytoplasmic STB in cultured cells. Furthermore, several fusions of tiny STBs cause the HAP1-induced STB to enlarge (Fujinaga et al., 2007). Interestingly, when co-expressed with HAP1A, HAP1B is linked to HAP1A and sequestered to STBs from the early occurrence of small STB particles (Fujinaga et al., 2007). Accordingly, it has been hypothesized that the HAP1A/HAP1B expression ratio controls the number of STBs that form or the size of the STBs that expand (Li et al., 1998b; Fujinaga et al., 2007).

It is postulated that STB/HAP1 acts as a protective barrier against apoptosis and cell death by raising the threshold of susceptibility to neurodegeneration and providing enhanced stability to neurons in HD or several other neurodegenerative disorders (NDs), such as spinal and bulbar muscular atrophy, spinocerebellar ataxia type 3 (SCA3), SCA17, and Joubert syndrome (Li et al., 2003; Fujinaga et al., 2004; Takeshita et al., 2006, 2011; Metzger et al., 2008; Islam et al., 2017). The striatum, thalamus, cerebral neocortex, cerebellum, and motor nuclei are considered neurodegenerative targets in various NDs, perhaps due to low or absent STB/HAP1 expression in these brain and spinal cord areas. Furthermore, it has been reported that the hypothalamus of HAP1 knockout mice brains is more predisposed to apoptosis/neurodegeneration (Li et al., 2003). However, in STB/HAP1-enriched brain areas such as the preoptic area, medial amygdala, and hypothalamic regions, NDs usually do not result in cell death (Fujinaga et al., 2004, 2009; Islam et al., 2017, 2020b). These results led to the idea called the “HAP1 protection hypothesis” against neurodegeneration (Fujinaga et al., 2004, 2009; Islam et al., 2017, 2020b, 2025; Wroblewski et al., 2018; Tarif et al., 2021, 2023).

Previous research has demonstrated that, in terms of physiological functions, hypothalamic HAP1 can regulate eating behavior and postnatal development (Chan, 2002; Li et al., 2003; Sheng et al., 2006; Lin et al., 2010; Xiang et al., 2014). The serotonin (5-hydroxytryptamine, 5-HT) can act as a growth factor during embryogenesis, and 5-HT receptor activation is a critical component of the series of events that lead to periodic changes in brain structure. Impaired brain development has been linked to CNS disorders, which may also occur or be exacerbated due to disruption of the 5-HT system (Sodhi and Sanders-Bush, 2004). Moreover, 5-HT neurons that are harbored within distinct subregions of raphe nuclei have unique electrophysiological, morphological, and receptor profiles that can be activated by stressors and could render them selectively sensitive to stress (Lukkes et al., 2011; Soiza-Reilly and Commons, 2011). Intriguingly, HAP1 can control hypothalamic

glucocorticoid receptors at the protein level to alter the hypothalamic function related to stress response (Chen et al., 2020). Thus, it is possible that HAP1 might be involved in modulating the functions of 5-HT in raphe nuclei.

There is mounting evidence that the dysfunction of the 5-HT system is responsible for certain NDs (Meltzer et al., 1998; Fox et al., 2009; Huot et al., 2011; Politis and Niccolini, 2015). It might be possible that, owing to its putative protective properties, HAP1 can protect the 5-HT system against neurodegenerative stresses. Thus, revealing the relationships of HAP1 with 5-HT throughout the raphe nuclei is essential. Though the expression of HAP1 was cursorily described in rodent brainstem (Fujinaga et al., 2004; Islam et al., 2012), to the best of our knowledge, the expression and detailed neuroanatomical distribution of HAP1 have remained indefinite never been studied throughout the rostral and caudal clusters of raphe nuclei. In this context, we attempted to elucidate the cytoarchitectonic boundary along with the comparative distribution of HAP1 and 5-HT in the raphe nuclei of adult mice. Here, we further made an effort to clarify the immunohistochemical relationship of HAP1 with 5HT in the raphe nuclei throughout the adult mouse brainstem (midbrain, pons, and medulla oblongata) using Western blotting and immunohistochemistry.

3.2. Materials and methods

3.2.1. Animals

In the present study, we used male C57BL/6J mice (8 weeks old; Japan SLC Inc., Shizuoka, Japan). The animals were housed (3 - 4 mice/cage) at 22°C constant temperature, kept in 12 h light-dark cycles (lights on 08:00–20:00) with free access to food and water independent of the diet. All efforts were made to reduce the number of mice used and to minimize animal suffering. The entire experimental protocol conformed to the guidelines for animal research by the Japanese government (Law No. 105, Notification No. 6) and was approved by the Committee on the Ethics of Animal Experimentation at Yamaguchi University School of Medicine.

3.2.2. Characterization of primary antibodies

The sources, immunogens, and working dilutions of primary antibodies used in the present study are summarized in Table 1. To explore the complete pattern and localization of HAP1, we used two different polyclonal antibodies against HAP1 in immunoperoxidase staining. One is a commercially produced goat polyclonal anti-HAP1 antibody (R19), and the other is our laboratory-raised rabbit polyclonal anti-HAP1 antibody (R131) (Table 1). Our prior research has confirmed the specificity of these two antibodies (Fujinaga et al., 2007; Islam et al., 2017, 2020b, 2020a, 2022). We have also evaluated the antibody's specificity in brainstem raphe nuclei using Western blotting

(Figure 1B) and immunoperoxidase staining (Supplementary Figure 1) in this study. In addition, a commercially produced anti-5-HT primary antibody was used here, and its specificity was demonstrated in our previous studies (Table 1). We have also assessed the specificity of this 5-HT antibody employing immunoperoxidase staining (Supplementary Figure 1) in the present study. 5-HT is a small molecule with a molar mass of about 0.176 kDa. In this study, we could not detect the 5-HT band using Western blot analysis. Prior research had established the specificity of the other commercially available antibodies employed in this study, most of which were selected from the Neuroscience Information Framework's Antibody Registry (Table 1).

3.2.3. Western blotting

Western blotting was accomplished as described in our previous studies (Islam et al., 2017, 2020a, 2021). In brief, mouse brains ($n = 6$) were harvested immediately after transcardial perfusion with ice-cold saline. After that, each brain was positioned into the stainless-steel mouse brain matrix (1 mm) with the hypothalamus facing up. Then, the brains were sectioned by placing chilled microtome blades along the extension of the hypothalamus (bregma, -0.34 to -2.46 mm), the rostral cluster (bregma, -4.04 to -5.20 mm), and the caudal cluster (bregma, -5.34 to -8.12 mm) of the brainstem raphe nuclei (Figure 1A). The hypothalamus (control) from the left and right sides of each individual mouse brain, as well as the rostral and caudal clusters of the raphe nuclei that are dispersed along the midline of the brainstem, were collected separately by micropunching under the microscope. Then the collected tissues were homogenized in tissue protein extraction reagent (T-PER; Thermo Scientific, Rockford, IL, USA) added with 5 μ l/ml of protease inhibitor (P8340; Sigma–Aldrich). The protein concentration for each sample was ascertained through the BCA Protein Assay method (Thermo Scientific, Waltham, MA, USA). 80 μ g of each protein was loaded, separated by 7.5% SDS-polyacrylamide gel electrophoresis, and then transferred onto a polyvinylidene difluoride membrane utilizing a wet transfer apparatus. Membranes were blocked for 2 h with 5% skimmed milk in Tris-buffered saline with 0.1% Tween-20 (TBST) and probed overnight with the primary antibodies: goat polyclonal anti-HAP1 (R19) (1:20,000), rabbit polyclonal anti-HAP1 (R131) (1:20,000), rabbit polyclonal anti-IgG (1:5000) or mouse monoclonal anti- α tubulin (1:200,000) (Table 1). To perform the HAP1 (R19) preadsorption test, the diluted antibody was treated with a particular blocking peptide (sc-8770P, Santa Cruz Biotechnology, Santa Cruz, CA, USA.) overnight at 4°C. Following several washes with TBST, the membrane was treated with horseradish peroxidase-conjugated secondary antibody: anti-goat (1:10,000), anti-rabbit (1:5,000), or anti-mouse (1:20,000) antibody for 2h at room temperature (Table 2). Enhanced chemiluminescence reagents (ECL select; GE Healthcare) and an Amersham Imager 600 (GE Healthcare) were used to visualize immunopositive protein bands after repeated washes in TBST. Densitometric analysis was conducted to quantify the immunoreactive band ratio of HAP1 R19 and R131 to α -tubulin between the rostral and caudal clusters using ImageJ software (NIH, MD, USA).

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

Antibody	Immunogen	Host / Antibody type	Catalogue number / Research resource identifiers (RRIDs)	Source	Dilution	References
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	Islam et al., 2017, 2025
Anti-HAP1 (R131)	GST-fused rat HAP1 ⁷⁰⁻⁴³³	Rabbit polyclonal	RRID: AB_2571562	Div. of Neuroanatomy, Yamaguchi University	1:20,000	Fujinaga et al., 2007
Anti-Serotonin (5-HT)	Serotonin covalently bound to bovine thyroglobulin with carboimide	Rabbit polyclonal	Cat# 066D, RRID: AB_2313879	Biomedica Corp. USA	1:5,000	Yanai et al., 2020; Sheng et al., 2004
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., 2012
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	Wroblewski et al., 2018
Anti-NeuN	Synthetic peptide of Human NeuN aa 1–100	Rabbit monoclonal	Cat# ab177487, RRID: AB_2532109	Abcam, Cambridge, UK	1:5,000	Islam et al., 2020a
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., 2021; Tarif et al., 2023

GFAP, Glial fibrillary acidic protein; HAP1, Huntingtin-associated protein-1; Iba1, Ionized calcium binding adaptor molecule 1; NeuN, Neuronal nuclei.

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

Secondary antibody and other reagents	Immunogen	Host	Catalogue number / Research resource identifiers (RRIDs) / 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, 2025
HRP-labelled anti-rabbit	Rabbit IgG F(ab') ₂	Donkey	Cat# NA934, RRID: AB_772206 Cytiva, Sigma-Aldrich, Germany.	Western blotting	1:5,000	Islam et al., 2017
HRP-labelled anti-mouse	Mouse IgG whole molecule	Sheep	Cat# NA931, RRID: AB_772210 GE Healthcare, Buckinghamshire, UK.	Western blotting	1:20,000	Islam et al., 2012
Biotinylated donkey anti-goat	IgG, isolated from goat serum	Donkey	Cat# AP180B, RRID: AB_92569 Millipore, Darmstadt, Germany.	Immunoperoxidase staining	1:1,000	Jahan et al., 2015
Biotinylated goat anti-rabbit	IgG, isolated from rabbit serum	Goat	Cat# AP180B, RRID: AB_92569 Millipore, Darmstadt, Germany.	Immunoperoxidase staining	1:1,000	Islam et al., 2017, 2021
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	Tarif et al., 2023
Alexa Fluor 488 donkey anti-rabbit	IgG, isolated from rabbit serum	Donkey	Cat# A32790, RRID: AB_2762833 Invitrogen, Eugene, OR, USA.	Immunofluorescence staining	1:1,000	Islam et al., 2020a
HRP-conjugated streptavidin	N/A	N/A	Cat# P0397, Agilent Dako, Glostrup, Denmark.	Immunoperoxidase staining	1:1,000	Yanai et al., 2020

HRP, Horseradish peroxidase; IgG, Immunoglobulin G.

3.2.4. Tissue preparation for immunohistochemistry

The mice were first anesthetized with an anesthetic mixture [MMB; (Kirihara et al., 2012)] of 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 was injected with 0.25-0.30 ml of MMB intraperitoneally and then transcardially perfused with ice-cold saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). After removing the brain, the same fixative used for perfusion was employed to postfix for 24 h. Then, the brain was cryoprotected with immersion in 30% sucrose in 0.1 M PB until it sank. The brainstem (midbrain, pons, and medulla oblongata) was sectioned serially from bregma -4.04 to -8.12 mm in the coronal plane at 30 µm intervals (Figure 1B) on a freezing microtome (Leica CM1900). After that, frozen sections were kept at 4°C in an ice-cold 0.02 M sodium phosphate-buffered solution (PBS; pH 7.4) with 0.1% sodium azide until used for immunohistochemistry.

3.2.5. Immunoperoxidase staining

Free-floating immunohistochemistry was performed using the brainstem (midbrain, pons, and medulla oblongata) section as previously described (Islam et al., 2012, 2017). Sections were first blocked and permeabilized in PBS containing 10% normal donkey serum (NDS) or 10% normal goat serum (NGS) and 0.3% triton X-100 (TX) for 2h. Following this, sections were pretreated with 50% cold methanol and 1.5% H₂O₂ at 4°C for 30 min. Sections were then incubated with primary antibodies to HAP1 R19 and R131 (1:20,000) or rabbit polyclonal anti-IgG (1:5000) for 5 d at room temperature or to 5-HT (1:5,000) at 37°C for 7 d. The blocking peptide (sc-8770P, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the diluted primary antibody (R19) to conduct the preadsorption staining. Following primary antibody immunoreaction, sections were washed and then incubated overnight at 4°C in PBS containing 1% NDS or NGS and biotinylated donkey anti-goat or goat anti-rabbit secondary antibody (1:1,000). This was followed by a 2 h incubation at 20°C with peroxidase-conjugated streptavidin (Dako, Glostrup, Denmark; 1:1000 diluted in PBS). Details on antibodies and selected reagents are included in Table 1 and Table 2. The sections were then washed in 0.05 M Tris-HCl buffer (pH 7.6) followed by a violet-to-black color reaction using 0.02% 3,3'-diaminobenzidine (DAB; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 0.6% nickel ammonium sulfate (Sigma-Aldrich, Tokyo, Japan) in 0.05 M Tris-HCl buffer containing 0.0008% hydrogen peroxide (nickel-enhanced DAB reaction) for approximately 10 min on ice. Sections were mounted on glass slides, dehydrated in ethanol following air drying, cleared in xylene, and coverslipped using Entellan Neu (Merck KGaA, Darmstadt, Germany).

3.2.6. Immunofluorescence staining

Double-label immunofluorescence staining was carried out in accordance with our earlier research ([Islam et al., 2012, 2017, 2023; Yanai et al., 2020](#)), and the list of antibodies and particular reagents were included in Table 1 and Table 2. For immunofluorescent staining, brainstem sections were blocked with PBS containing 10% NDS and 0.3% Triton X-100 for 2 h at 4°C. Sections were then washed, followed by incubation with goat anti-HAP1 antibody (R19, 1:6000) and rabbit anti-5-HT (1:2000) for 7 d at 37°C or goat anti-HAP1 antibody (R19, 1:10000) in combination with rabbit anti-neuronal nuclei (NeuN) (1: 5000), rabbit anti-GFAP (1:1000) or rabbit anti-Iba1 (1:1000) in PBS containing 1% NDS and 0.3% Triton X-100 for 5 d at 20°C. After repeated washing, sections were incubated with a mixture of Alexa Fluor 594-conjugated donkey anti-goat and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Molecular Probes, Eugene, OR, USA; 1:1000 diluted in PBS containing 1% NDS) at 20°C for 2 h. After that, sections were washed with PBS, mounted on glass slides, air-dried, and embedded with Fluoromount Plus (K048, Diagnostic Biosystems, Pleasanton, CA, USA). Before being used, slides were kept at 4°C in a light-tight slide box.

3.2.7. Cytoarchitectonic analysis and terminology

The cytoarchitectonic assessment was completed using Nissl staining (cresyl violet; Merck) of sections neighboring or adjoining those stained for 5-HT or HAP1. Along the midline of the brainstem, raphe nuclei are allocated based on their distribution into two clusters: the rostral cluster, confined to the mesencephalon (caudal midbrain) and rostral pons, and the caudal cluster, extending from the caudal pons to the caudal portion of the medulla oblongata ([Alonso et al., 2013](#)). The center part of the brainstem has been separated into two zones: the median zone and the paramedian zone, within which raphe nuclei are included ([Alonso et al., 2013](#)). Additionally, the hindbrain and a portion of the midbrain are temporarily split into many areas known as rhombomeres. The rostral cluster of raphe nuclei is generated by rhombomeres 1, 2, and 3, whereas the caudal raphe cluster is generated by rhombomeres 5–7 ([Weissbourd et al., 2014](#)). Nomenclature for the entire raphe nuclei area in the mouse brain was confirmed by previous research and subsequently adopted by the mouse brain atlas ([Paxinos and Franklin, 2008](#)), which was followed in our current study.

3.2.8. Cell counting

For cell counting, 40x objective immunofluorescence images were taken and imported into ImageJ software (NIH, Bethesda, MD, USA). The actual number of HAP1 or 5-HT-immunoreactive (ir) neurons and those double-stained for HAP1 and 5-HT were used to compute co-expression ratios for HAP1 in 5-HT, or 5-HT in HAP1 (Table 3), in accordance with the counting protocol outlined in our earlier study ([Nagano and Shinoda, 1994; Jahan et al., 2015](#)). $TN = N \times D / \{(T + W) \times S\}$ [TN, total

number of 5-HT in HAP1-ir cells or HAP1 in 5-HT-ir cells in individual raphe nuclei of rostral and caudal clusters; N, 5-HT or HAP1-ir neurons along with HAP1 and 5-HT double-stained neurons observed in sections; D, distance between the first and last sections in which raphe nuclei were counted (μm); T, section thickness (30 μm in the present study); W, mean diameter of the raphe nuclei (8 μm for the mouse in the present study); S, number of sections in which distinct raphe nuclei were counted. Four sections from a single mouse were used for each nucleus, with no closure of 60 μm between sections. Six mice were utilized to count the rostral and caudal clusters of raphe nuclei.

3.2.9. Statistical analysis

The Statistical Package for Social Sciences (SPSS), version 25.0, was used to examine every data set. Instead of performing a formal sample size calculation, for statistical analysis, the number of animals used ($n = 6$ mice for each analysis) was determined based on prior published studies in the brainstem (Islam et al., 2022). The design follows the three 'R' approach (replacement, reduction, refinement), focusing on minimizing animal usage while upholding scientific integrity and statistical rigor. For the computation and graphical representation of Western blot protein intensity, the relative band optical density ratio of HAP1 R19/ α -tubulin or HAP1 R131/ α -tubulin data was imported into MS Excel (Microsoft Office Excel 2019, USA). Subsequently, one-way Analysis of Variance (ANOVA) was conducted, followed by a post-hoc test (Tukey's test) to compare the means using SPSS v. 25. Additionally, the other chosen sample size ($n = 6$) permitted parametric statistical analysis of HAP1/5-HT and 5-HT/HAP1 cell count ratios. The student's t-test was then used for pairwise comparisons of the HAP1/5-HT and 5-HT/HAP1 cell count ratios, and the one-way ANOVA was followed by Tukey's post hoc test for regional comparisons. For every test, p-values <0.01 or <0.05 were deemed significant. To verify inter-animal variability, the statistical unit used in our data is the average co-expression ratio per animal ($n = 6$), which is derived from numerous sections for each raphe nucleus.

3.2.10. Photomicrographs

For light microscopic images, a color digital Lumenera USB 2.0 camera (Lumenera Corporation, Ottawa, Canada) equipped with an Eclipse E80i photomicroscope (Nikon) and image tiling software (Mitani Corporation, Tokyo, Japan) was used to take photomicrographs. For fluorescence images, a confocal laser microscope (Leica Microsystems, STELLARIS 8; Wetzlar, Germany) with LAS X software was used to attain single optical sections (4296×4296 pixels). The original photographs were left unaltered, with the exception of adjusting the brightness and contrast using Adobe Photoshop CS 8.0 (Adobe Systems, Inc. San Jose, CA, USA).

3.3. Results

3.3.1. General expression of HAP1 immunoreaction in the raphe nuclei

The expression of HAP1 in the raphe nuclei was examined in immunohistochemistry and Western blotting (Figures 1A-D). Both HAP1A and HAP1B isoforms (about ~90 kDa for HAP1B; ~80 kDa for HAP1A) were detected in the rostral and caudal clusters of mouse brainstem raphe nuclei (Figure 1B) in Western blotting using goat polyclonal anti-HAP1 (R19) or rabbit polyclonal anti-HAP1 (R131) antibody. The immunoreactivity was substantially abolished after using a specific blocking peptide against the anti-HAP1 (R19) primary antibody or using normal rabbit IgG as a negative control against the HAP1 (R131) antibody (Figure 1B). The protein band ratio of HAP1 R19 and R131 to α -tubulin (Figure 1C) was used to quantitatively analyze the relative strength of HAP1 immunoreactivity in both clusters of raphe nuclei, yielding results indicative of at least six separate analyses. Our results showed that HAP1 immunoreactivity between the rostral and caudal clusters of the mouse brainstem raphe nuclei did not vary significantly ($p>0.05$) using both HAP1 R19 and HAP1 R131 antibodies (Figure 1C). In immunohistochemistry, both R19 and R131 antibodies showed a similar expression pattern of HAP1 in the raphe nucleus (Figures 1D-E). Most HAP1-ir cells contained STBs in diffusely HAP1-ir cytoplasm, whereas few showed only diffuse cytoplasmic immunoreactivity for HAP1 with no clearly visible STBs (Figures 1D-E). These suggest that most HAP1-ir cells transcript and translate both HAP1A and HAP1B isoforms in the raphe nucleus. Smaller HAP1-ir STBs, however, were often masked by diffusely expanding intense HAP1 immunoreaction in the cytoplasm. Therefore, in immunohistochemistry, it was challenging in light microscopy to determine whether cytoplasmic diffuse HAP1 particles are unbound to the minor STB units.

Double-label immunohistochemistry for HAP1 and nuclear neuronal marker (NeuN), astrocyte marker (GFAP), or microglia marker (Iba1) was carried out to uncover the type of HAP1-ir cells in brainstem raphe nuclei. Significant NeuN immunoreactivity was seen in most of the HAP1-ir cells of the dorsal raphe nucleus (Figures 1F-H). On the other hand, GFAP (Figures 1I-K) and Iba1-ir cells (Figures 1L-N) never contained HAP1 immunoreactivity. These findings confirmed that HAP1-ir cells in the brainstem raphe nuclei shared no characteristics with glial cells, but they did show characteristics of neurons.

Next, to examine the detailed distribution of HAP1-ir neurons in the rostral and caudal clusters of the raphe nuclei, we mapped the comparative neuroanatomical distribution of 5-HT and HAP1 using neighbouring sections. The regional distribution of 5-HT (Figures 2A-C, 3A-C, 4A-E) and HAP1-ir cells (Figures 2D-F, 3D-F, 4F-J) was summarized in a non-consecutive series of maps throughout the rostral and caudal clusters of the raphe nuclei.

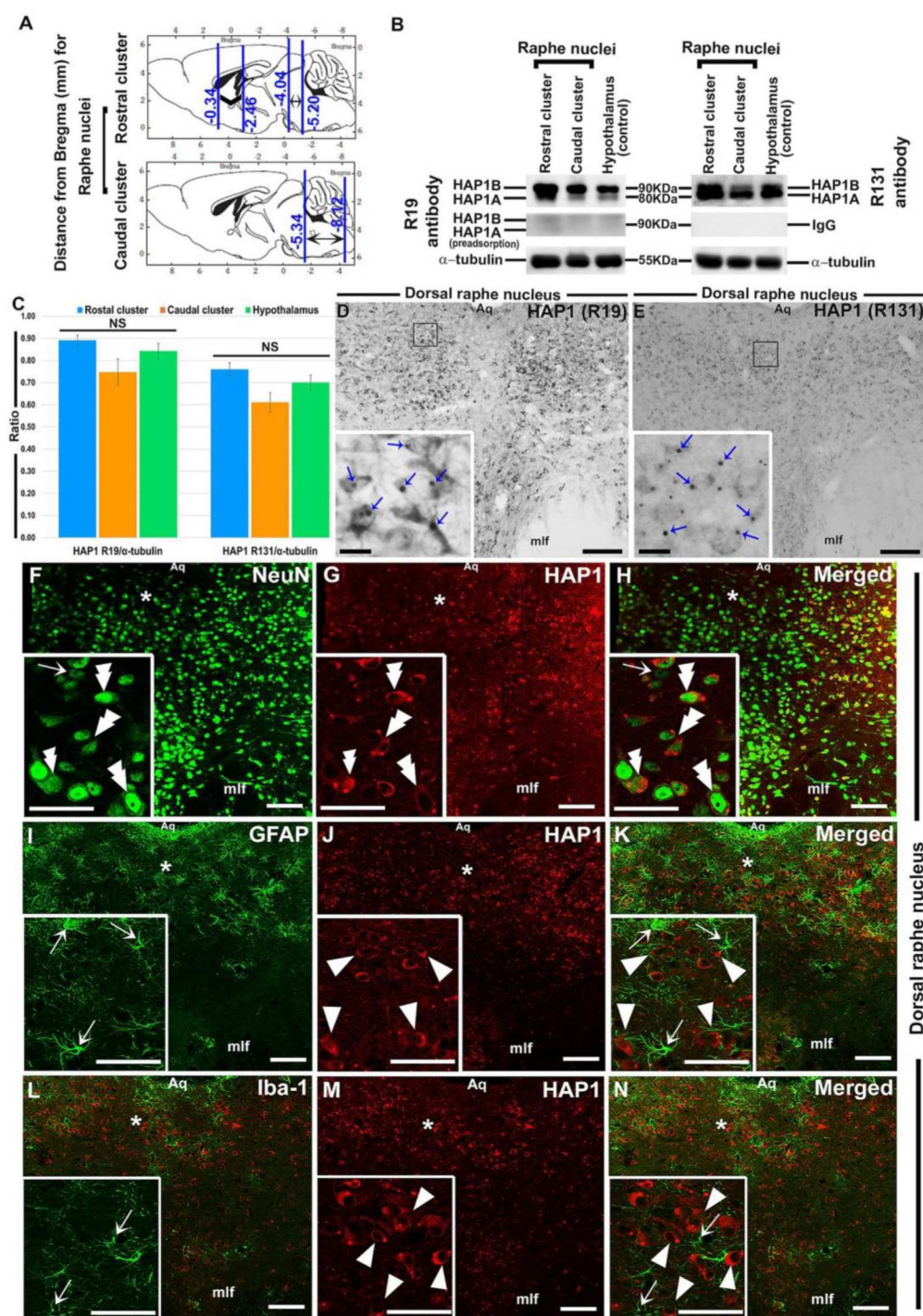


Figure 1. Western blotting and immunoperoxidase immunohistochemistry for HAP1 and double-label immunofluorescence immunohistochemistry for HAP1 with neuronal or glial cell markers. (A) Brain sections were examined for rostral (Bregma -4.04 to -5.20 mm) and caudal (Bregma -5.34 to -8.12 mm) clusters of raphe nuclei. **(B)** Western blot analysis showing the HAP1 R19 and R131 immunoreactive (ir) bands; Pre-adsorption of the anti-HAP1 R19 antibody or using normal rabbit IgG resulted in the disappearance of HAP1-ir bands. **(C)** The ratio (mean) of HAP1 R19 and HAP1 R131-ir bands to α -tubulin on the Western blots (n=6). NS=non-significant. **(D-E)** Immunoperoxidase staining shows HAP1 immunoreactivity using R19 and R131. Blue arrows indicate the HAP1-ir stigmoid body (STB) in the insets of D and E. Photomicrograph showing double-label immunofluorescence staining of HAP1 and NeuN **(F-H)**, GFAP **(J-K)**, and Iba1 **(L-N)**, in which insets are the enlargement of respective areas indicated by asterisks. 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 NeuN, GFAP, or Iba1. Scale bar = 20 μ m in **D-N** and insets. For abbreviations, see “List of Glossary.”

3.3.2. Distribution of HAP1-ir neurons in the rostral cluster of raphe nuclei: a comparison with 5-HT

In the rostral cluster of raphe nuclei, both 5-HT and HAP1-ir cells were highly distributed in the caudal linear nucleus (CLi), median raphe nucleus (MnR), dorsal raphe nucleus (DR), supramedian raphe nucleus (SuL), caudal part of dorsal raphe nucleus (DRC), prepontine raphe nucleus (PPnR), and pontine raphe nucleus (PnR) (Figures 2A-C, 3A-C, 4A; Figures 2D-F, 3D-F, 4F). DR is the largest of all raphe nuclei. There are five subregions of DR, including the dorsal raphe nucleus dorsal part (DRD), dorsal raphe nucleus ventral part (DRV), dorsal raphe nucleus lateral part (DRL), posterodorsal raphe nucleus (PDR), and dorsal raphe nucleus interfascicular part (DRI). Strongly stained 5-HT (Figures 5A-E) and HAP1-ir neurons (Figures 5G-I) were uniformly disseminated in the DRD, DRV, DRI, DRL, and PDR. In the MnR, 5-HT cell bodies were distributed unevenly (Figures 5A, F). 5-HT neurons were more prevalent in the rostro-dorsal part of the MnR than in the ventro-caudal region. Moderately stained HAP1-ir neurons, however, were evenly distributed throughout the MnR (Figures 5G, J). Interestingly, 5-HT neurons appeared to be absent in the paramedian raphe nucleus (PMnR) (Figures 5A, F). In contrast, HAP1-ir neurons were strongly expressed in the PMnR (Figures 5G, J). Dot-like HAP1-ir STBs were observed in most of the raphe nuclei of the rostral cluster (Figures in insets of 5G-J).

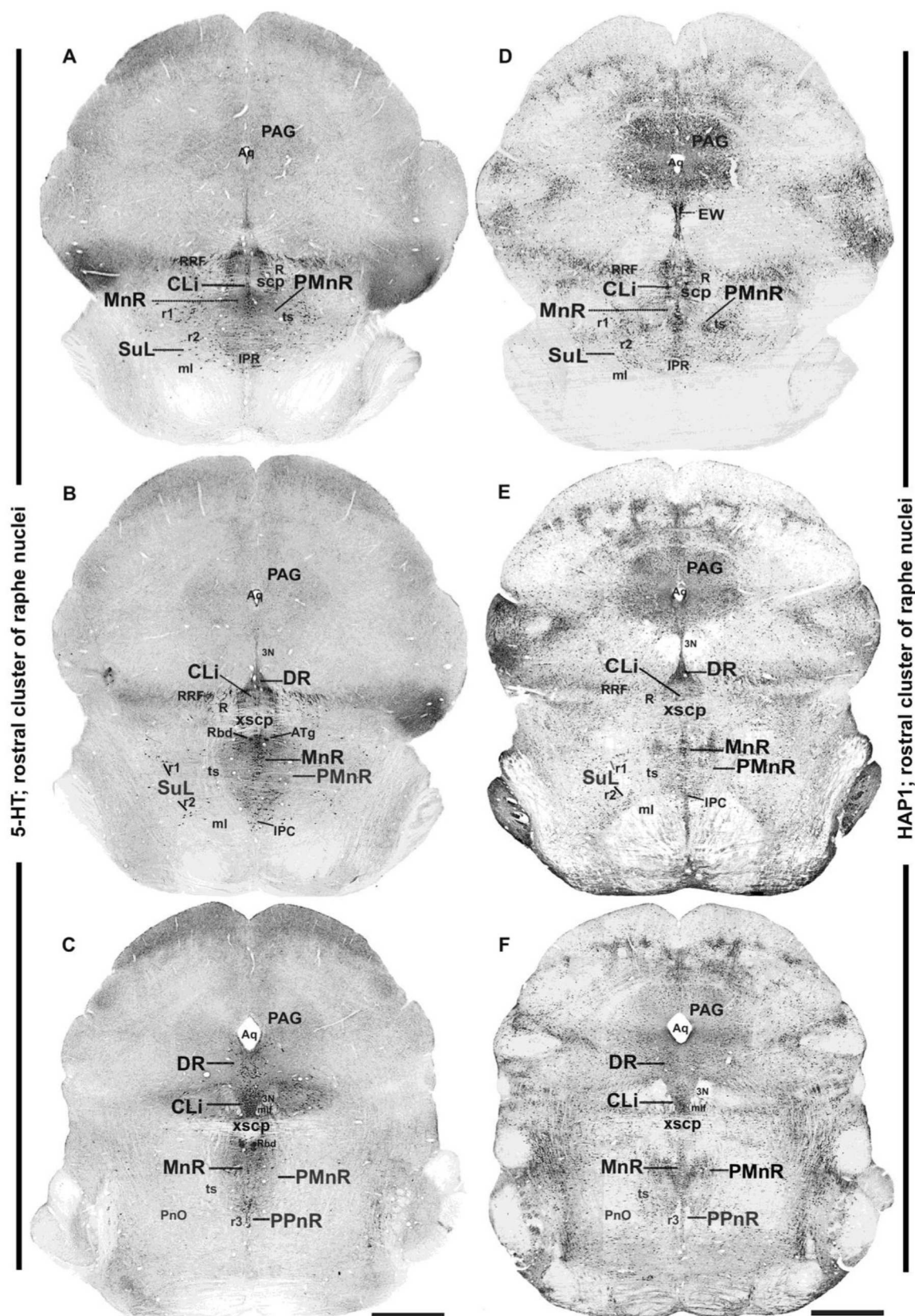


Figure 2. Distribution of 5-HT and HAP1 immunoreactivity across the caudal midbrain raphe nuclei (rostral cluster). Photomicrographs showing the 5-HT (A-C) and HAP1 (D-F) immunoreaction throughout the raphe nuclei in caudal midbrain. Selected structures are identified for topographic reference. Scale bar = 125 μ m. For abbreviations, see “List of Glossary.”

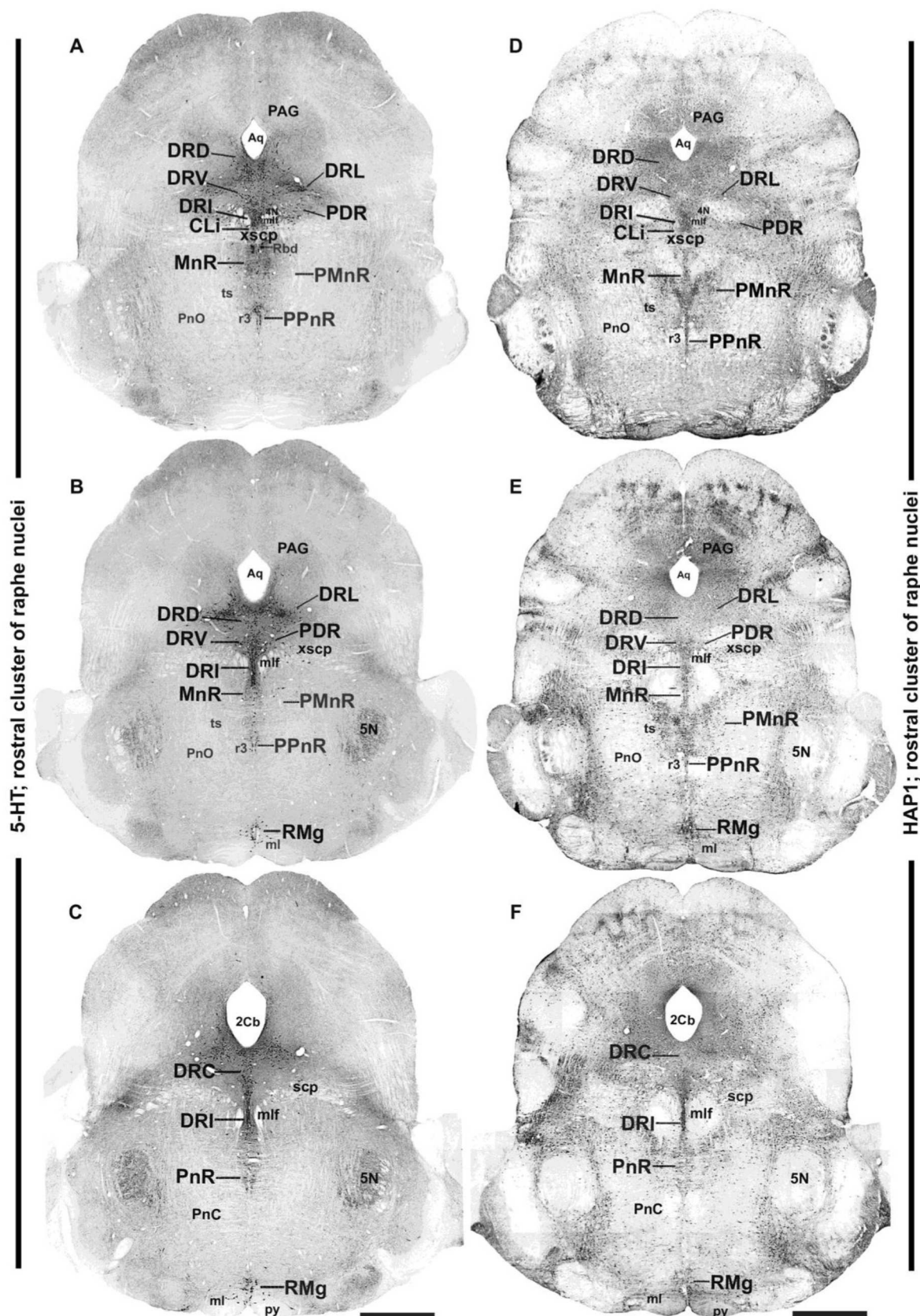


Figure 3. Distribution of 5-HT and HAP1 immunoreactivity across the rostral pons raphe nuclei (rostral cluster). Photomicrographs showing the 5-HT (A-C) and HAP1 (D-F) immunoreaction throughout the raphe nuclei in rostral pons. Scale bar = 125 μ m. For abbreviations, see “List of Glossary.”

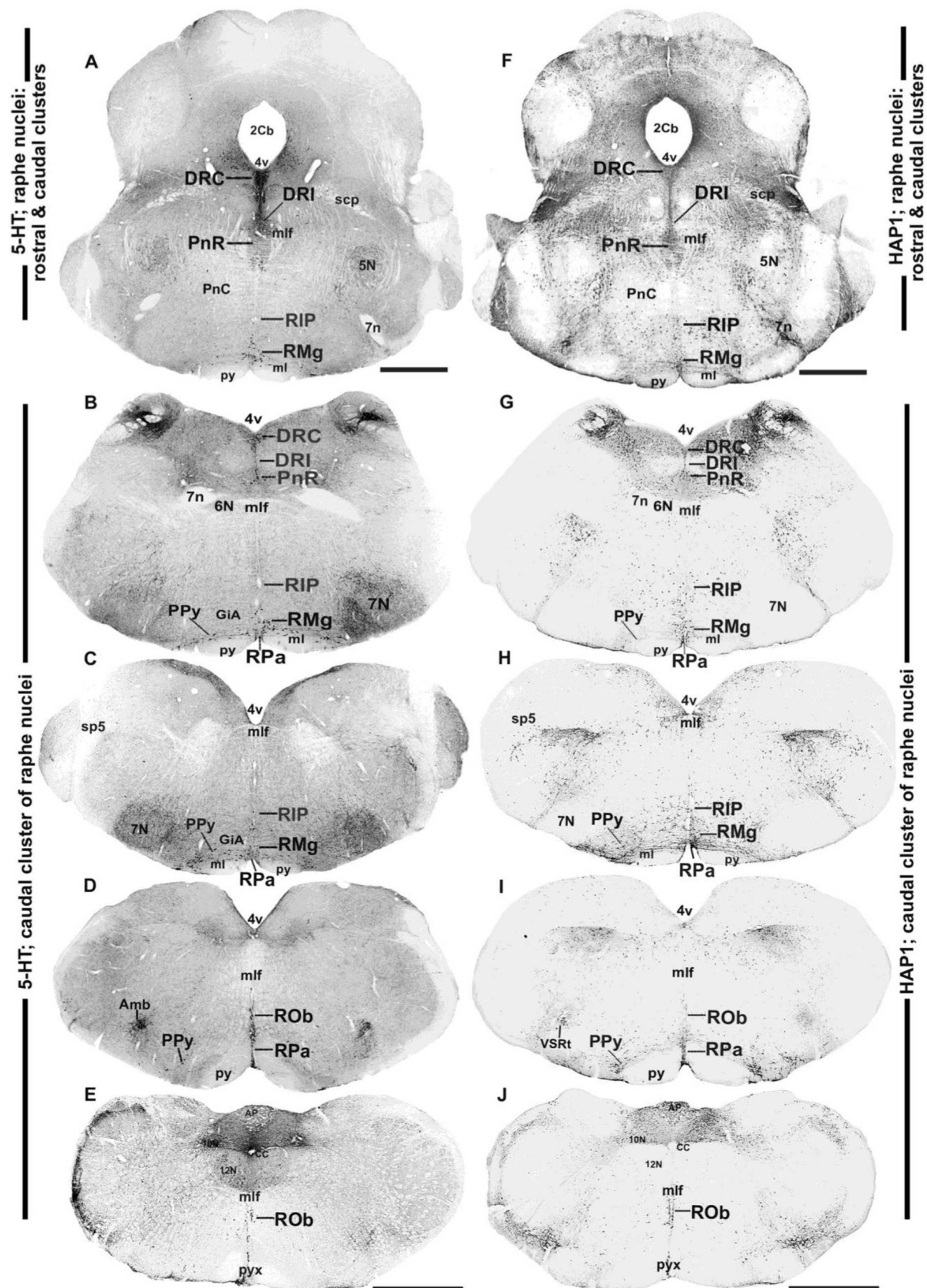


Figure 4. Distribution of 5-HT and HAP1 immunoreactivity across the caudal pons and medullary raphe nuclei (caudal cluster). Photomicrographs showing the 5-HT (A-E) and HAP1 (F-J) immunoreaction throughout the raphe nuclei in the caudal pons and medulla. Scale bar = 125 μ m. For abbreviations, see “List of Glossary.”

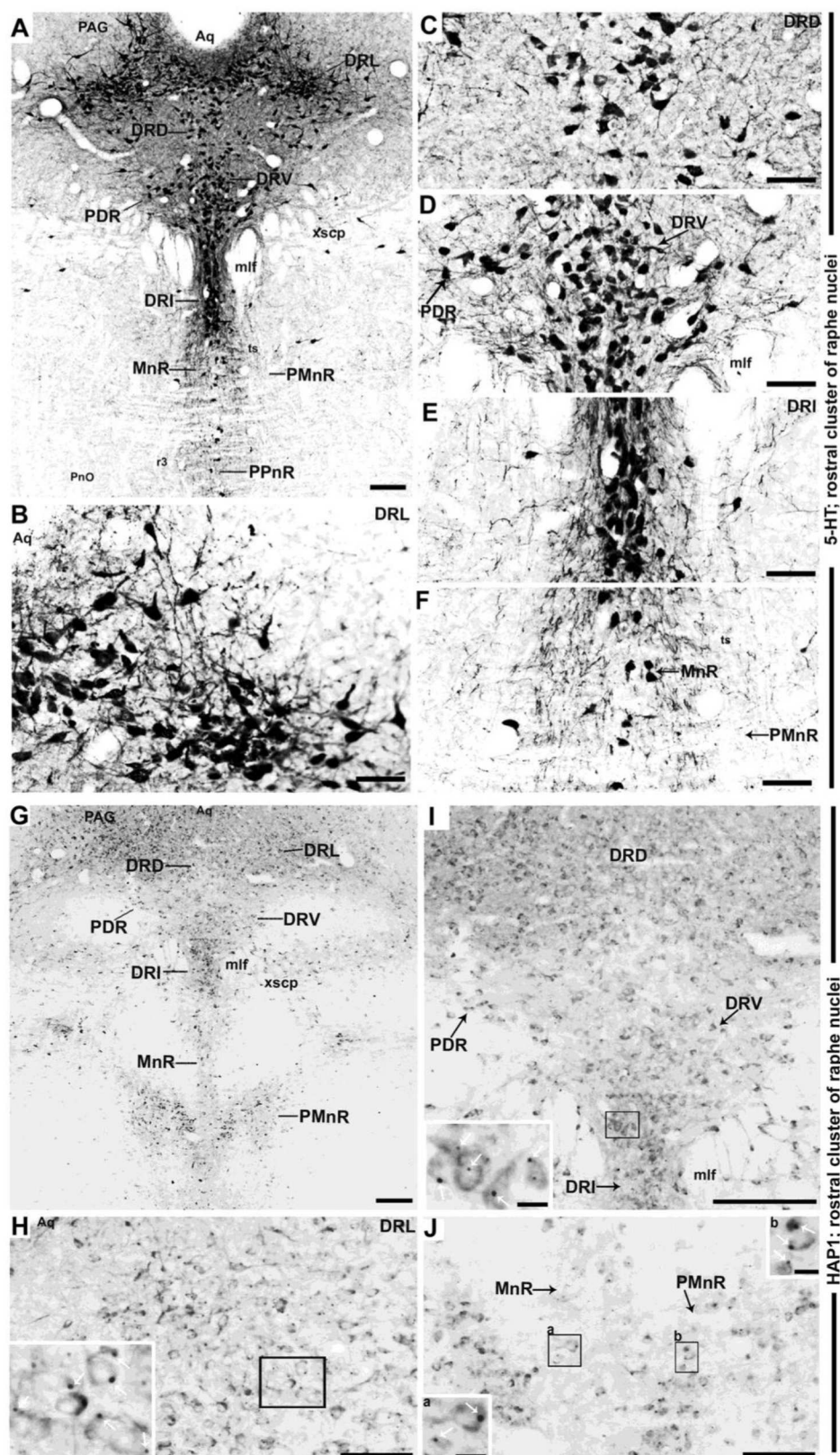


Figure 5. Selected high magnification immunoperoxidase images of 5-HT and HAP1 immunoreactivity in the rostral cluster of raphe nuclei. Photomicrographs showing the strong 5-HT (A-F) and HAP1-ir cells (G-J) in DRL, DRD, DRV, PDR, DRI, and MnR. The insets in H-J are the enlargement of the box areas, and the white arrows indicate STB. Scale bar = 120 μm in A; 100 μm in B-F, G-J; 20 μm in insets. For abbreviations, see “List of Glossary.”

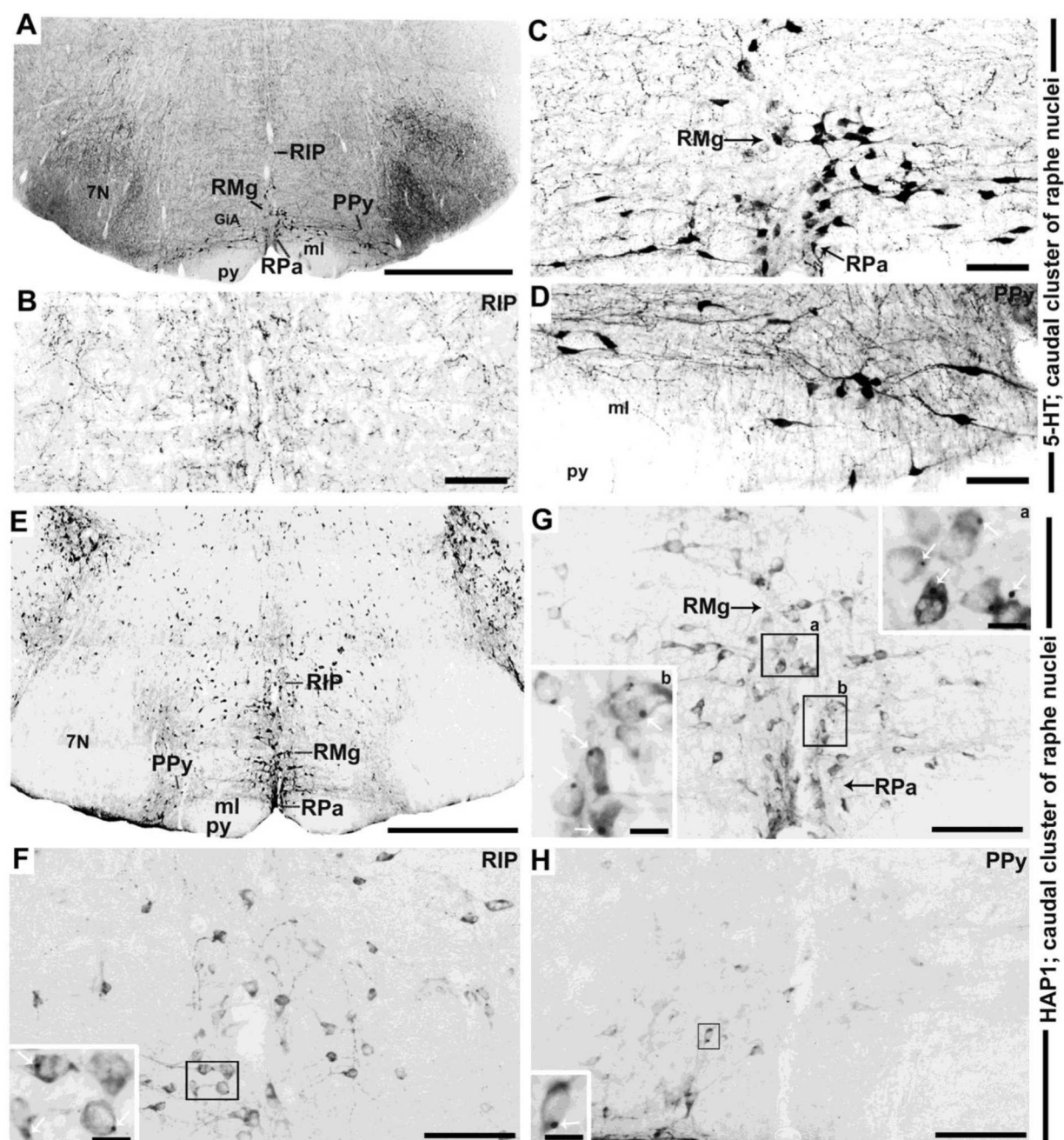


Figure 6. Selected high magnification immunoperoxidase images of 5-HT and HAP1 immunoreactivity in the caudal cluster of raphe nuclei. Photomicrographs showing the strong 5-HT (A-D) and HAP1-ir cells (E-H) in RIP, RMg, RPa, and PPy. The insets in F-H are the enlargement of the box areas, and the white arrows indicate STB. Scale bar = 50 μ m in A, E; 100 μ m in B-D, F-H; 20 μ m in insets. For abbreviations, see “List of Glossary.”

3.3.3. Distribution of HAP1-ir neurons in the caudal cluster of raphe nuclei: a comparison with 5-HT

In the caudal cluster of raphe nuclei, both 5-HT (Figures 6A-D) and HAP1-ir (Figures 6E-H) cells were disseminated in the raphe magnus nucleus (RMg), raphe obscurus nucleus (ROb), and raphe pallidus nucleus (RPa) with its lateral expansion referred to as the medullary parapyramidal nucleus (PPy). Raphe interpositus (RIP) is a tiny nucleus in the brainstem that is located on the midline of the caudal pons. The RIP is located where the rootlets of the abducens nerve pass through the brainstem, lying beneath the mlf (Figures 4B-C, 4G-H). Intriguingly, the RIP was devoid of 5-HT neurons (Figures 6A-B), while intensely stained HAP1-ir neurons were detected in the RIP (Figures 6E-F). RMg is considered the most rostral and the largest of the caudal cluster. In RMg, 5-HT neurons were polygonal and perpendicularly oriented in close proximity to the midline (Figures 6A, C). 5-HT neurons seemed to be dispersed unevenly across the RMg nucleus (Figures 6A, C). Strongly stained HAP1-ir neurons were also disseminated in the RMg (Figures 6E, G). RPa is dispersed rostrocaudally throughout the paramedian basal plate of the caudal medulla oblongata (Figures 4B-D, 4G-I). A much smaller dispersion of the 5-HT neurons was observed in RPa (Figures 6A, C), while moderately stained HAP1-ir neurons were found there (Figures 6E, G). Additionally, the lateral expansion of RPa, referred to as PPy, encapsulated the pyramidal tract located laterally (Figures 4B-D, 4G-I). This PPy contained very scattered 5-HT neurons (Figures 6A, D). In contrast, HAP1 showed weak staining in the PPy, which sporadically extended toward the lateral side (Figures 6E, H). Dot-like HAP1-ir STBs were observed in most of the raphe nuclei of the caudal cluster (Figures in insets of 6F-H).

Our immunoperoxidase staining results revealed an almost similar distribution pattern of HAP1 and 5-HT across the rostral and caudal clusters of the raphe nuclei. To further investigate their immunohistochemical relationships, we subsequently conducted double-label immunofluorescent immunohistochemistry for HAP1 and 5-HT.

3.3.4. Immunohistochemical relationships of 5-HT and HAP1 in the rostral and caudal cluster

Our double-labeled immunofluorescence histochemistry indicated that the 5-HT immunopositive cells showed evident HAP1 immunoreactivity in the nuclei of both rostral (Figures 7-10) and caudal clusters (Figures 11-12), including MnR (Figures 7A-F), SuL (Figures 7A-C, G-L), CLi (Figures 8A-F), PPnR (Figures 8A-C, 8G-I), five subregions within the DR (Figures 9A-L, 10G-I); DRC (Figures 10A-F); PnR (Figures 10A-C, 10J-L); RMg (Figures 11A-C, 11G-I); RPa from caudal pons (Figures 11A-C, 11J-L) and medulla (Figures 12A-C, 12G-I) [to which a group of PPy included, Figures 11M-O]; and ROb (Figures 12A-F and upon formation of two parallel vertical laminae Figures 12J-O). Our double-label immunofluorescence staining results, however, confirmed that the HAP1-ir neurons in the mesencephalic PMnR (Figures 7A-C, 7M-O) and RIP of the caudal pons (Figures 11A-F) never contained 5-HT immunoreactivity.

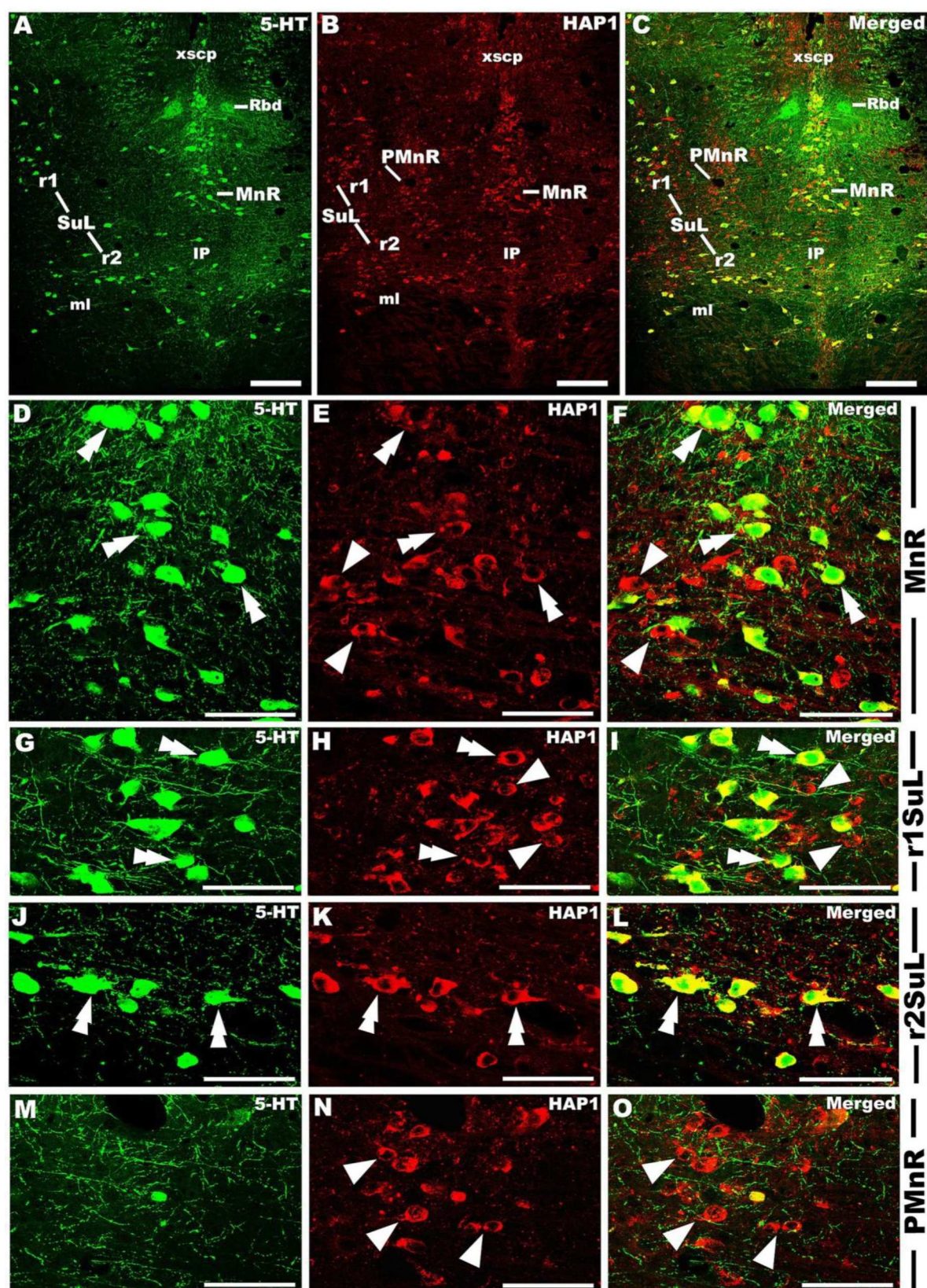


Figure 7. Double-label immunofluorescent immunohistochemistry for 5-HT and HAP1 in the median raphe nucleus and its lineage. Photomicrographs showing double-label immunofluorescence staining of 5-HT and HAP1 in MnR, PMnR, and SuL (A-C). D-F, G-I, J-L, M-O are the enlargement of MnR, r1SuL, r2SuL, and PMnR in A-C. Double arrowheads indicate the cells positive for HAP1 and 5-HT, white arrows and single white arrowheads indicate the cells positive only for 5-HT and HAP1, respectively. Scale bar = 20 μ m in A-O. For abbreviations, see “List of Glossary.”

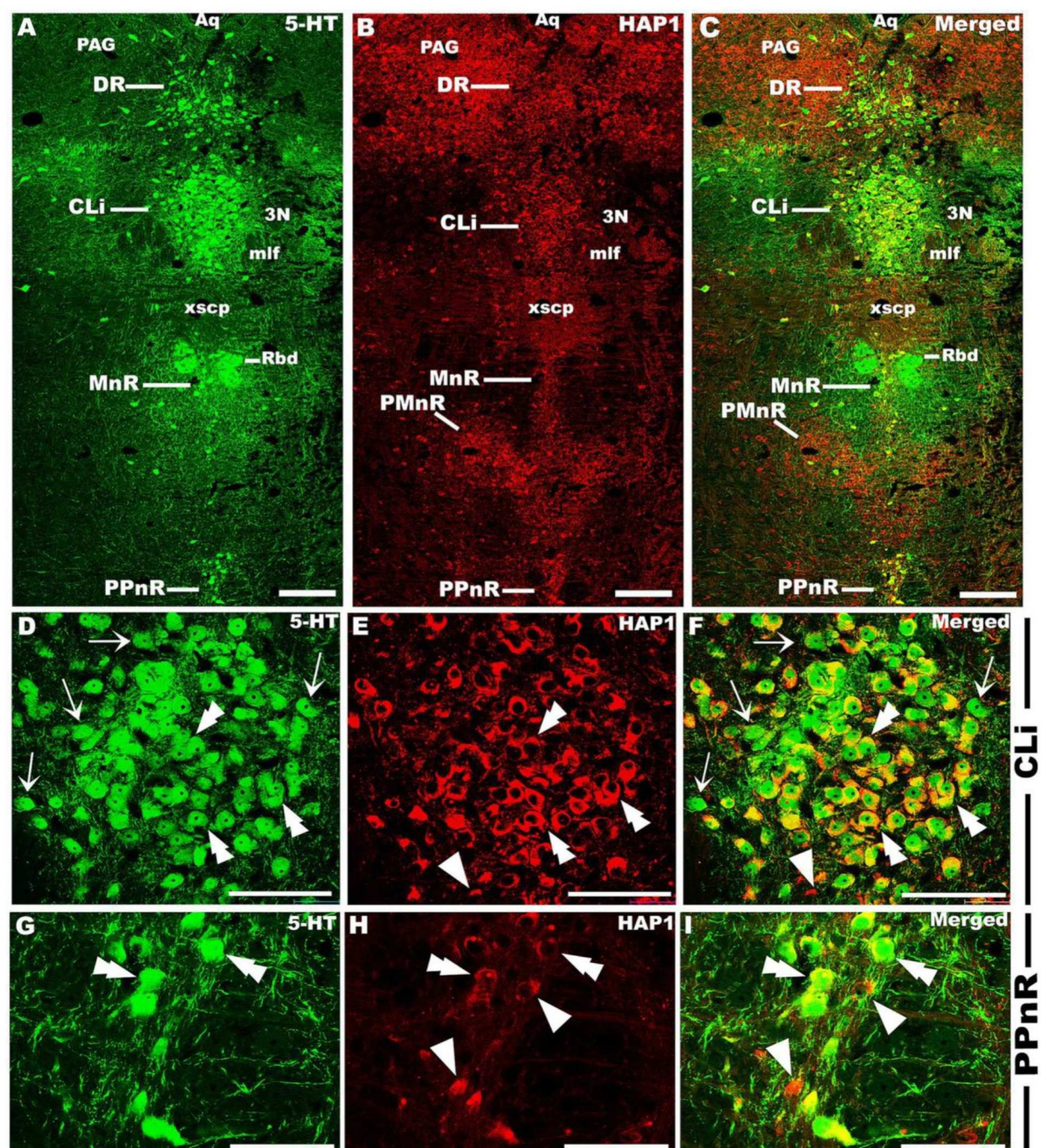


Figure 8. Double-label immunofluorescent immunohistochemistry for 5-HT and HAP1 in the caudal linear and prepontine raphe nuclei. Photomicrographs showing double-label immunofluorescence staining of 5-HT and HAP1 in CLi and PPnR (A-C). D-F and G-I are the enlargement of CLi and PPnR in A-C. Double arrowheads indicate the cells positive for HAP1 and 5-HT, white arrows and single white arrowheads indicate the cells positive only for 5-HT and HAP1, respectively. Scale bar = 40 μ m in A-I. For abbreviations, see “List of Glossary.”

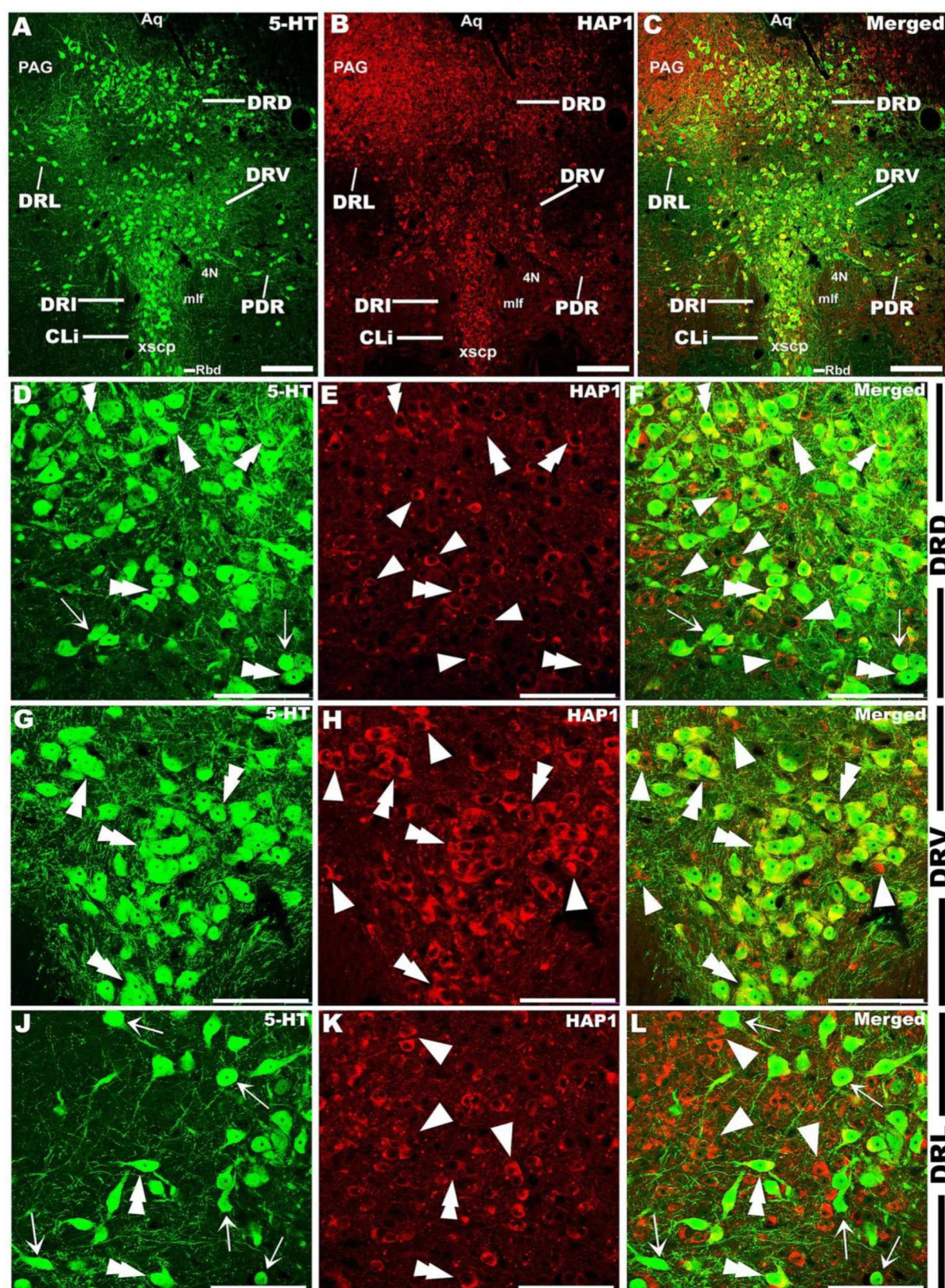


Figure 9. Double-label immunofluorescent immunohistochemistry for 5-HT and HAP1 in the dorsal raphe nucleus at the level of trochlear (4N) nerve nucleus. Photomicrographs showing double-label immunofluorescence staining of 5-HT and HAP1 in the sub-division of DR (A-C). D-F, G-I, J-L are the enlargement of DRD, DRV, and DRL in A-C. Double arrowheads indicate the cells positive for both HAP1 and 5-HT, white arrows and single white arrowheads indicate the cells positive only for 5-HT and HAP1, respectively. Scale bar = 40 μ m in A-L. For abbreviations, see “List of Glossary.”

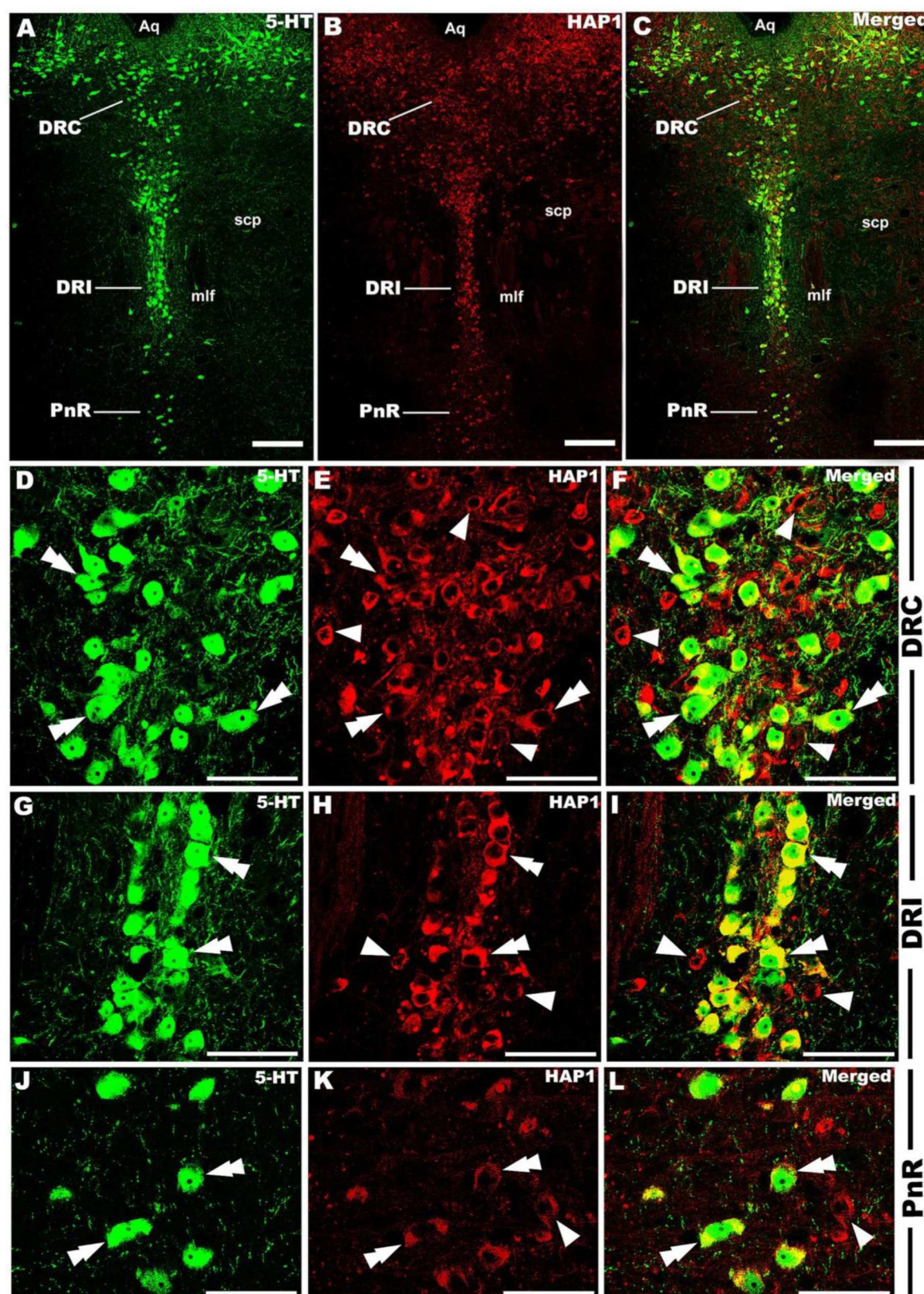


Figure 10. Double-label immunofluorescent immunohistochemistry for 5-HT and HAP1 in the caudal division of the rostral cluster. Photomicrographs showing double-label immunofluorescence staining of 5-HT and HAP1 in the caudal division of the rostral cluster (A-C). D-F, G-I, J-L are the enlargement of DRC, DRI, and PnR in A-C. Double arrowheads indicate the cells positive for HAP1 and 5-HT, white arrows and single white arrowheads indicate the cells positive only for 5-HT and HAP1, respectively. Scale bar = 40 μ m in A-L. For abbreviations, see “List of Glossary.”

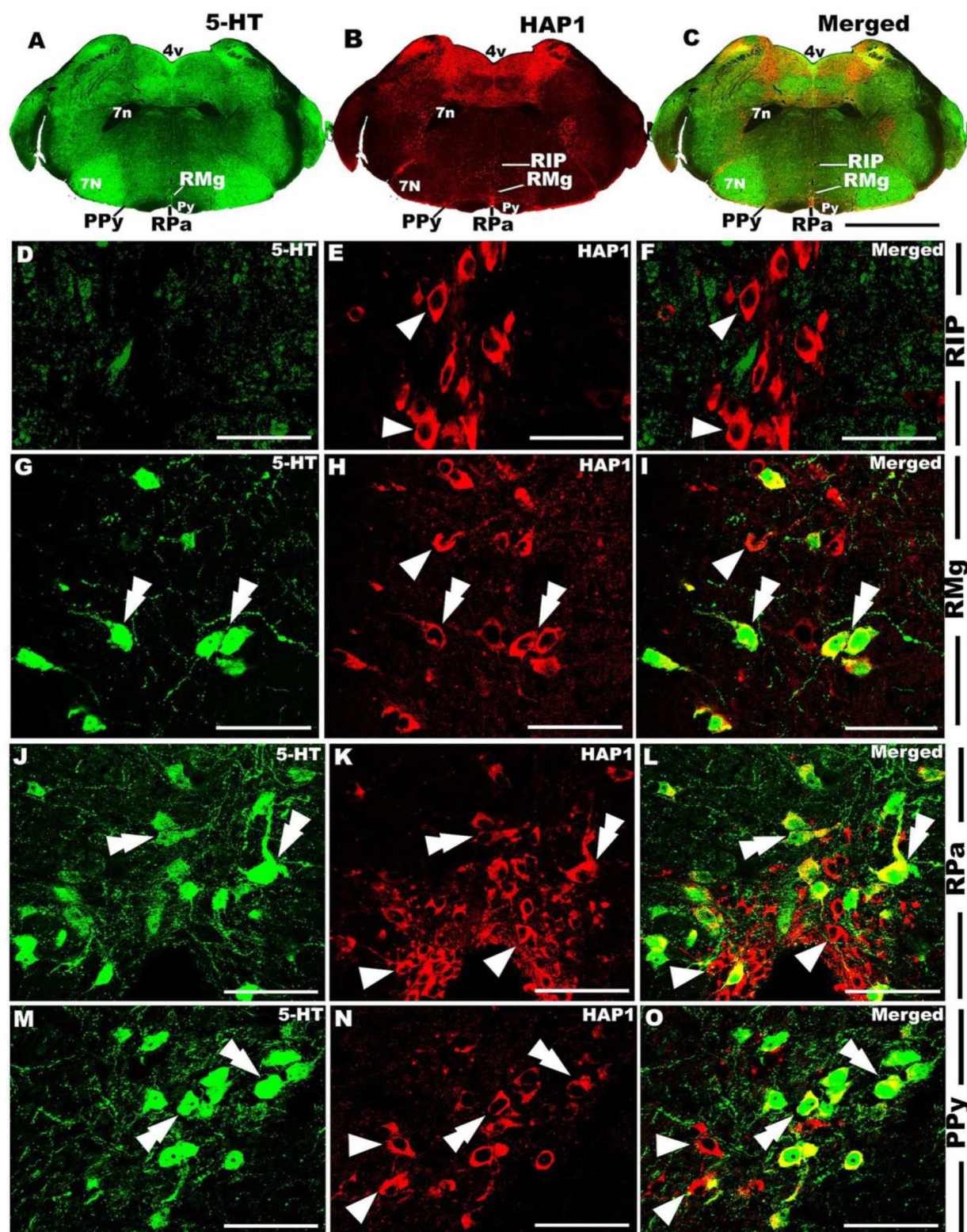


Figure 11. Double-label immunofluorescent immunohistochemistry for 5-HT and HAP1 in the caudal pons. Photomicrographs showing double-label immunofluorescence staining of 5-HT and HAP1 in caudal cluster in caudal pons (A-C). D-F, G-I, J-L, M-O are the enlargement of RIP, RMg, RPa, and PPY in A-C. Double arrowheads indicate the cells positive for HAP1 and 5-HT, white arrows and single white arrowheads indicate the cells positive only for 5-HT and HAP1, respectively. Scale bar = 40 μm in A-O. For abbreviations, see “List of Glossary.”

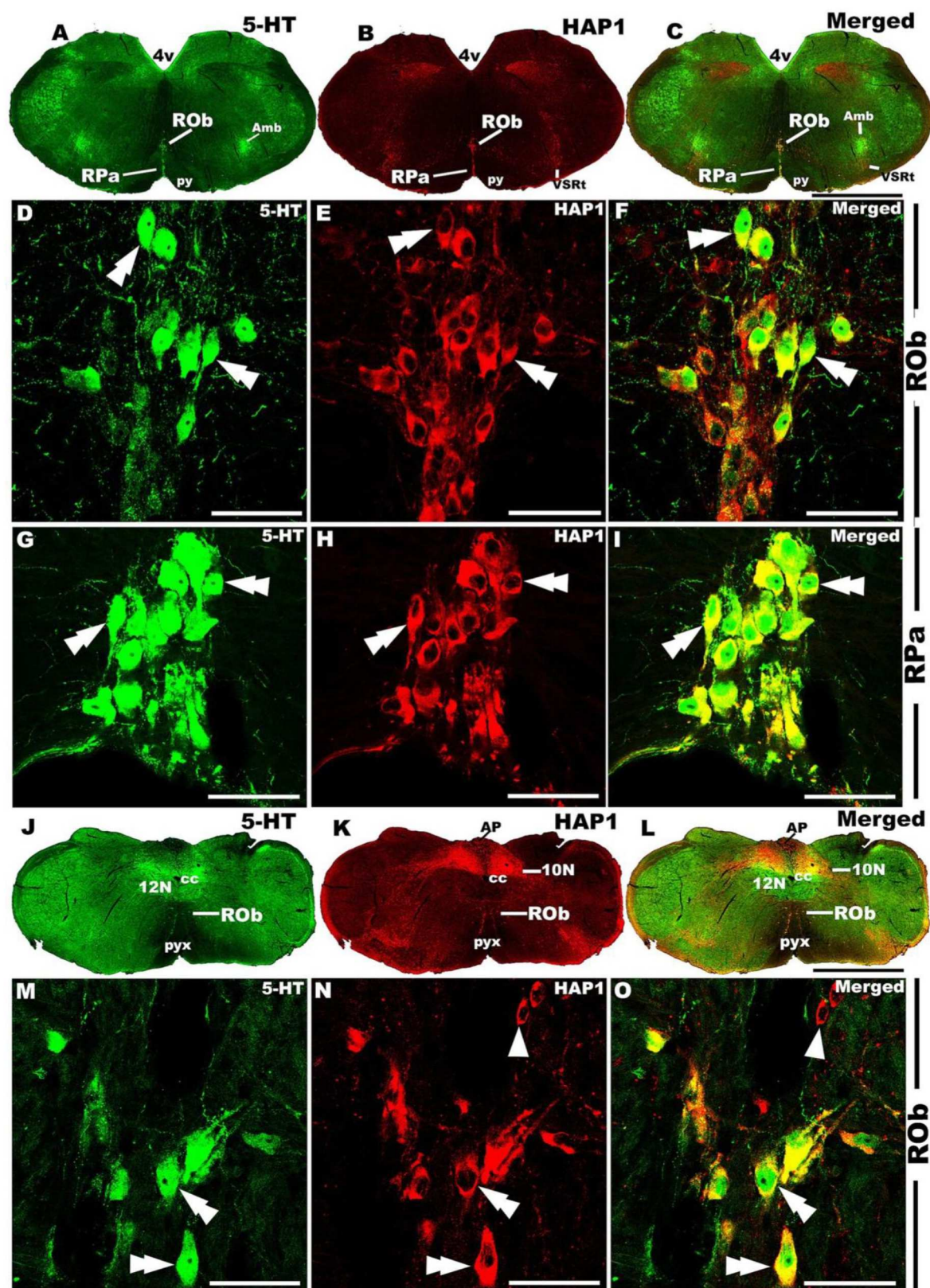


Figure 12. Double-label immunofluorescent immunohistochemistry for 5-HT and HAP1 in the medulla. Photomicrographs showing double-label immunofluorescence staining of 5-HT and HAP1 in caudal cluster in medulla (A-C, J-L). D-F and G-I are the enlargement of ROb and RPa in A-C. M-O is the enlargement of Rob in J-L, where the Rob gradually splits into two strings. Double arrowheads indicate the cells positive for HAP1 and 5-HT, white arrows and single white arrowheads indicate the cells positive only for 5-HT and HAP1, respectively. Scale bar = 40 μm in A-O. For abbreviations, see “List of Glossary.”

Our cell counting results showed higher coexpression ratios for HAP1 in 5-HT than those of 5-HT in HAP1 coexpression ratios in most of the rostral and caudal clusters of raphe nuclei, suggesting that the majority of 5-HT neurons contained HAP1 immunoreactivity (Table 3). In midbrain and rostral Pons, CLi exhibited identical HAP1 in 5-HT and 5-HT in HAP1 co-expression ratios of about 97% (Table 3). The DRV also showed high coexpression, with approximately 96.00% of 5-HT-ir neurons containing HAP1 immunoreactivity, while 85.98% of the HAP1-ir neurons had 5-HT immunoreactivity (Table 3). Other nuclei, such as the DRL, exhibited coexpression of HAP1 in 5-HT at 85.65% and of 5-HT in HAP1 at 80.36% (Table 3). However, the MnR displayed a slightly lower coexpression of 5-HT in HAP1 at 70.45%, compared to HAP1 in 5-HT at 95.56% (Table 3).

In the rostral pons, the PnPR exhibited a high coexpression ratio of HAP1 in 5-HT (98.43%) and 5-HT in HAP1 (96.02%). 97.84% of 5-HT-ir neurons showed HAP1 immunoreactivity in the PnR nuclei, while approximately 96.91% of HAP1-ir neurons displayed 5-HT immunoreactivity in that region (Table 3).

The caudal cluster (caudal pons and medulla) of raphe nuclei also exhibited a substantial coexpression ratio of HAP1 with 5-HT. Notably, the RMg had a high coexpression ratio for both HAP1/5-HT (97.42%) and 5-HT/HAP1 (90.68%) (Table 3). In the ROb and RPa, 99.67% and 97.38% of 5-HT neurons contained HAP1 immunoreactivity, respectively. Meanwhile, 94.66% of HAP1-ir cells in ROb and 81.19% of HAP1-ir cells in RPa displayed 5-HT immunoreactivity (Table 3). The RIP, however, stood out by showing no HAP1 coexpression with 5-HT due to a lack of 5-HT neurons (Table 3).

The statistical analyses show significant regional variation in the co-expression ratios of HAP1 and 5-HT throughout the mouse brainstem raphe nuclei. The highest HAP1/5-HT ratios were found in both the rostral and caudal pons and medulla, while fluctuations in co-expression were detected in the midbrain raphe nuclei. Considering inter-animal variation among mice, the significant differences were confirmed by one-way ANOVA followed by Tukey's test, and specific pairwise comparisons were made using Student's t-test, showing multiple significant results at both 1% and 5% levels of significance.

Table 3 Co-expression ratios of HAP1/5-HT or 5-HT/HAP1 in the brainstem raphe nuclei of adult mice.

Brainstem area			Mean ± SD		LS (Student's t test)
			Raphe Nucleus	HAP1/5-HT co-expression ratio (%)	
Midbrain	Rostral cluster	Median raphe (MnR)	95.56 ^d ±0.76	70.45 ^g ±0.6	**
		Paramedian raphe (PMnR)	HAP1/ 0 [#]	0 [#] / HAP1	N/A
		Supralemniscal raphe (SuL)	98.44 ^{ab} ±±0.59	90.66 ^d ±0.52	**
		Caudal linear raphe (CLi)	97.76 ^{abc} ±0.66	97.78 ^a ±0.36	NS
		Dorsal raphe dorsal (DRD)	90.61 ^e ±1.46	82.59 ^f ±0.73	**
		Dorsal raphe ventral (DRV)	96 ^{cd} ±2.29	85.98 ^e ±0.52	**
		Dorsal raphe interfascicular (DRI)	97.6 ^{bc} ±0.71	94.73 ^{bc} ±0.64	**
		Dorsal raphe lateral (DRL)	85.65 ^f ±0.68	80.36 ^f ±0.5	**
Rostral pons	Posterodorsal raphe (PDR)	98.45 ^{ab} ±0.61	93.66 ^c ±0.47	**	
	Dorsal raphe caudal (DRC)	96.49 ^{bcd} ±1.11	86.19 ^e ±0.37	**	
	Prepontine raphe (PPnR)	98.43 ^{ab} ±0.85	96.02 ^{abc} ±0.76	*	
Caudal pons	Caudal cluster	Pontine raphe (PnR)	97.84 ^{abc} ±0.72	96.91 ^{ab} ±0.45	NS
		Raphe magnus (RMg)	97.42 ^{bcd} ±0.43	90.68 ^d ±0.41	**
		Raphe interpositus (RIP)	0 [#] / HAP1	HAP1/ 0 [#]	N/A
Medulla		Raphe obscurus (ROb)	99.67 ^a ±0.44	94.66 ^{bc} ±0.91	**
		Raphe pallidus (RPa)	97.38 ^{bcd} ±0.65	81.19 ^f ±0.4	**
		Parapyramidal raphe (PPy)	98.2 ^{ab} ±1.52	88.96 ^d ±0.48	**
LS (One way ANOVA followed by Tukey's test)			**	**	

Values represent the mean $\pm$ SD of co-expression ratios (%) in adult mice ($n = 6$). ^{a,b,c,d,e,f,g} denotes the Means within the same column with different superscript letters that differ significantly. ** Significant at 1% ($p < 0.01$); * Significant at 5% ($p < 0.05$); [#] No co-expression, 5-HT immunoreactivity was absent in both PMnR and RIP nuclei; N/A, not applicable; NS, non-significant; LS, Level of significance; SD, Standard deviation; 5-HT, Serotonin; HAP1, Huntingtin-associated protein 1.

3.4. Discussion

The current study, employing Western blot and immunohistochemistry, to the best of our knowledge, represents the first detailed investigation of HAP1 expression and precise distribution in the raphe nuclei throughout the mouse brainstem. As far as we are aware, this study is also the first to map the comparative distribution of HAP1 with 5-HT and their morphological relationships in rostral and caudal clusters of raphe nuclei. The expression of HAP1 in the rodent brainstem has been briefly investigated previously using *in situ* hybridization (Fujinaga et al., 2004) and immunohistochemistry (Islam et al., 2022). However, to our understanding, its cellular localization, comprehensive comparative distribution, and neurochemical characterization in relation to 5-HT have never been investigated in the raphe nuclei.

3.4.1. Comparative distribution of HAP1 and 5-HT-ir neurons in the raphe nuclei

The current study shows that HAP1 immunoreactivity is expressed in the neurons, not glial cells, across the raphe nuclei. These results agree with previous works in other regions of the brain or spinal cord (Islam et al., 2017, 2022; Wroblewski et al., 2018). The midbrain, pons, and medulla have been considered the primary brainstem subdivisions, and serotonergic raphe populations are assigned to these territories (Figure 13A). Our current comparative mapping of HAP1 and 5-HT neurons suggests that both the HAP1 and serotonergic populations present in the rostral cluster essentially belong to different, separate subgroups of nuclei, including CLi, diverse parts of the DR, MnR with ventrolaterally placed SuL, PPnR, and PnR that express high 5-HT immunoreactivity. The PMnR, however, is devoid of 5-HT neurons. The rostral cluster nuclei correspond to the above-mentioned definite brainstem raphe nuclei, as confirmed by prior research (Alonso et al., 2013). Furthermore, five subregions of the DR had been found in rodents by Steinbusch in the early 1980s (Steinbusch, 1981). Our study also offers a more detailed description of the segmental arrangement of the raphe groups, including RMg, ROb, RPa, and medullary PPy, in the caudal cluster that expresses both 5-HT and HAP1. Indeed, our current research clarifies the presence of RIP in the caudal cluster, which shows no 5-HT immunoreactivity but does exhibit HAP1 immunoreactivity. The previous literature (Büttner-Ennever et al., 1988) also aligns with this finding, where RIP lacks 5-HT neurons.

Our earlier research found no apparent sex differences in HAP1 expression in adult mice's hippocampal region (Islam et al., 2012). Sex variations in the control of serotonergic transmission have been shown in earlier studies, especially in genetically altered models like 5-HT receptor knockout (KO) mice (John et al., 1991). Therefore, to completely clarify the cytoarchitectonic and functional variability of HAP1 in the raphe system, additional research, including in-depth investigations of sex as a biological variable across various species, is necessary.

3.4.2. Pathophysiology and possible impact of HAP1 on 5-HT neurons in the raphe nuclei

5-HT neurotransmission generally regulates neurogenesis, synaptogenesis, corticogenesis, neuronal migration, neuronal maturation, and axonal network formation (Ulhaq and Kishida, 2018). Alterations in DR 5-HT neurotransmission have been linked to a number of neuropsychiatric and neurological disorders, including major depressive disorder, bipolar disorder, schizophrenia, obsessive-compulsive disorder, and movement disorders in Parkinson's disease (PD) (Mahmood and Silverstone, 2001; Vaswani et al., 2003; Huot et al., 2011; Politis and Niccolini, 2015). When it comes to PD, patients' brains predominantly suffer from increasing dopaminergic denervation, but other systems, such as the 5-HT, are also impacted. 5-HT typically stimulates dopamine release via a range of 5-HT receptors in the normal, non-Parkinsonian brain (Alex and Pehek, 2007). 5-HT activity is generally lower in PD patients, serving as a compensatory strategy for the lower dopaminergic activity (Leentjens et al., 1998). Currently, a number of studies indicate that 5-HT terminal degeneration can cause the development of both motor and non-motor symptoms commonly associated with PD (Halliday et al., 1990; Paulus and Jellinger, 1991; Kish et al., 2008). Braak's staging describes how the degenerative process in PD progresses gradually upwards (Voon et al., 2006). The rostral raphe nuclei, which house the 5-HT neurons of the brainstem, undergo Lewy body and Lewy neurite deposition during Braak stage II (Braak et al., 2003). Generally, neurodegeneration in the raphe nuclei may be the secondary cause of the loss of striatal 5-HT innervation in PD (D'Amato et al., 1987; Braak et al., 2003). Brainstem serotonergic neurons, however, are anticipated to be affected prior to midbrain dopaminergic neurons in PD. Additionally, a couple of research reveal that when 5-HT level is decreased that cause increased tau expression and cellular aggregation (John et al., 1991) which have been associated with the Alzheimer's disease (AD) (Yang and Schmitt, 2001; Doraiswamy, 2003; Gudelsky and Yamamoto, 2003; Kovacs et al., 2003).

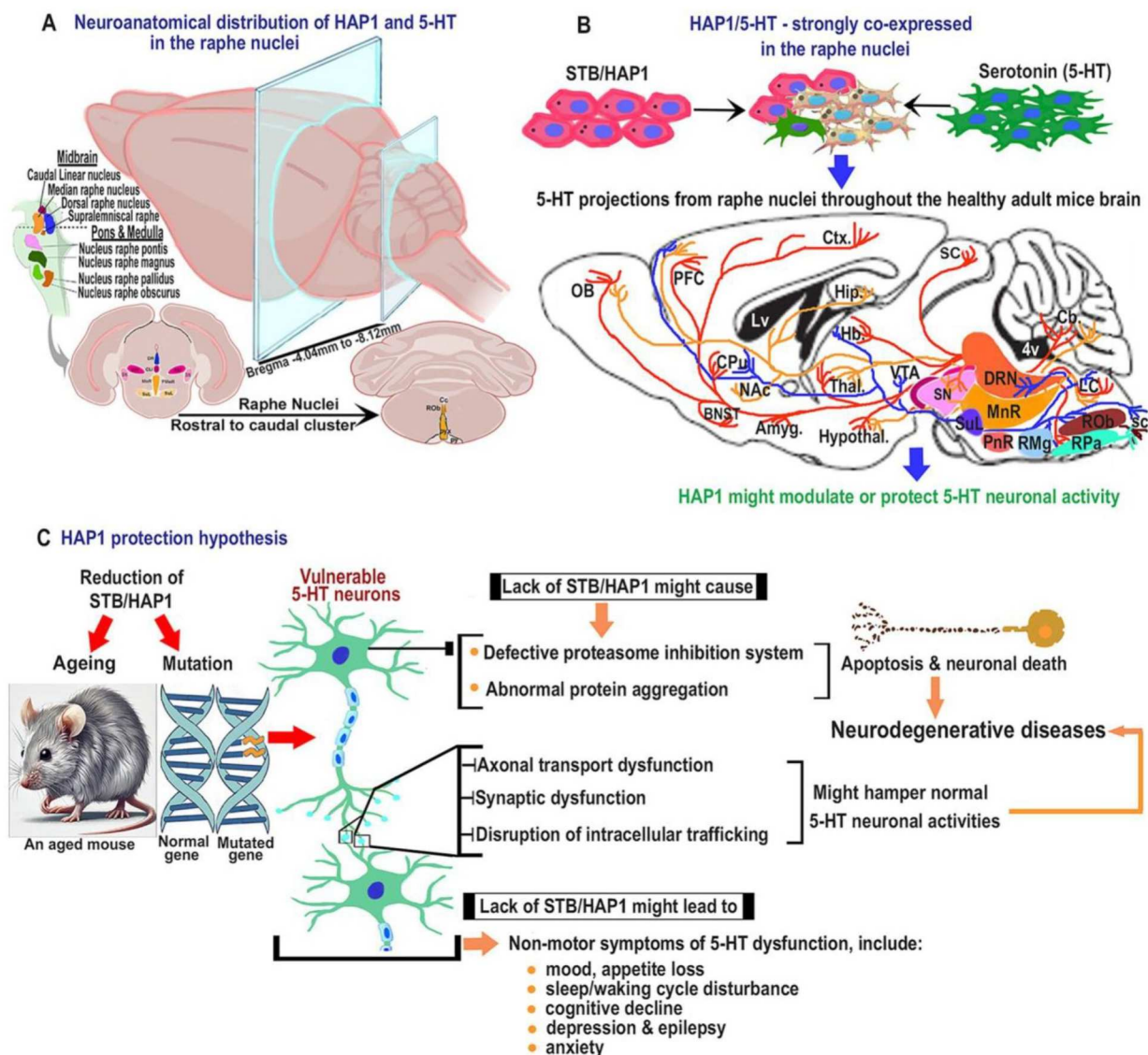


Figure 13. Schematic diagram for the plausible effects of HAP1 in the pathophysiology of serotonin neurons. **A)** Representative coronal sections for neuroanatomical distribution of HAP1 and 5-HT. **B)** Illustration of the projections of 5-HT neurons that contain strong HAP1 immunoreactivity in healthy adult mice, which is indicated by the blue arrow. The 5-HT neurons might be protected due to having putative HAP1 protectivity. **C)** Hypothetical pathways indicate that due to ageing or mutation in HAP1, 5-HT neurons may lack HAP1 protection, which is annotated by the red arrow. The connection between neurodegenerative diseases and the emergence of non-motor symptoms due to 5-HT neuron dysfunction may become more evident in the absence of the putative HAP1 protectivity. Dysfunction of 5-HT neurons and plausible neurodegenerative pathways are indicated by the yellowish arrow. For abbreviations, see “List of Glossary.”

Besides a robust topographic distribution, our cell counting results suggest that almost all of the 5-HT neurons in raphe nuclei contained STB/HAP1 immunoreactivity (Figure 13B). This implies that HAP1 might modulate 5-HT neuronal activity throughout the healthy brain (Figure 13 B). Previous research has suggested that 5-HT, as a neurotransmitter, exerts its effects via membrane receptors in the brain and spinal cord (Hannon and Hoyer, 2008). It is possible that HAP1 could regulate the release of 5-HT from axon terminals by preserving the stability of 5-HT membrane receptors. Intriguingly, it has been reported that membrane receptors cannot be effectively delivered to the proper cellular locations if the association of HAP1 with microtubule-dependent transporters is inhibited (Chen et al., 2023). Additionally, prior studies have shown that HAP1 is connected to synaptic vesicles (Gutekunst et al., 1998) and transfers of different vesicular proteins or membrane receptors, such as vesicles that encompass BDNF (Gauthier et al., 2004). In fractions containing synaptosomes, synaptic vesicle-associated protein (SVP38) and BDNF are reduced in HAP1-KO mouse brain lysate (Lin et al., 2010). HAP1 is particularly crucial for TrkB internalization following BDNF binding (Lim et al., 2018), which is critical for neuron survival in the pathogenesis of AD and PD (Kang et al., 2017). Likewise, synaptic plasticity, neurogenesis, and neuronal survival in the brain are regulated by the 5-HT system, which is acknowledged to interact with the BDNF pathway to create a chain of positive feedback (Mattson et al., 2004). The alteration of vesicular dynamics could be a critical factor in the onset of ND. Hence, it might be postulated that modifying HAP1 expression in 5-HT neurons within the raphe nuclei may cause synaptic disruption, hindering 5-HT neurotransmitter release or neuronal plasticity, which could contribute to neurodegeneration in certain NDs (Figure 13C).

It has been reported that HAP1 can modulate intracellular trafficking (Chen et al., 2023). It is possible that HAP1 could regulate 5-HT neuronal activity via intracellular trafficking, which is crucial for supplying nutrients, molecules, and organelles to nerve terminals. The previous electron microscopy findings demonstrate that HAP1 is dispersed throughout microtubules and membrane-bound organelles (Gutekunst et al., 1998). Consistently, HAP1 may form protein complexes with the trafficking proteins like kinesin light chain and microtubule-dependent transporters, such as dynactin p150, which facilitate the anterograde and retrograde distribution of various cargos or proteins in neuritic processes and nerve terminals (Chen et al., 2023; Xiong and Sheng, 2024). It is plausible that a deficiency in HAP1 could lead to a decrease in the quantity of various proteins in the synaptosome-enriched axon terminal, which could potentially impact 5-HT neuronal interactions. Protein mislocalization and the build-up of undegraded proteins in neurons are often caused by abnormalities in intracellular trafficking (Inoshita and Imai, 2015). Therefore, ND pathogenesis may be exacerbated by the reduction in HAP1-mediated intracellular trafficking in 5-HT neurons, especially in long-

projecting neurons, which might lead to neuronal dysfunction (Figure 13C). In addition, defective axonal transport of presynaptic cargos or proteins can also cause neurodevelopmental disorders accompanying 5-HT dysfunction, including autism spectrum disorders, attention-deficit/hyperactivity disorder, disruption of learning and motor function, developmental epilepsies, and schizophrenia (Xiang et al., 2015; Thapar et al., 2017; Sydnor et al., 2021). However, identifying serotonergic boutons inside serotonin transporter (SERT) axons utilizing pre- (synaptophysin) and postsynaptic components of excitatory (PSD95) or inhibitory (gephyrin) synapses, and the interaction between HAP1 deficiency and SERT trafficking has yet to be revealed.

Sleep-wake cycle disturbance is the non-motor symptom of PD. The DR is thought to be involved in the sleep-wake cycle control. The activity of the DR is high during waking, low during slow-wave sleep, and abolished during rapid eye movement sleep (Bodner et al., 1995). This notion arises from animal research findings showing that lesions in the 5-HT neurons lead to reduced sleep, which is associated with decreased 5-HT expression in the brain (Bodner et al., 1995) and is responsible for the development of PD. Interestingly, HAP1 is highly expressed in the locus coeruleus, the pedunculopontine nucleus, and the lateral dorsal tegmental nuclei (Islam et al., 2022, 2025). These nuclei are interconnected with the 5-HT neurons from the DR and may play a role in regulating the sleep-wake cycle. Other non-motor symptoms of 5-HT dysfunction include mood disorder, appetite loss, pain, depression, anxiety, fatigue, cognitive decline, hallucinations, sensory and autonomic dysfunction, olfactory deficit, and constipation. Our current immunohistochemical results may indicate that HAP1 possibly controls the above-mentioned non-motor symptoms via modulation of 5-HT neurons in the raphe nuclei (Figure 13C).

Abundant expression of HAP1-ir neurons, other than 5-HT, in PMnR was observed in our study. Neurons of the PMnR are projected to the dorsal horn of the spinal cord and inhibit nociceptive transmission (Miller and Van Vactor, 2009). It is noteworthy that our previous studies have demonstrated that HAP1 is highly expressed in the dorsal root ganglion (Islam et al., 2020a) and the superficial layers of the dorsal horn of the spinal cord (Islam et al., 2017). This could either help to inhibit descending pain or modulate specific nociceptive stimuli.

RIP in the caudal cluster exhibited HAP1-ir neurons in our current study, which are omnipause neurons (Rüb et al., 2003) and have a potential role in the premotor network for saccades (Langer and Kaneko, 1990). Horizontal saccade slowdown may be a result of the noticeable loss of RIP neurons caused by neurodegeneration in the brainstem of terminal SCA3 patients (Rüb et al., 2003). Through the Josephin domain, HAP1/STB has been shown to interact with both normal and polyglutamine-expanded ataxin-3 mutants (Takeshita et al., 2011). Future studies may focus on how

HAP1 alters the physiological function of normal ataxin-3 or how it modifies the toxicity of mutant ataxin-3, influencing the degree of neuronal damage in RIP.

Evidence is mounting that patients with PD, AD, or HD exhibit persistently elevated cortisol levels during stressful situations due to downregulation of glucocorticoid receptor (GR) expression. This suggests that the hypothalamic-pituitary-adrenal (HPA) axis is altered in NDs (Oakley and Cidlowski, 1993; Karanth et al., 1997; Uys et al., 2006; Vyas and Maatouk, 2013; Boero et al., 2018). Another study indicates that a malfunction in 5-HT receptor activity may result from the hypersecretion of cortisol (Pitchot et al., 2001), indicating a reciprocal interaction between the 5-HT and HPA axis. Intriguingly, it has been reported that HAP1 can stabilize the expression of hypothalamic GR (Chen et al., 2020), which is crucial for regulating the HPA axis and alleviating major depression. In addition, the hypothalamus regulates 5-HT neurons through a push-pull mechanism (Zhou et al., 2017), and HAP1 is highly expressed in hypothalamic regions (Fujinaga et al., 2004, 2009). Taken together, our current findings suggest that HAP1 may stabilize 5-HT neurons and enhance or restore 5-HT functions to mitigate depression-like symptoms in PD (Figure 13C).

3.4.3. Plausible HAP1 protective effects in the serotonergic system

Our present research is tempting to speculate that HAP1 might be involved in modulating the serotonergic effects throughout the brain as a “knock-on effect” via the HAP1-expressing 5-HT neuronal projections from the rostral and caudal clusters of the raphe nuclei (Figure 13B). It has been known that HAP1 transfection inhibits neuronal apoptosis triggered by the proteasome inhibitor MG132 in cultured cells (Fujinaga et al., 2011). This highlights the importance of HAP1 in protecting neurons against neurodegenerative apoptosis. It is known that the proteasome inhibition system gradually declines in activity during aging (Zhao et al., 2016). Intriguingly, it has been reported that the expression of HAP1 mRNA is downregulated in the aged rodent brain (Page et al., 1998). Thus, changes in HAP1 expression levels or modifications in the distribution patterns of HAP1 may relate to the reduction in proteasome activity in aged mice brains, which could lead to alterations in HAP1's protective role against neurodegeneration. Given that HAP1 is also known to be essential for controlling embryonic development, restoring HAP1 expression in neuronal cells throughout developmental stages might prevent early lethality (Dragatsis et al., 2004). In the future, further detailed research should be carried out to examine the HAP1 expression in the raphe nuclei that are subject to temporal regulation throughout the embryonic or aging phases. However, it is intriguing to conjecture that the lower expression of HAP1 immunoreactivity in elderly populations may alter the HAP1 and 5-HT co-expression ratio in the brain, potentially leading to decreased HAP1 protective effects and possibly contributing to neurodegeneration. It is also anticipated that exposure

to environmental stress factors may lead to mutations in HAP1, which is likely linked to several NDs due to 5-HT neuronal dysfunctions resulting from changes in HAP1 protectivity (Figure 13C).

3.4.4. Possible effects of HAP1 on other neuronal types: coexist at the raphe nuclei

Though our present study focused on the relationships of HAP1 with 5-HT neurons only, it is well-known that other neuronal types (dopaminergic, peptidergic, GABAergic, and glutamatergic) are present in the raphe nuclei (Huang et al., 2019). 5-HT can regulate the effects of these neurotransmitters in raphe nuclei (Fox et al., 2009). Notably, our current results indicate that the number of HAP1-ir cells might exceed that of 5-HT neurons in the raphe nuclei. Nevertheless, it is possible that HAP1-ir cells found in all raphe nuclei may potentially have an influence on other neuronal types mentioned above. Future studies, however, need to focus on the cytoarchitectonic characterization of HAP1 concerning the dopaminergic, peptidergic, GABAergic, and glutamatergic neurons within specific raphe nuclei.

3.4.5. Limitations of the current study and future perspective

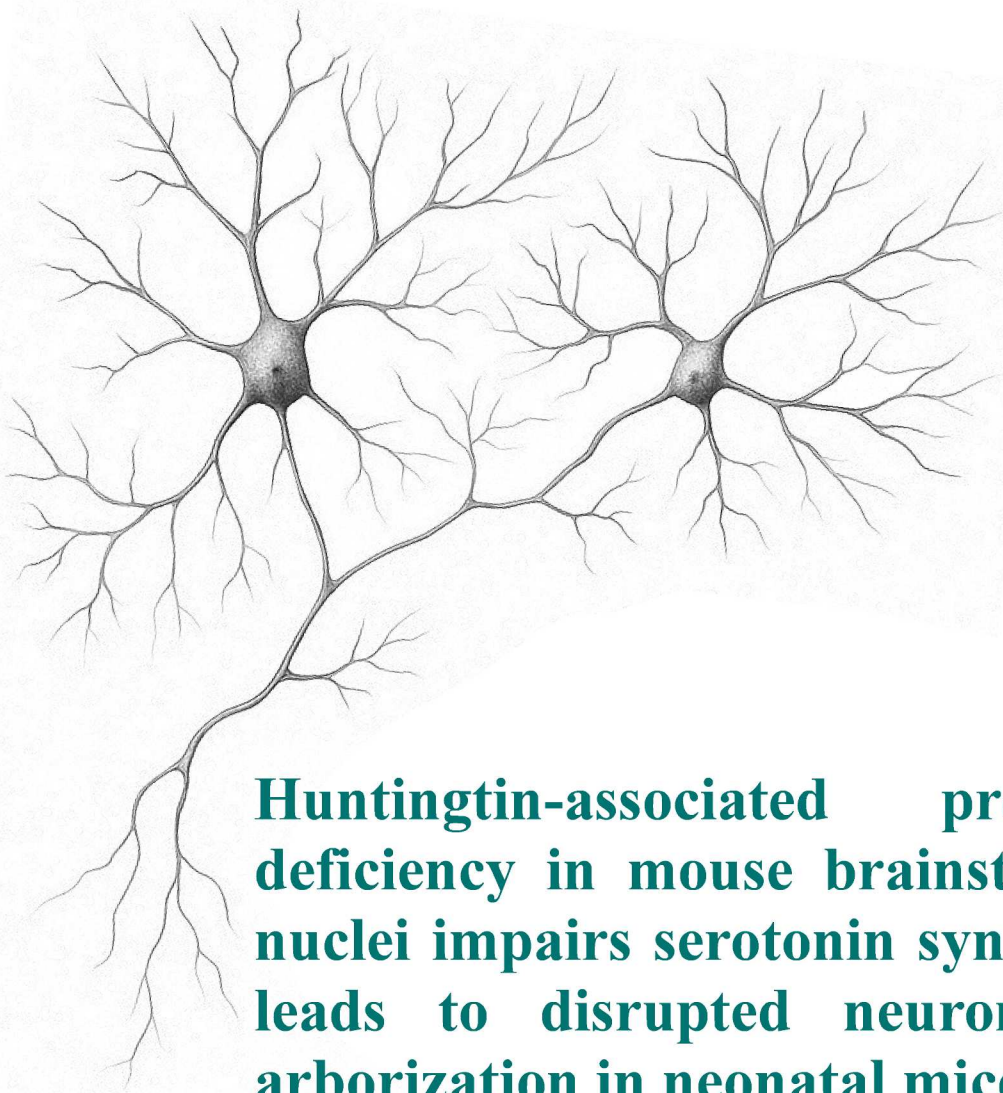
Our current study examined the immunohistochemical relationships between HAP1 and 5-HT in the raphe nuclei of adult male mice. As discussed above, there are potential sex variations in the control of serotonergic transmission; future studies should clarify the relationship between HAP1 and serotonin in female mice across various species. Future research using HAP1-KO mice may shed light on the potential connections between serotonergic neuron growth and functions and the pathophysiology of various NDs. In addition, using animal models that mimic the conditions of certain neurodegenerative diseases (e.g., PD, HD, AD), further study is required to elucidate the precise molecular mechanisms underlying the functional aspects of HAP1, which could be employed in prospective therapeutic applications.

3.5. Conclusion

HAP1 has recently emerged as a neuroprotective binding partner with causal agents of several NDs; however, our neuroanatomical knowledge of HAP1 with 5HT in raphe nuclei remains in its infancy. Our current study reveals the abundant expression of HAP1 immunoreactivity in all the raphe nuclei. This study also clarifies that almost all 5-HT neurons contain HAP1 immunoreactivity in rostral and caudal clusters of raphe nuclei. These findings may provide significant insights into unraveling the role of HAP1 in regulating 5-HT neuronal activity, where HAP1 could act as a neuroprotector that may preserve the integrity of either 5-HT neuron soma or its processes.



Chapter 4



Huntingtin-associated protein 1 deficiency in mouse brainstem raphe nuclei impairs serotonin synthesis and leads to disrupted neuronal fiber-arborization in neonatal mice



Abstract

Huntingtin-associated protein 1 (HAP1) is known as a possible neuroprotective interactor with causative proteins for several neurodegenerative diseases. We have previously reported that HAP1 is highly expressed across the brainstem raphe nuclei, and these represent the origin of central serotonergic (5-HT) projections. Since nearly 99% of 5-HT neurons displayed an immunoreactivity of HAP1 in adult mice, indicating that HAP1 might play a crucial role in regulating or protecting 5-HT functions of the area. We employed CRISPR-Cas9 genome editing to generate genetically modified HAP1-homozygous knockout (HAP1^{-/-}) pups in order to explain this. Unfortunately, HAP1^{-/-} pups succumbed to death without the known etiology within 24 h of birth. Hence, in the present study, we assessed the neurochemical relationship of HAP1 with 5-HT in postnatal day 0 wild (HAP1^{+/+}) pups, which implied that about 98% of 5-HT neurons contained HAP1 immunoreactivity in both clusters of raphe nuclei. Despite the absence of significant alterations in the quantity of 5-HT neurons, the somatic volume was significantly decreased in HAP1^{-/-} pups. Notably, HAP1^{-/-} pups had considerably fewer serotonergic fiber varicosities in the raphe nuclei, cortex, hippocampus, hypothalamus, and amygdala. Even HAP1-heterozygous knockout (HAP1^{+/-}) pups also demonstrated moderated varicosities in comparison to wild-type pups. Moreover, HAP1^{-/-} pups exhibited decreased expression of tryptophan hydroxylase-2, a crucial enzyme in the synthesis of serotonin in the brain. These findings can, thus, yield valuable insights into deciphering the role of HAP1 as a major regulator supporting the preservation of the structural and synthesizing stability of the serotonergic system.

Keywords: *HAP1-knockout pups, serotonergic neuron, fiber arborization, varicosity, 5-HT synthesis, neurodegeneration.*

4.1. Introduction

Huntingtin-associated protein 1 (HAP1) is a neuron-specific cytoplasmic protein that was first identified as a binding partner of huntingtin (HTT), a protein produced by Huntington's disease (HD) gene (Li et al., 1995a). HAP1 is prominently expressed in stigmoid body (STB), a non-membrane bound cytoplasmic inclusion that is spherical in shape and has a diameter of 0.5-3 μ m, which is a characteristic feature of STB inside the neuron (Shinoda et al., 1992, 1993). In addition to its relationship with HD pathology, HAP1 is demonstrated as a multifunctional protein with strong expression in the brainstem and hypothalamus that participates in vesicular transport, receptor stabilization, and intracellular trafficking of neuronal survival and differentiating cargo proteins (Gutekunst et al., 1998; Sheng et al., 2006). The transport of neuronal vesicular proteins or membrane receptors, such as brain-derived neurotrophic factor (BDNF)-containing vesicles, γ -aminobutyric acid type A receptor (GABAA), and tropomyosin-related kinase A receptor tyrosine kinase (TrkA),

can be facilitated by HAP1's interaction with microtubule-based motor complexes, such as dynactin p150 and kinesin light chain (Engelender, 1997; Li et al., 1998a; Gauthier et al., 2004; Kittler et al., 2004; McGuire et al., 2006; Rong et al., 2006). The pathophysiology of several neurodegenerative diseases is influenced by disruption of these transport mechanisms (Huang et al., 2015). Knockout (KO) mice studies have demonstrated that HAP1 plays a significant role in the maintenance of neurons and their postnatal survival, thereby demonstrating its involvement in brain development and formation of circuits (Chan, 2002; Li et al., 2003). Notably, its concentration in neuroendocrine and neuromodulatory regions implies that HAP1 supports the stability of the neurotransmitter system by regulating the intracellular signalling and receptor trafficking (Chan, 2002; Sheng et al., 2006; Xiang et al., 2014; Chen et al., 2020). For instance, hypothalamic HAP1 stabilizes the glucocorticoid receptor (GR) in the cytoplasm, influences the activity of GABAergic neurons which are involved in regulating stress responses, feeding, and is important for trafficking proteins in orexin neurons (Chan, 2002; Li et al., 2003; Sheng et al., 2006; Lin et al., 2010; Xiang et al., 2014; Chen et al., 2020).

There is growing evidence in support of the HAP1 protection hypothesis, according to which HAP1 is considered as a molecular shield against apoptosis and neurodegeneration (Fujinaga et al., 2004, 2009; Islam et al., 2017, 2022, 2025; Wroblewski et al., 2018; Tarif et al., 2023). Though neurodegenerative diseases, including HD, spinal and bulbar muscular atrophy, spinocerebellar ataxia, and Joubert syndrome, are selective in areas with low or absent HAP1 expression (striatum, thalamus, cerebellum, and motor nuclei). However, HAP1-rich regions, including the hypothalamus, preoptic area, and medial amygdala, may be resistant to loss of neurons and neurodegeneration (Li et al., 2003; Fujinaga et al., 2004, 2009; Takeshita et al., 2006, 2011; Metzger et al., 2008; Islam et al., 2017, 2022). These findings endorse the protection hypothesis and suggest that HAP1 is an essential regulator of neuronal homeostasis during pathogenic stress.

The brainstem raphe nucleus is the main source of the serotonergic system (5-hydroxytryptamine; 5-HT), which controls some critical physiological and behavioural functions, including feeding, stress response, mood regulation, circadian rhythm, and autonomic control (Jacobs and Azmitia, 1992; Hornung, 2003). Many forebrain and brainstem targets are innervated by highly branching axonal arbors that are extended from the serotonergic neurons in the dorsal and median raphe nuclei (Muzerelle et al., 2016). Because alteration in axonal branching or synaptic connection may impair serotonergic transmission and lead to altered stress reactivity, neuropsychiatric, and neurodegenerative phenotypes, this arborization pattern is critical for the modulatory effects of 5-HT (Gaspar and Lillesaar, 2012). Overall, 5-HT is a neurotransmitter that acts on the brain and spinal cord through its effect mediated by membrane receptors (Hannon and Hoyer, 2008). According to

earlier research, impairments in the cytoskeletal regulators and traffic proteins have a significant potential to change the density and patterns of the neuron fibres, thereby affecting the growth of neurites as well as the axonal transport and release of neurotransmitters (Buckley et al., 2000; Muñoz-Lasso et al., 2020; Alberti et al., 2022). HAP1 might be involved in all of these paths, and its absence might jeopardize the integrity of serotonergic neurons in raphe nuclei. Besides, HAP1 and 5-HT neurons are widely co-expressed in the adult mouse raphe nuclei, according to a recent immunohistochemistry study (Afrin et al., 2025), indicating a functional connotation between HAP1 and serotonergic neurons.

The first stage, "synthesis," is a crucial modulator of the many sequential processes that make up 5-HT neurotransmission (Walther and Bader, 2003). Specifically, the primary rate-limiting step in the production of 5-HT is catalysed by the brain isoform called tryptophan hydroxylase 2 (TPH2) that directly relates to stress regulation (Chen and Miller, 2012). Dysregulation of this TPH2 expression is associated with numerous neurodevelopmental and psychiatric disorders (Jahanshahi et al., 2011). Since HAP1 affects hypothalamic stress circuits (Chen et al., 2020), the lack of HAP1 may secondarily affect the 5-HT synthesis.

HAP1 is generally regarded as a neuroprotective factor that provides the long-term stability of neuronal circuits. Although there is growing evidence that ties the functions of HAP1 to stress-related signaling and neuroprotection, it is ambiguous how the deficiency of HAP1 affects the development and function of serotonergic neurons. Considering the significant expression of HAP1 in the raphe nuclei, we aimed to ascertain whether the absence of HAP1 induces changes in serotonergic neuronal structure in postnatal day 0 (P0) pups. By analyzing serotonin synthesis and fiber arborization patterns in HAP1-KO mice, we further intended to discover if HAP1 is important for the establishment of serotonergic circuits during early postnatal development. Consequently, our results may elucidate the significance of HAP1 in serotonergic network formation and its potential implication in diseases that may be associated with serotonin dysregulation.

4.2. Materials and methods

4.2.1. Animal rearing and generation of HAP1^{-/-} pups

HAP1-homozygous knockout (HAP1^{-/-}) C57BL/6J mice were obtained with the assistance of CRISPR-Cas9-based genome editing in the Department of Neuroscience, Graduate School of Medicine, Yamaguchi University, Japan. To generate HAP1^{-/-} mice, the gRNA1 and gRNA2 sites on exon-2 were chosen to delete DNA fragment (Figure 1A). A complex of gRNA and Cas9 was injected into C57BL/6J zygotes for homologous recombination, and resultant 2-cell embryos were

transplanted to recipient surrogate mothers. The gene sequence of founder mice was examined to confirm the occurrence of the frameshift in the exon-2 of HAP1. The resulting 8 weeks old male and female HAP1- heterozygous knock out (HAP1+/-) mice were then subjected to matings to produce homozygous or heterozygous offspring and wild-type (HAP1 +/+) littermates. These pups were genotyped using the PCR method, which amplified the mouse HAP1 DNA fragment flanking the targeted region isolated from tail biopsies using two primers: a forward primer (5'-GAGTCCAGTCTCACAATCTTTGTT-3') and a reverse primer (5'-GTCCAGATTGGTCCTCTGTAATCTA-3'). There was a match among all the animals used in the experiments in terms of age and weight. Mice were kept five per cage in a room with a 12:12-hour light/dark cycle (light on at 08:00), controlled temperature (21 ± 1 °C), and controlled humidity ($55 \pm 10\%$), and they were given feed and water *ad libitum*. Procedures were performed on animals, as per the Japanese Government Regulations of Animal Research (Law No. 105, Notification No. 6), and were given authorization by the Yamaguchi University Graduate School of Medicine Animal Ethics Committee.

4.2.2. Characterization of primary antibodies

The origins, immunogens, and working dilutions of the primary antibodies used in this study are listed in Table 1. The genetic ablation of HAP1 was verified by Western blot using goat polyclonal anti-HAP1 antibody (R19). A rabbit polyclonal anti-5-HT antibody was utilized in immunoperoxidase staining to map the serotonergic raphe nuclei. The specificity of these two commercially developed antibodies has already been confirmed in our earlier study (Afrin et al., 2025). Additionally, the level of the enzyme expression in 5-HT synthesis was assessed using a commercially produced rabbit polyclonal anti-TPH2 antibody, which was selected based on the Neuroscience Information Framework Antibody Registry and whose specificity has been documented in earlier research (Table 1).

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

Antibody	Immunogen	Host / Antibody type	Catalogue number / Research resource identifiers (RRIDs)	Source	Dilution	References
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	Islam et al., 2025
Anti-Serotonin (5-HT)	Serotonin covalently bound to bovine thyroglobulin with carboimide	Rabbit polyclonal	Cat# 066D, RRID: AB_2313879	Biomeda Corp. USA	1:5,000	Marya et al., 2025; Sheng et al., 2004; Yanai et al., 2020
Anti-TPH2	recombinant monoclonal targets TPH2	Rabbit monoclonal	Cat# ab184505, RRID: AB_2892828	Abcam, Japan	1:10,000	(Cheung et al., 2023)
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	Tarif et al., 2023

HAP1, Huntingtin-associated protein-1; TPH2, Tryptophan hydroxylase-2.

4.2.3. Western blot analysis

Western blotting was conducted in accordance with the protocols that were already established in our previous studies (Islam et al., 2017, 2020a; Afrin et al., 2025). The P0 mouse brains (3 replicates per genotype) were taken right after transcardial perfusion with ice-cold saline. Homogenization of brain tissues was done using T-PER buffer (Thermo Scientific) containing protease inhibitors (Sigma-Aldrich), followed by the determination of protein concentrations by the BCA assay. Equal parts of proteins (80 ug) were loaded on 7.5% SDS-PAGE gels and transferred to polyvinylidene difluoride membrane by a wet transfer system. It was followed by the blockage of membranes in 5% milk/Tris-buffered saline with 0.1% Tween-20 (TBST) at 20°C and overnight incubation at 4°C with primary antibodies against HAP1 (1:20,000), TPH2 (1:10,000), or α -tubulin (1:200,000) (Table 1). Membranes were washed with TBST and then incubated with HRP-conjugated anti-goat (1:10,000), anti-rabbit (1:5,000), and anti-mouse (1:20,000) antibodies for 2 h at 20°C (Table 2). ECL (GE Healthcare) was used to visualize the immunoreactive bands and imaged on an Amersham Imager 600. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for densitometric quantification of TPH2 to α -tubulin in brain lysate of HAP1^{+/+} and HAP1^{-/-} mice.

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

Secondary antibody and other reagents	Immunogen	Host	Catalogue number / Research resource identifiers (RRIDs) / 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	Marya et al., 2025 Islam et al. 2025
HRP-labelled anti-rabbit	Rabbit IgG F(ab') ₂	Donkey	Cat# NA934, RRID: AB_772206 Cytiva, Sigma-Aldrich, Germany.	Western blotting	1:5,000	Islam et al., 2022
HRP-labelled anti- mouse	Mouse IgG whole molecule	Sheep	Cat# NA931, RRID: AB_772210 GE Healthcare, Buckinghamshire, UK.	Western blotting	1:20,000	Meher et al., 2025a
Biotinylated goat anti-rabbit	IgG, isolated from rabbit serum	Goat	Cat# AP180B, RRID: AB_92569 Millipore, Darmstadt, Germany.	Immunoperoxidase staining	1:1,000	Meher et al., 2025b
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	Tarif et al., 2023
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	Islam et al., 2020a
HRP-conjugated streptavidin	N/A	N/A	Cat# P0397, Agilent Dako, Glostrup, Denmark.	Immunoperoxidase staining	1:1,000	Yanai et al., 2020

HRP, Horseradish peroxidase; IgG, Immunoglobulin G.

4.2.4. Tissue preparation and sectioning for immunohistochemical analysis

P0 mice were first placed under mild hypothermic anesthesia, and ice-cold saline was perfused transcardially and thereafter fixed using 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Then brains were carefully dissected and immersed in the same fixative for 24 h at 4°C. Following the fixation step, samples were cryoprotected by soaking them in 30% of sucrose dissolved in 0.1 M PB until the tissue sank to the bottom completely. The forebrain (bregma 1.11 to 4.71 mm), brainstem rostral and caudal clusters (bregma 4.83 to 7.59 mm) were sectioned serially along coronal and transverse line to the rhombomeres at 40 µm thickness in a cryostat (Leica CM1900; Leica Microsystems, Germany). The sections of the brainstem were kept at 4°C in 0.02 M phosphate-buffered saline (PBS; pH 7.4) with 0.1% sodium azide prior to immunohistochemical analysis.

4.2.5. Immunoperoxidase staining

The immunoperoxidase staining was performed on free-floating brainstem sections of P0 mice according to already set-out procedures (Afrin et al., 2025; Islam et al., 2025; Meher et al., 2025a, 2025b). In brief, tissue sections were blocked and permeabilized for 2 h at 4°C in PBS that contained 10% normal goat serum (NGS) and 0.3% Triton X-100. Pretreatments of sections were then carried out using 50% cold methanol and 1.5% hydrogen peroxide over a period of 30 min at 4°C to quench endogenous peroxidase activity. Sections were washed and incubated with 1:7000 rabbit polyclonal anti-5-HT antibody for 7 days at 37°C in PBS with 1% NGS and 0.3% Triton X-100. Sections were then rinsed and incubated overnight at 4°C with biotinylated goat anti-rabbit IgG (1:1000) in PBS containing 1% NGS. After that, sections were incubated for 2 h at 20°C with peroxidase-conjugated streptavidin (1:1000; Dako, Glostrup, Denmark). Following extensive washing in 0.05 M Tris-HCl buffer (pH 7.6), immunoreactivity was detected by a nickel-enhanced DAB reaction (0.02% DAB, 0.6% nickel ammonium sulfate, and 0.0008% hydrogen peroxide in 0.05 M Tris-HCl) for about 10 minutes on ice (very chilled condition). The stained sections were placed on glass slides, allowed to air dry, dehydrated using graded ethanol, cleared using xylene, and cover-slipped using Entellan Neu (Merck KGaA, Darmstadt, Germany). Tables 1 and 2 contained the list of antibodies and reagents.

4.2.6. Immunofluorescence staining

The co-expression of HAP1 and 5-HT in the brainstem sections was visualized by utilizing the established steps and procedures of the previous studies (Afrin et al., 2025; Islam et al., 2025; Meher et al., 2025a, 2025b). Tables 1 and 2 contained the details of antibodies and reagents. Concisely, 2 h incubation of sections was performed in PBS diluted blocking solution (10% normal donkey serum, NDS, and 0.3% Triton X-100) at 4°C. Incubation was done with the addition of goat anti-HAP1 (R19, 1:6000) and rabbit anti-5-HT (1:5000) primary antibodies diluted in 1% NDS and 0.3% Triton X-100

in PBS for 7 days at 37°C. Sections were then washed and incubated with the Alexa Fluor 594-conjugated donkey anti-goat IgG and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:1000; Molecular Probes, Eugene, OR, USA) over 2 h at 20°C. Lastly, PBS was used to rinse sections, after which they were placed on glass slides, air-dried, and surface-mounted using Fluoromount Plus (K048, Diagnostic Biosystems, Pleasanton, CA, USA). Slides were refrigerated at 4°C in a dark slide box until their imaging.

4.2.7. Cytoarchitectonic analysis and terminology

The cytoarchitectonic assessment was completed using Nissl staining (cresyl violet; Merck) of sections neighboring or adjoining those stained for 5-HT in P0 wild mice. The 5-HT raphe neurons are well organized and topographically distributed along the caudal to rostral axis of the hindbrain as different raphe subnuclei (Dahlstroem and Fuxe, 1964). Along the midline of the brainstem, raphe nuclei are allocated based on their distribution into the rostral and the caudal cluster (Afrin et al., 2025). Recent genetic studies defined the rhombomeric origin of the different raphe nuclei (Jensen et al., 2008) and provided a useful description of the 5-HT axon projections arising from the different rhombomeres (Bang et al., 2012), that was subsequently adapted in our study. Terminology for the entire raphe nuclei area in the P0 wild-type pup's brainstem was confirmed by previous research (Alonso et al., 2013) and then approved from the developing mouse brain atlas (Paxinos et al., 2020), which was followed in our current study.

4.2.8. Quantification of immunoreactive neurons

Sections of P0 HAP1^{+/+} mice (n = 6) stained with immunofluorescence were visualized with a 40x objective lens to determine the cellular co-localization. Photomicrographs were obtained with the same exposure conditions and then imported to ImageJ software, where they were quantitatively analyzed using the labeled cells. The total numbers of HAP1 or 5-HT- immunoreactive (ir), and HAP1/5-HT double-labeled neurons were quantified to determine the proportion of HAP1 in 5-HT, or 5-HT in HAP1 (Table 3). The standardized counting procedure as outlined in our earlier work was used to compute co-expression ratios (Afrin et al., 2025); $TN = N \times D / \{(T + W) \times S\}$ [TN, total number of 5-HT in HAP1-ir cells or HAP1 in 5-HT cells in individual raphe nuclei of rostral and caudal clusters; N, 5-HT or HAP1-ir neurons along with HAP1 and 5-HT double-stained neurons observed in sections; D, distance between the first and last sections in which raphe nuclei were counted (μm); T, section thickness (40 μm in the present study); W, mean diameter of the raphe nuclei (6 μm for the mouse in the present study); S, number of sections in which distinct raphe nuclei were counted. In addition, immunoperoxidase stained sections from P0 HAP1^{+/+} and HAP1^{-/-} mice (n = 6 per genotype) were taken using a 20× objective lens. Then, the 5-HT immunoreactive neuronal

population were manually counted within the definite anatomical limits of the raphe nuclei. Each nucleus in the rostral and caudal cluster of raphe nuclei was counted from at least three sections of a single pup without an 80 μm closure in between sections. After that, the somatic volume of 5-HT cells was measured using ImageJ/ morphometric tracing in 2D images with the following formula:

$$\text{The estimate cell (soma) volume: } V_{\text{cell}} = \frac{4}{3} \pi \left(\frac{d_1}{2}\right) \left(\frac{d_2}{2}\right) \left(\frac{d_3}{2}\right)$$

d_1 = major diameter in the XY plane

d_2 = minor diameter in the XY plane

d_3 = vertical diameter (serial section thickness, 40 μm)

4.2.9. Statistical analysis

In each of the experiments, the samples of the transgenic and wild type mice were processed simultaneously. The mean and standard deviation of means (SD) are used to present the data. Each animal ($n = 6$ for each genotype) used in the statistical analysis was selected according to previous published studies in the brainstem raphe nuclei instead of the formal calculation of the sample size (Afrin et al., 2025). For the computation and graphical representation, data was imported into MS Excel (Microsoft Office Excel 2019, USA). The quantitative data were analyzed using two-way ANOVA and statistically significant differences were evaluated further by a Tukey's post hoc test. All statistical analyses were performed with SPSS 25.0 version for Windows. P -values <0.01 or <0.05 were deemed significant in every assessment. Note that the Western blot and immunohistochemistry for each replication were carried out independently at separate times. Therefore, the baseline of the optical density signal differs in different sets of experiments.

4.2.10. Microscopic imaging

The light microscopic observations were conducted using the Eclipse E80i photomicroscope (Nikon, Tokyo, Japan) that had a Lumenera color digital USB 2.0 camera (Lumenera Corporation, Ottawa, Canada). The image tiling software (Mitani Corporation, Tokyo, Japan) was employed to produce high-resolution composite images that will cover the entire area of interest. A confocal laser-scanning microscope (STELLARIS 8, Leica Microsystems, Wetzlar, Germany) was used to obtain fluorescence images under the operation of LAS X software. Individual optical sections were captured with a resolution of 4296 x 4296 pixels using the same acquisition parameters for all specimens to guarantee comparability. The photomicrographs were all processed equally. There were no structural changes, with only slight adjustments in brightness and contrast done in Adobe Photoshop CS 8.0 (Adobe Systems, San Jose, CA, USA) to augment the overall image clarity to prepare the figures.

4.3. Results

Serotonin neurons in distinct parts of the raphe nuclei have a very particular cytoarchitectonic layout reflecting their unique connection patterns that disclose function and their developmental origin. In our study, we had performed genotype-dependent comparisons within a uniform genetic context; we employed postnatal day 0 (P0) wild-type (HAP1^{+/+}), HAP1-homozygous knockout (HAP1^{-/-}), and HAP1-heterozygous knockout (HAP1^{+/-}) littermates in all immunohistological, morphometric, and biochemical investigations.

4.3.1. Phenotypic characterization and validation of HAP1^{-/-} pups

We accomplished CRISPR-Cas9-mediated genome editing to produce HAP1^{-/-} pups for the purpose of clarifying the functional importance of HAP1 in serotonergic neurons (Figure 1A). The deletion of the HAP1 gene in HAP1^{-/-} pups was confirmed by Western blot analysis of brain lysate, and the gene-dependent changes in protein expression were indicated by the fact that a reduced band intensity was observed in HAP1^{+/-} pups in contrast to the wild-type littermates (Figure 1B). HAP1A and HAP1B isoforms (approximately 80 kDa and 90 kDa, respectively) were identified in the HAP1^{+/+} and HAP1^{+/-} pups by the use of goat polyclonal anti-HAP1 (R19) antibody (Figure 1B). Genotyping using PCR was done before analysis to verify each genotype. Remarkably, there were no discernible variations in body weight and gross morphology between HAP1^{+/+}, HAP1^{-/-}, and HAP1^{+/-} littermates just after birth (Figure 1C, D). The identical neonatal body weights in the genotypes suggest that gross prenatal growth, and survival before delivery were not affected in the HAP1-deficient mice. Nevertheless, HAP1^{-/-} neonates died within 24 hours of birth, although they could be morphologically normal at birth, suggesting that HAP1 is necessary to survive after birth (Figure 1E). HAP1 heterozygous mice, on the other hand, showed no noticeable phenotypic anomalies in their adulthood. Though, from one week after birth to four weeks, delayed growth and locomotor activity were observed. After that, the mice slowly started growing at the same rate as their littermates.

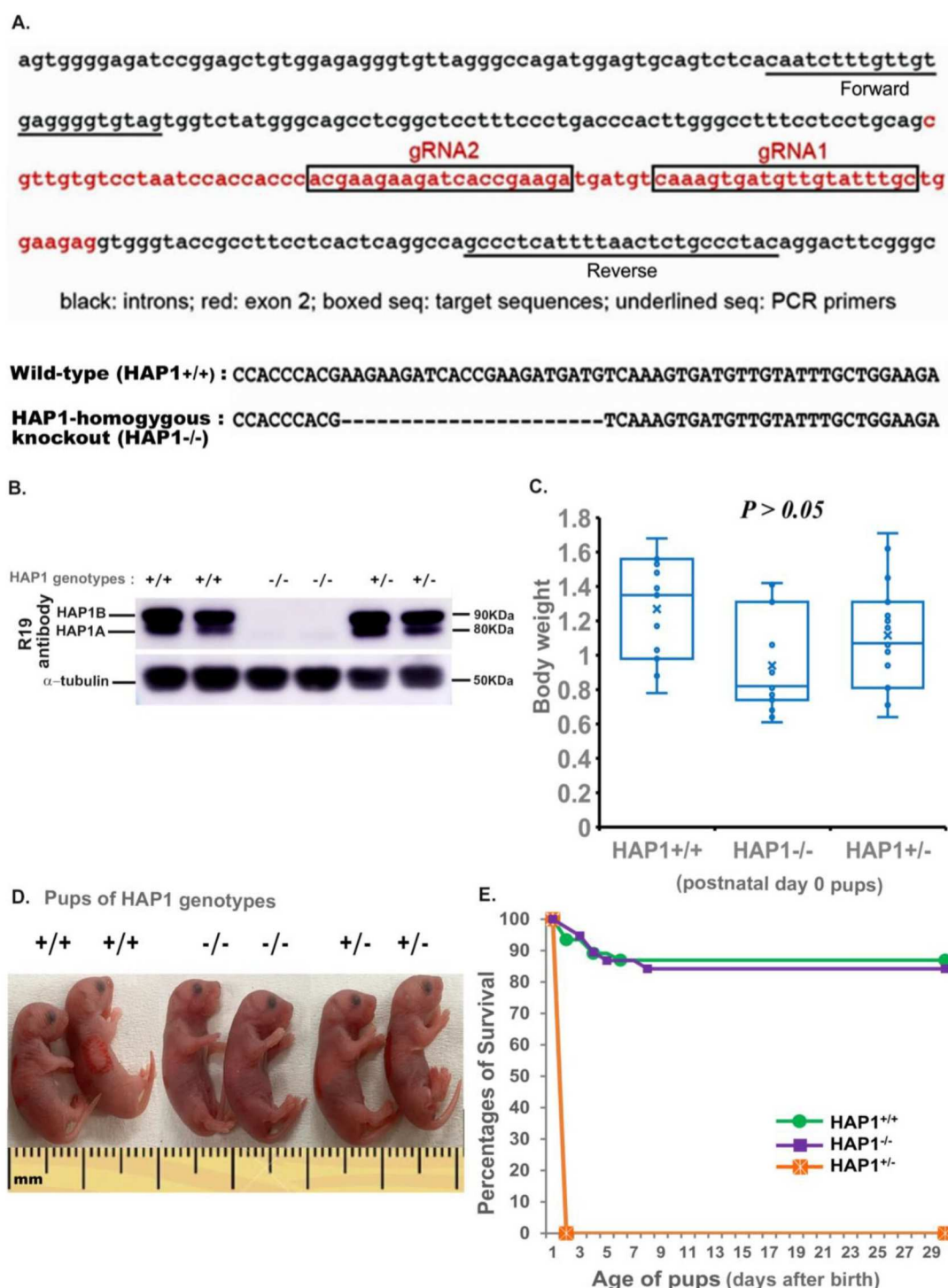


Figure 1. CRISPR-Cas9 genome editing, genotypic validation and phenotypic characterization of HAP1 genotypes. (A) Schematic presentation depicts the target sequences (boxed seq.) for genome editing in order to generate HAP1-homozygous knockout (HAP1^{-/-}) pups. Pups were then genotyped using PCR amplification of the mouse HAP1 genomic region flanking the edited locus using the forward and reverse primers (underlined seq.) (B) Western blot analysis demonstrated the HAP1 R19 and α -tubulin immunoreactive (ir) bands in brain lysate of P0 wild pups. Disappearance of HAP1-ir band supported the effective deletion of HAP1 gene in HAP1^{-/-} pups (C) Representative images showing the gross morphologically alike neonates in all HAP1 genotypes (D) Body weight quantification just after birth revealed no significant differences among HAP1^{+/+}, HAP1^{-/-}, and HAP1^{+/-} littermates (E) Survival percentage quantification suggested that all HAP1^{-/-} neonates died within 24 hours of birth, even though their corresponding littermates survived past the first postnatal day.

4.3.2. Neuroanatomical distribution of serotonergic neurons in the rostral and caudal cluster of raphe nuclei in P0 wild pups

P0 wild mouse encompassed central serotonergic cell groups in the brainstem raphe nuclei. These 5-HT cells were arranged into discrete rostro-caudal cell clusters with clear cytoarchitectonic boundaries along the rhombomeric (r) segments (Figures 2A-F; 3A-G). The immunoperoxidase studies of the raphe nuclei showed that the types of neurons expressing 5-HT had large, medium, or tiny soma (Figures 6-10; A, C, E, G).

The midbrain dorsal raphe nucleus (mDR) was the primary serotonergic region of the dorsal raphe (DR) at the rostral pole within the rostral cluster (Figures 2A-E). The isthmic dorsal raphe nucleus (isDR) was found in the caudal midbrain (Figures 2C-F; 3A). Rostrally, it was also subdivided into dorsal (isDRd), ventral (isDRv), lateral (isDRl), and wing (isDRw) parts of raphe nuclei (Figures 2C-F; 3A). DR was further segmented into dorsal (DRd), ventral (DRv), and interfascicular (DRi) parts at the level of r1, caudal to the midbrain (Figures 3B-C). The 5-HT neurons were strongly labeled and widely expressed in the DR (Figures 7A, C, E, G; 8E, G). On the contrary, the caudal linear nucleus (CLi) was smaller in terms of serotonergic population (Figures 2A-D; 8A), which complements the isDR complex. It had also a laterally scattered wing (CLiw) part (Figures 2A-E; 8C). The median raphe nucleus (MnR), which had rostral (MnRr) and caudal (MnRc) subdivisions, was present immediately ventral to the DR (Figures 2A-F; 3A). 5-HT neurons were dispersed irregularly in the MnR (Figures 2A-F; 3A). The MnRr (Figures 9A) was slightly larger and had more 5-HT neurons than its caudal counterpart (Figures 9C). These central groups were accompanied by a large ventrolaterally relocated serotonergic aggregate (supralemniscal raphe nucleus, SuL) that spans the rhombomeres r1 to r3 (Figures 2A-B; 6A, C). Hence, SuL 5-HT neurons had an irregular and scattered spatial distribution without classical nuclear allocation (Figures 2A-B). They were situated laterally of the interpeduncular nucleus (IPR and IPC) and immediately adjacent to the trigeminal lemniscus decussation (Figures 2A-B). On the other hand, the paramedian raphe nucleus (PMnR) lacked 5-HT neurons (Figures 2A-F; 3A).

The serotonergic populations of the prepontine raphe nucleus (PPnR) were positioned at the dorsal of the trigeminal lemniscal decussation in the r2 area of the rostral pons and proximally caudal to the MnR (Figures 2B-E). The PPnR was spanned in the median plane relative to the pontine rhombomere (r3) and abundantly expressed 5-HT (Figures 2B-E; 9E). Moreover, rostral pons includes pontine raphe nucleus (PnR). PnR is described as a tiny, discrete, radially extended cellular aggregation/column immediately caudal to the PPnR (Figures 2D-F). PnR had also expanded across the r4 (Figures 3A-B). In PnR, 5-HT cell bodies were dispersed and generally flanked on either side of the median plane (Figures 9G). A high number of 5-HT cells were accumulated at its caudal limit.

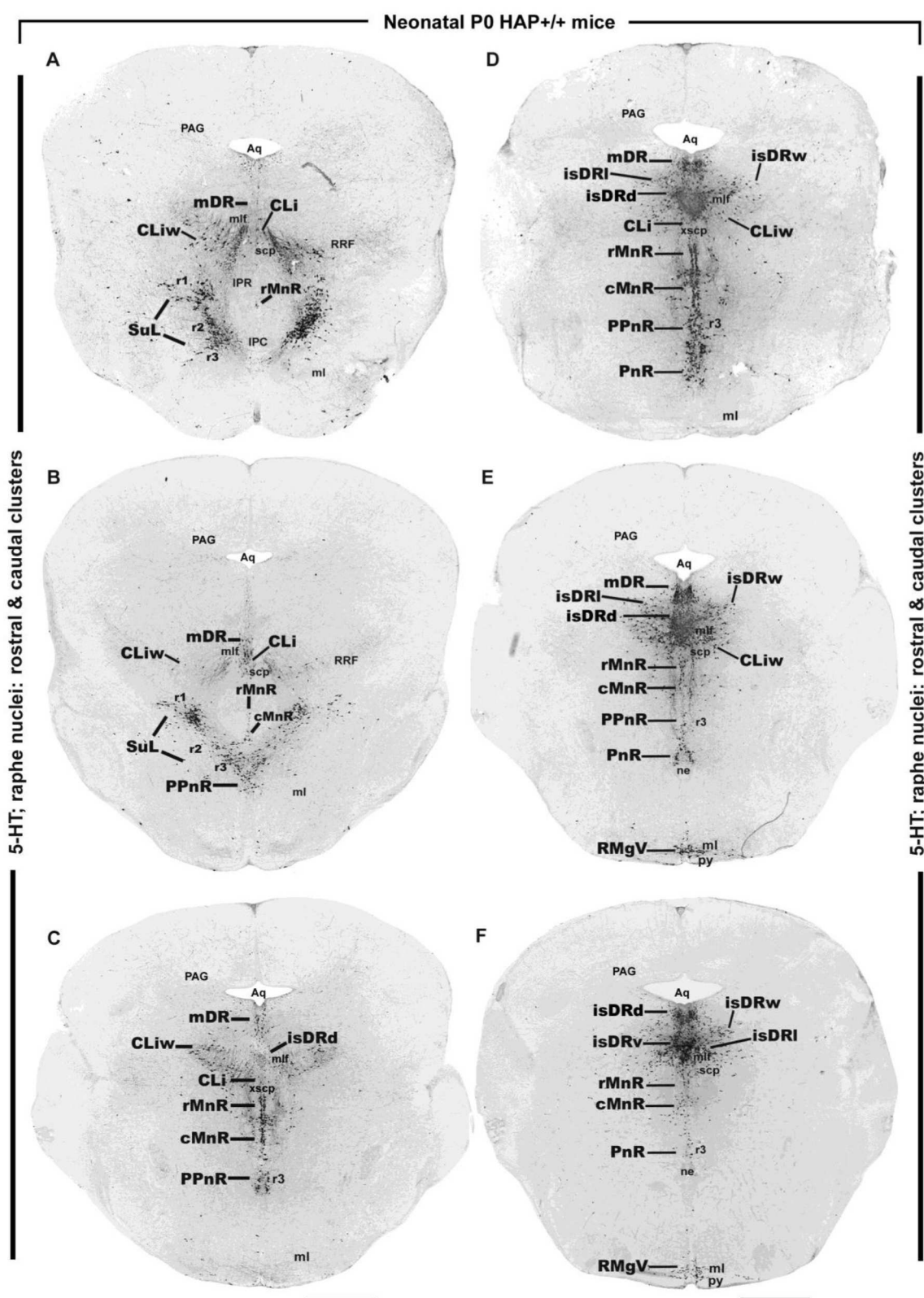


Figure 2. Distribution of 5-HT immunoreactivity revealed by immunoperoxidase staining across the midbrain–pons continuum in P0 wild pups. (A–F) Representative photomicrographs from six nonconsecutive coronal brainstem sections illustrating the topographical organization of serotonergic neurons at birth. Immunoperoxidase staining for 5-HT delineates the major raphe nuclei and associated serotonergic projections extending from the midbrain and pontine regions. Some key selective anatomical structures were highlighted to facilitate locating between rostrocaudal levels. Scale bar = 125 μm. For abbreviations, see “List of Glossary.”

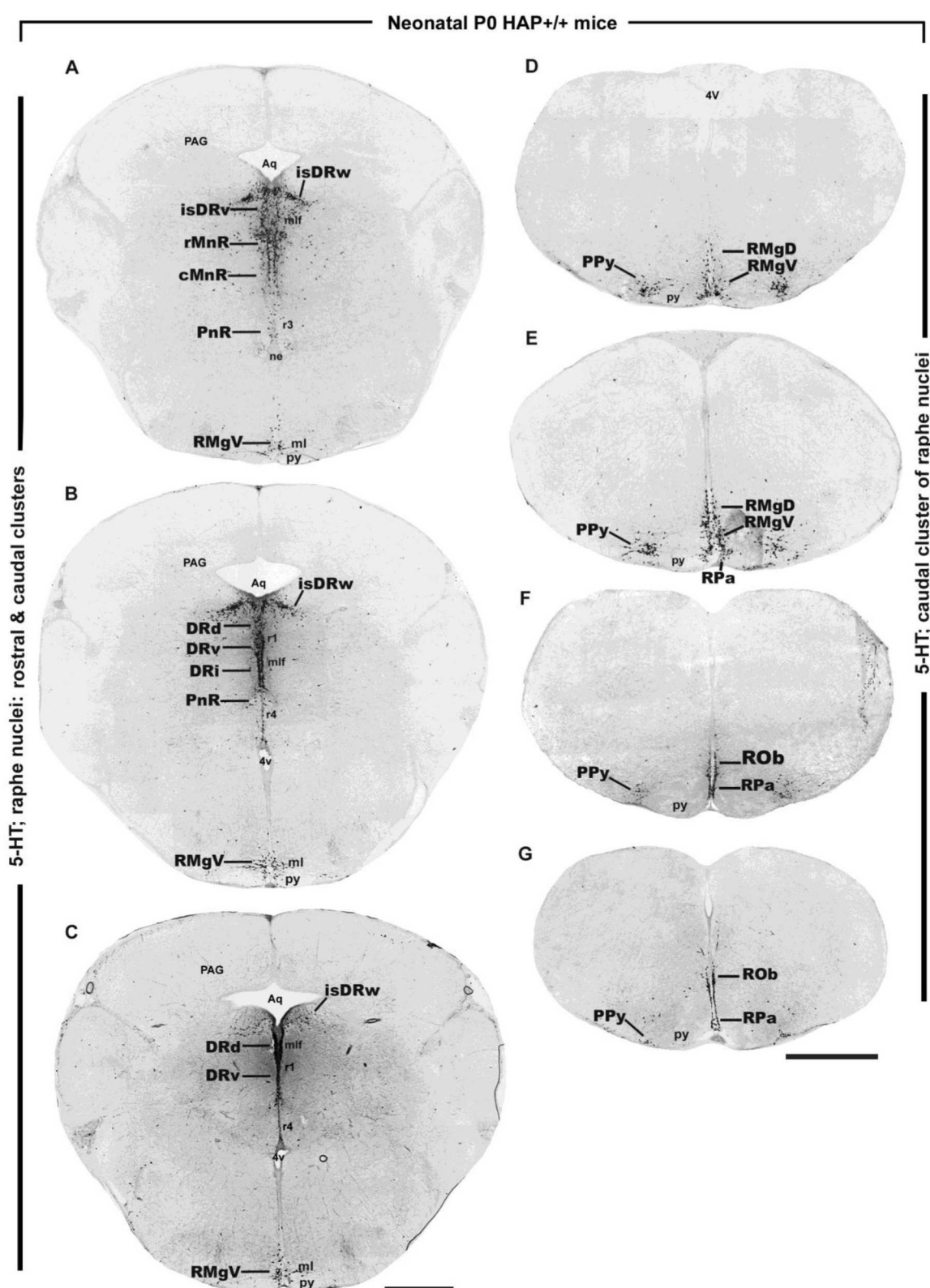


Figure 3. Distribution of 5-HT immunoreactivity revealed by immunoperoxidase staining across the pons-medulla continuum of P0 wild pups. (A-G) Representative photomicrographs from seven nonconsecutive sections through the brainstem (these are first coronal and then transversal to the rhombomeres) showing the topographical organization of serotonergic neurons at birth. **(D-G)** through retropontine and medullary hindbrain (transverse sections). Immunoperoxidase staining for 5-HT delineates the major raphe nuclei and associated serotonergic projections from the pontine and the medullary regions. Some key selective anatomical structures were highlighted to facilitate locating between rostrocaudal levels. Scale bar = 125 μ m. For abbreviations, see “List of Glossary.”

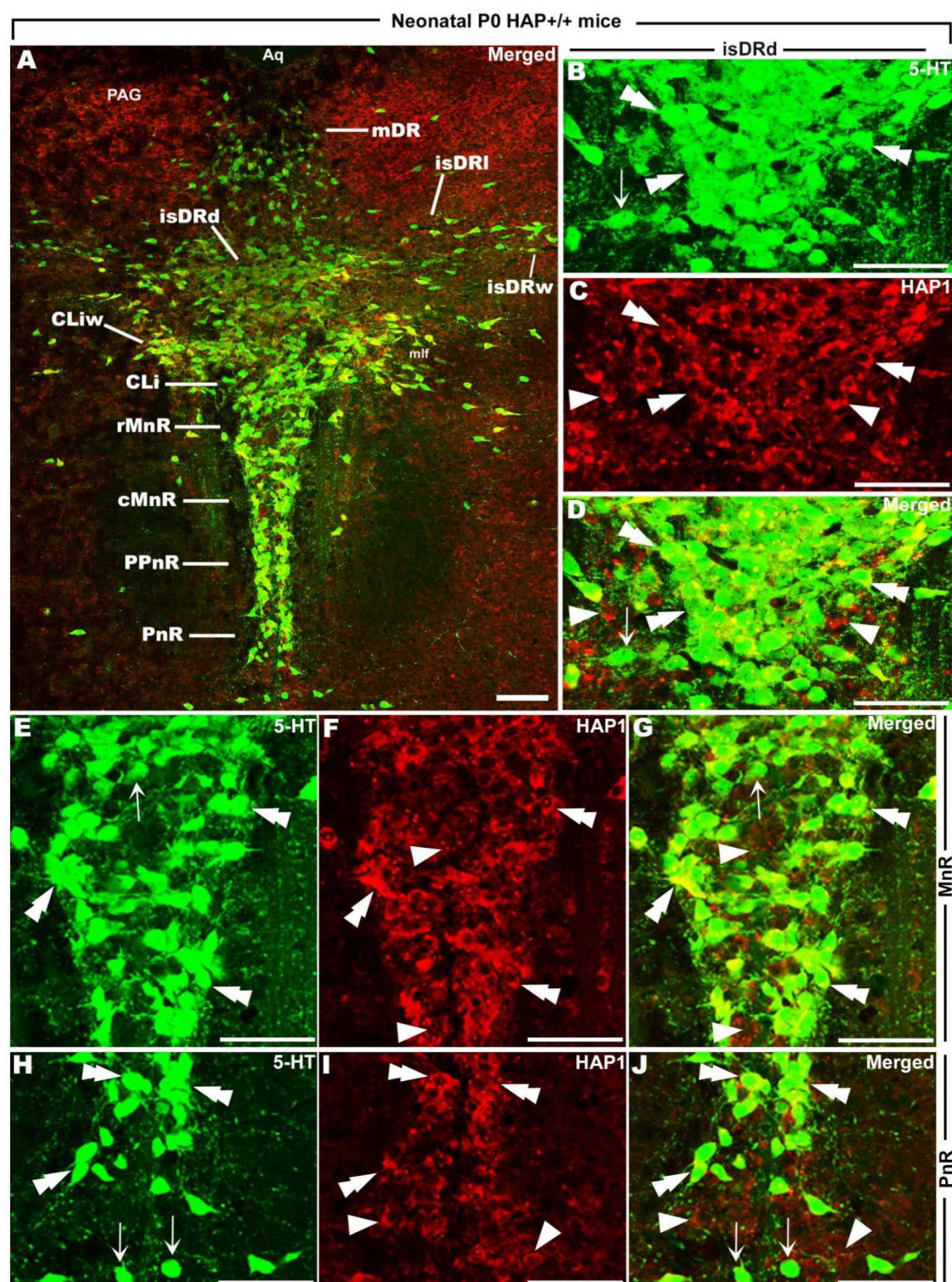


Figure 4. Double-label immunofluorescent immunohistochemistry of 5-HT and HAP1 in the rostral cluster of brainstem raphe nuclei in P0 wild pups. (A) Representative photomicrograph of dual immunofluorescence showing 5-HT and HAP1 immunoreactivity in the region of the caudal midbrain. (B–J) Higher-magnification images from the isthmus dorsal raphe dorsal nucleus (isDRd), median raphe nucleus (MnR), and pontine raphe nucleus (PnR) corresponding to panel (A), illustrating cellular colocalization patterns. Double arrowheads indicate neurons exhibiting co-expression of HAP1 and 5-HT, whereas white arrows and single arrowheads identify neurons immunopositive only for 5-HT or only for HAP1, respectively. Scale bar = 20 μ m in (A–J). For abbreviations, see “List of Glossary.”

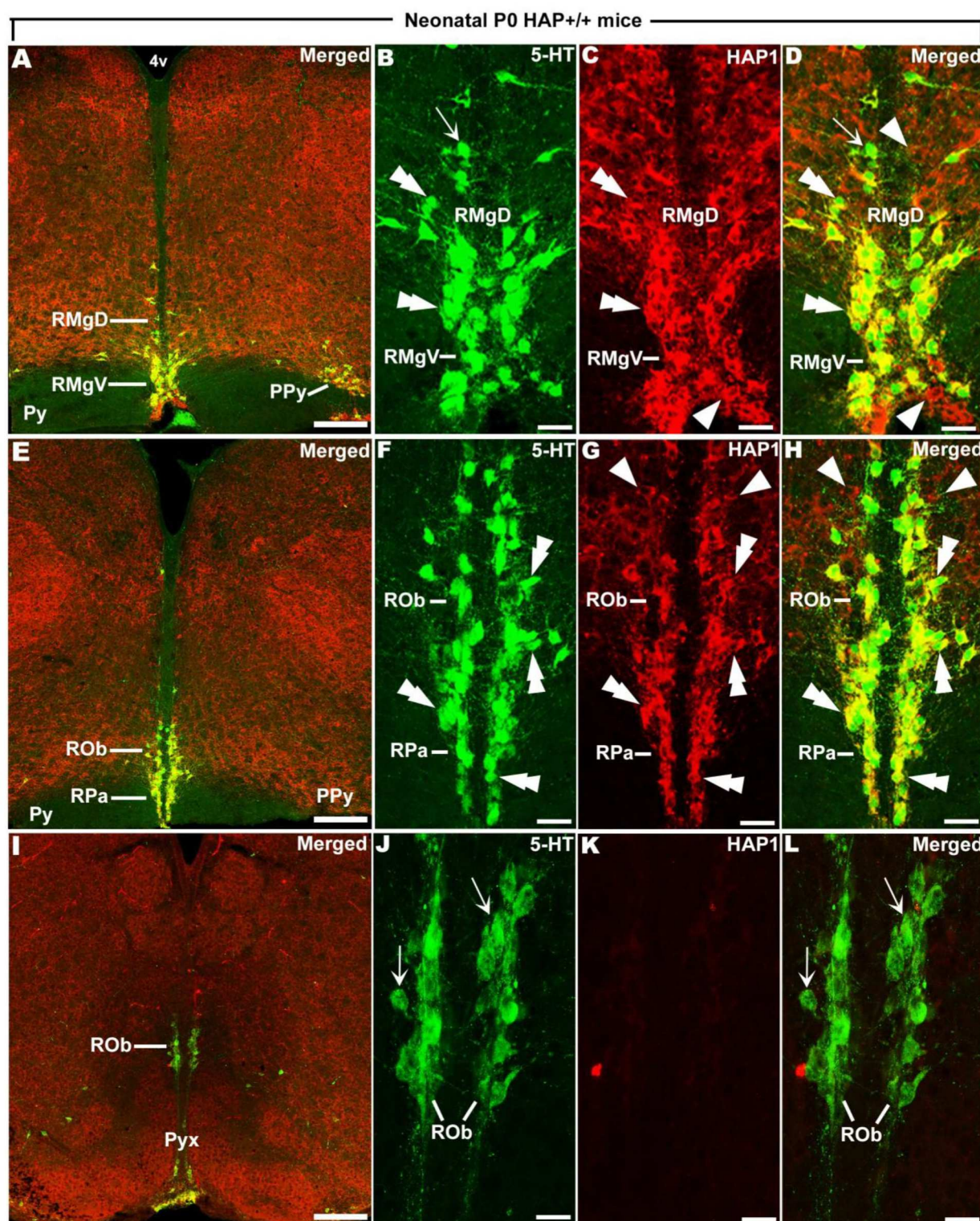


Figure 5. Double-label immunofluorescent immunohistochemistry of 5-HT and HAP1 in the caudal cluster of brainstem raphe nuclei in P0 wild pups. (A, E, I) Representative photomicrographs of the immunofluorescence for 5-HT and HAP1 in the caudal pons and medulla. Higher-magnification images from the (B–D) raphe magnus (RMg), (F–H; J–L) raphe obscurus (ROb) and (F–H) raphe pallidus (RPa) nuclei corresponding to panel (A, E, I) respectively, illustrating cellular colocalization patterns. Double arrowheads indicate neurons exhibiting co-expression of HAP1 and 5-HT, whereas white arrows and single arrowheads identify neurons immunopositive only for 5-HT or only for HAP1, respectively. Scale bar = 20 μ m in (A–J). For abbreviations, see “List of Glossary.”

The 5-HT raphe nuclei of the caudal cluster (Figures 3D-G) consisted of the raphe magnus nucleus (RMg), raphe obscurus nucleus (ROb), raphe pallidus nucleus (RPa), and the laterally dispersed parapyramidal raphe nucleus (PPy). There were no 5-HT neurons in the raphe interpositus nucleus (RIP) (Figures 3D-E).

The RMg was extended over the pons (Figures 2E-F; 3A-E), where the high density of ventral serotonergic cells was distinguished by more scattered dorsal cells (Figures 3D-E). Further, there was a ventrolateral continuation of the parapyramidal raphe nucleus (PPy) (Figures 3D-E).

The ROb and RPa nuclei were part of the medulla and spanned across it (Figures 3E-G). RPa was found more ventrally along the midline and directly above the pyramidal tract (py), with ROb found in the intermediate stratum (Figures 3F-G). Caudally, 5-HT neurons of ROb progressively formed two parallel vertical laminae adjacent to the midline (Figures 3G). Lateral extension of serotonergic cells that enclose the pyramidal tract formed the medullary PPy (Figures 3F-G). There were intensely stained, but with a considerably smaller unequal distribution of 5-HT neurons in the RMg (Figures 10A), ROb (Figures 10C), and RPa (Figures 10E). Also, the PPy contained a small cluster of 5-HT neurons (Figures 10G).

The neuroanatomical distribution of serotonergic neurons across the rostral and caudal clusters of the raphe nuclei was demonstrated by our immunoperoxidase results. To further investigate the immunohistochemical associations between HAP1 and 5-HT in P0 HAP1^{+/+} pups, we subsequently performed double-label immunofluorescent immunohistochemistry (Figures 4A-J; 5A-L).

4.3.3. Co-localization of HAP1 with serotonergic neurons in the brainstem raphe nuclei of P0 wild pups

Double immunofluorescence labeling with anti-HAP1 and anti-5-HT antibodies demonstrated extensive co-expression of HAP1 and 5-HT throughout the rostral (Figures 4A-J) and caudal (Figures 5A-L) clusters and its substantial ratio in both clusters of the raphe nuclei (Table 3).

In the caudal midbrain from the rostral cluster nuclei, the co-expression of HAP1/5-HT was high across most nuclei, particularly in the CLi (97%), SuL (97.15%), and MnR (94.57%). Other nuclei within the caudal midbrain, such as the DR maintain a relatively high co-expression, with ratios of HAP1/5-HT ranging from 93.78%. Meanwhile, 5-HT/HAP1 co-expression was slightly lower in these regions, with the MnR showing 70.55% and DR, 85.76%. However, in the case of the PMnR, a lack of co-expression in either direction is significant, as indicated by a value of 0.

Table 3. Co-expression ratios of HAP1/5-HT or 5-HT/HAP1 in the brainstem raphe nuclei of P0 wild (HAP1+/+) pups.

Brainstem area		Raphe Nucleus	Mean $\pm$ SD		LS (Student t test)
			HAP1/5-HT ratio (%)	5-HT/HAP1 ratio (%)	
Midbrain	Rostral cluster	Median raphe (MnR)	94.57 ^{cd} $\pm$ 0.51	70.55 ^e $\pm$ 1.35	**
		Paramedian raphe (PMnR)	0 [#]	0 [#]	N/A
		Supralemniscal raphe (SuL)	97.15 ^a $\pm$ 0.57	90.7 ^c $\pm$ 1.28	**
		Dorsal raphe (DR)	93.78 ^d $\pm$ 0.87	85.76 ^e $\pm$ 0.74	**
		Caudal linear raphe (CLi)	97 ^a $\pm$ 0.68	97.84 ^a $\pm$ 0.89	NS
		Prepontine raphe (PPnR)	95.38 ^{bcd} $\pm$ 0.79	96.25 ^a $\pm$ 1.91	NS
Rostral pons		Pontine raphe (PnR)	93.83 $\pm$ 0.67	97.24 ^{ab} $\pm$ 1.14	**
Caudal pons	Caudal cluster	Raphe magnus (RMg)	93.74 ^d $\pm$ 0.64	91.23 ^c $\pm$ 1.07	**
		Raphe interpositus (RIP)	0 [#]	0 [#]	N/A
Medulla		Raphe obscurus (ROb)	95.96 ^b $\pm$ 0.42	95.33 ^b $\pm$ 2.35	NS
		Raphe pallidus (RPa)	95.35 ^{bc} $\pm$ 0.72	81.33 ^f $\pm$ 1.02	**
		Parapyramidal raphe (PPy)	96.04 ^b $\pm$ 1.6	89.24 ^d $\pm$ 1.17	**
LS (One way ANOVA followed by Tukey's test)			**	**	

Values represent the Mean $\pm$ SD of co-expression ratios (%) in adult mice ($n = 6$). ^{a,b,c,d,e,f,g} Denote the means within same column with different superscript letters differ significantly. **, Significant at 1% ($p < 0.01$); [#] No co-expression, 5-HT immunoreactivity was absent in both PMnR and RIP nuclei; N/A, not applicable; NS, non-significant; LS, Level of significance; SD, Standard deviation; 5-HT, Serotonin; HAP1, Huntingtin-associated protein 1.

Moving to the rostral pons, the PPnR had a high ratio for both markers (HAP1/5-HT: 95.38%, 5-HT/HAP1: 96.25%), thus further confirming a significant co-expression of these two markers in this rostral pons region. The PnR had exhibited a similarly high ratio of 5-HT/HAP1 (97.24%), but its HAP1/5-HT ratio was somewhat smaller (93.83%), suggesting the presence of a greater population of 5-HT neurons expressing HAP1.

In the caudal cluster of the caudal pons, RMg showed co-expression ratios of HAP1/5-HT (93.74%) & 5-HT/HAP1 (91.23%) which suggested high degree of overlap. In contrast, in the RPI, there was a lack of co-expression, which indicates that there was no significant interaction between these markers in this particular nucleus.

Finally, in the medulla, the ROb and RPa displayed the same ratio of co-expression, especially in HAP1/5-HT (95%). However, 5-HT neurons of ROb in the caudal part of the medulla did not have co-expression with HAP1. Though RPa and PPy showed high co-expression ratios of HAP1/5-HT but comparatively lesser ratios of 5-HT/HAP1 (81.33% & 89.24%, respectively).

Collectively, HAP1/5-HT co-expression was consistently high in most raphe nuclei in both rostral and caudal clusters, notably in the CLi, SuL, and PPy, suggesting a robust presence of HAP1 in serotonergic neurons. However, 5-HT/HAP1 co-expression ratios were commonly lower than HAP1/5-HT ratios, notably in the MnR and RPa, which suggested that although HAP1 expression was widespread in serotonergic neurons, not all HAP1-expressing neurons were serotonergic. Nuclei like the PMnR and RIP exhibited an absolute absence of co-expression, suggesting region-specific heterogeneity of the interaction between 5-HT and HAP1. These robust HAP1/5-HT co-expression patterns indicated that HAP1 could be a constitutive component within raphe 5-HT neurons at an early postnatal developmental stage. These results have identified the particular brainstem raphe nuclei areas where HAP1 may have a crucial neuroprotective impact in serotonergic neurons.

4.3.4. Morphological alterations of serotonergic neuronal soma in HAP1^{-/-} pups

The number of 5-HT-positive neurons in HAP1^{-/-} pups across the raphe nuclei was decreased, but there was no significant difference between HAP1^{+/+} and HAP1^{-/-} pups in the rostral and caudal clusters of raphe nuclei (Figure 12A). HAP1^{-/-} pups showed a significant variation in cellular shape with smaller, more compact cell bodies (Figures 6-10; B, D, F, H) even though there were no obvious alterations in the quantity of 5-HT neurons. The somatic volume of HAP1^{-/-} pups' serotonergic neurons was significantly lower in the rostral (Figure 12C) and caudal (Figure 12CD) clusters of raphe nuclei than that of wild-type littermates, according to morphometric analysis. This alteration revealed that HAP1-deficient pups may have somatic atrophy or altered cellular geometry, which may be brought about by disrupted cytoskeletal structure or intracellular trafficking channels.

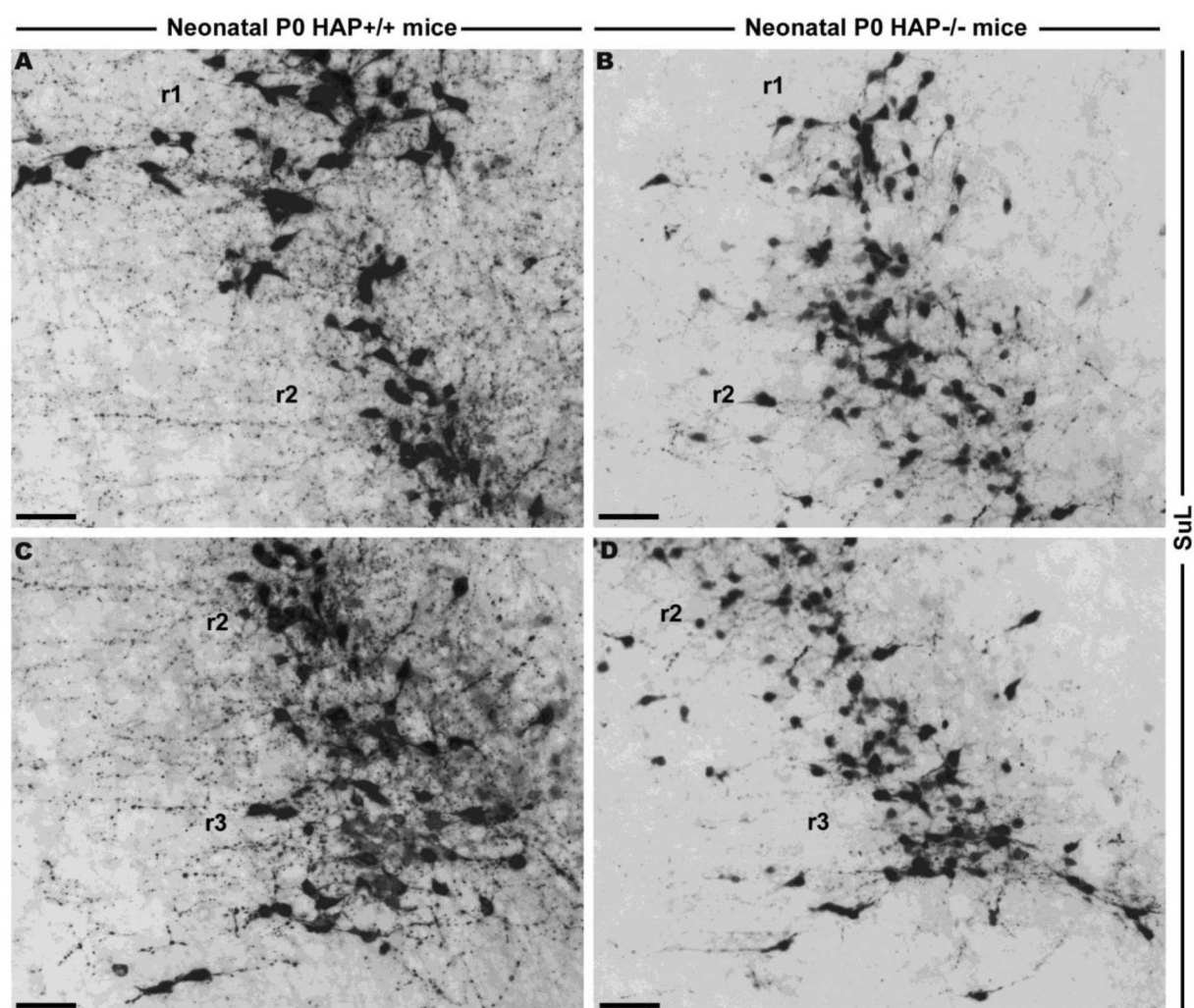


Figure 6. Comparative high magnification immunoperoxidase images of 5-HT immunoreactivity in the suprallemniscal raphe nucleus (SuL). (A–D) Photomicrographs showing serotonergic neuronal soma and fibers in the SuL of P0 wild (A, C) and HAP1–/– (B, D) pups across the rhombomeres (r1, r2 and r3). Images signify representative differences in somatic morphology and fiber arborization with varicosities between the genotypes at neonatal age. Scale bar = 20 μ m in (A–D). For abbreviations, see “List of Glossary.”

4.3.5. Impaired axonal varicosities and serotonergic fiber arborization in HAP1^{-/-} pups

The serotonergic fibers that originate at the raphe nuclei had different but distinct morphological profiles in HAP1^{+/+} mice (Figures 6-10; A, C, E, G). The axons developing off the mDR (Figures 7A), isDRd (Figures 7C), isDRv (Figures 7G), and CLi (Figures 8A) had small, beaded, and irregularly spaced varicosities. Conversely, the serotonergic axons of the SuL (Figures 6A, C), isDRI (Figures 7E), isDRw (Figures 8E, G), CLiw (Figures 8C), rMnR (Figures 9A), cMnR (Figures 9B), PPnR (Figures 9E), and PnR (Figures 9G) had larger and more rounded varicosities but were closely spaced along the fiber pathway. Axons of large varicosity were seen to form baskets by enveloping the soma and the proximal dendrites of a few interneurons around the CLiw (Figures 8C), rMnR (Figures 9A), cMnR (Figures 9B), PPnR (Figures 9E), and PnR (Figures 9G). Accordingly, axons from the caudal cluster of RMg (Figures 10A), ROb (Figures 10C), RPa (Figures 10E), and PPy (Figures 10G) showed medium-sized rounded varicosities that were also closely spaced from each other. Evenly scattered, either finely beaded or the dense clustering of large varicosities with dense terminal arborization was also observed throughout the cortex, hippocampus, hypothalamus, and amygdala of HAP1^{+/+} mice (Figures 11A, C, E, G). The combined arrangement and selective morphology of serotonergic varicosities in HAP1^{+/+} mice were manifestations of the typical topographical organization of raphe serotonergic fiber innervation of the brainstem and forebrain areas.

In stark contrast, HAP1^{-/-} mice showed a marked decrease in serotonergic varicosities across the raphe nuclei in addition to their forebrain projection regions. Additionally, the serotonergic fibers in the raphe nuclei seemed grossly atrophic and less complex with scarce and unevenly distributed varicosities (Figures 6-10; B, D, F, H). Quantitative analysis showed that there was a significant reduction in the number of varicosities per unit length of serotonergic axon in comparison to wild-type control throughout the rostral and caudal clusters of raphe nuclei and their surroundings (Figure 12B). Other corresponding changes were observed in areas of the forebrain to which serotonergic projections were innervated (Figures 11B, D, F, H). Serotonergic fibers were poorly branched in the cortical areas of HAP1^{-/-} mice, and the typical fine beaded varicosities that appeared in the wild-type mice were significantly fewer and irregularly spaced (Figures 11A, B). There was also a significant reduction in hippocampal serotonergic varicosities, where serotonergic innervation was usually very high in HAP1^{+/+} mice (Figures 11C, D). Moreover, HAP1^{-/-} brains exhibited prominent perturbed serotonergic fiber arborization of the hypothalamus (Figures 11E, F) and amygdala (Figures 11G, H) compared to wild-type controls. However, HAP1^{+/-} mice displayed an intermediate phenotype in that they had partially decreased serotonergic fiber arborization and varicosities when compared to HAP1^{+/+} mice but moderately increased relative to HAP1^{-/-} littermates.

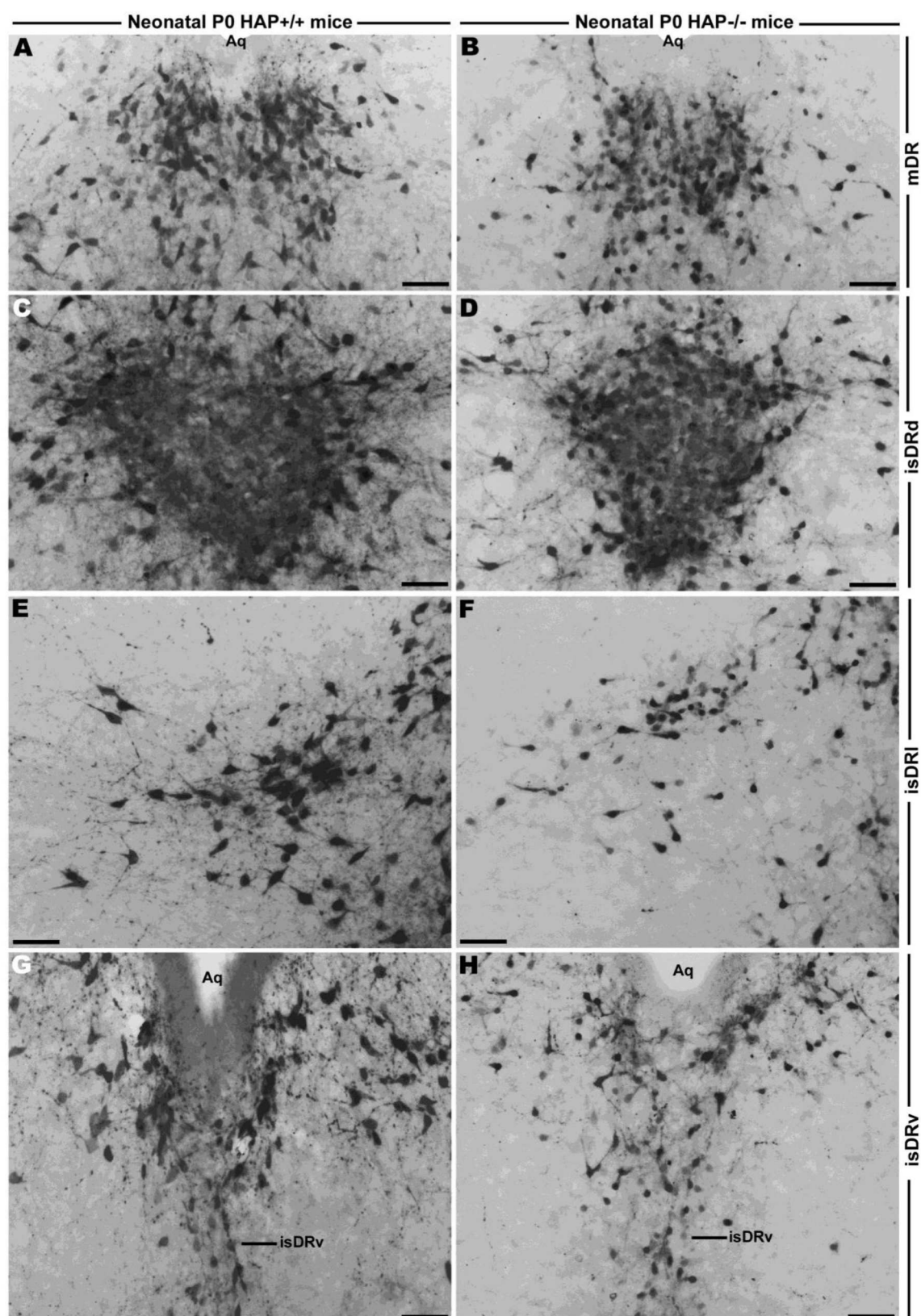


Figure 7. Comparative high magnification immunoperoxidase images of 5-HT immunoreactivity in the subdivisions of dorsal raphe nucleus (DR). (A–H) Photomicrographs showing serotonergic neuronal soma and fibers in the mDR, isDRd, isDRI, and isDRv of P0 wild (A, C, E, G) and HAP1^{-/-} (B, D, F, H) pups respectively. Images signify representative differences in somatic morphology and fiber arborization with varicosities between the genotypes at neonatal age. Scale bar = 20 μ m in (A–H). For abbreviations, see “List of Glossary.”

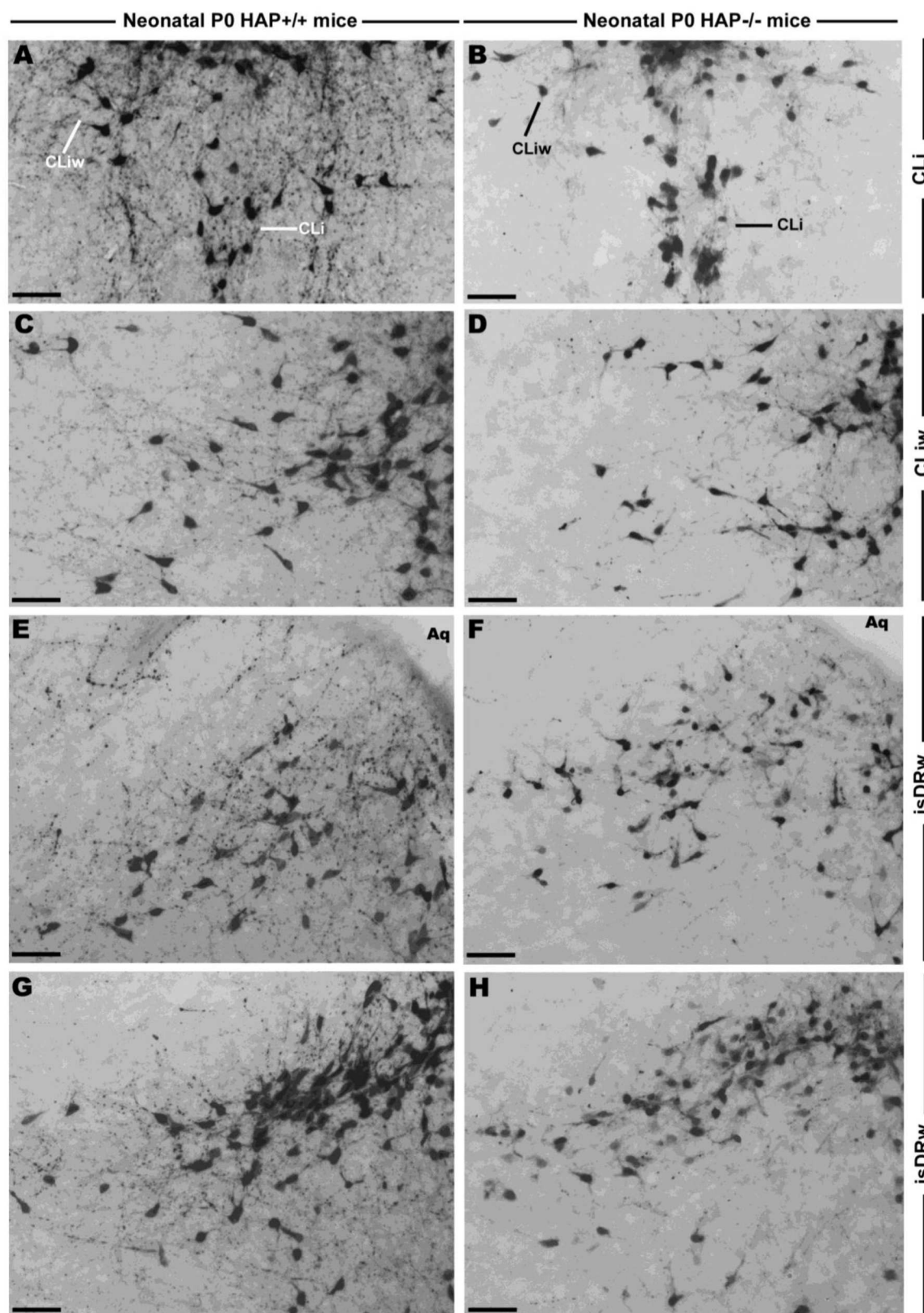


Figure 8. Comparative high magnification immunoperoxidase images of 5-HT immunoreactivity in the rostral cluster of raphe nuclei (caudal midbrain). (A–H) Photomicrographs showing serotonergic neuronal soma and fibers in the CLi, CLiw, and isDRw (rostral to caudal) of P0 wild (A, C, E, G) and HAP1–/– (B, D, F, H) pups respectively. Images signify representative differences in somatic morphology and fiber arborization with varicosities between the genotypes at neonatal age. Scale bar = 20 μ m in (A–H). For abbreviations, see “List of Glossary.”

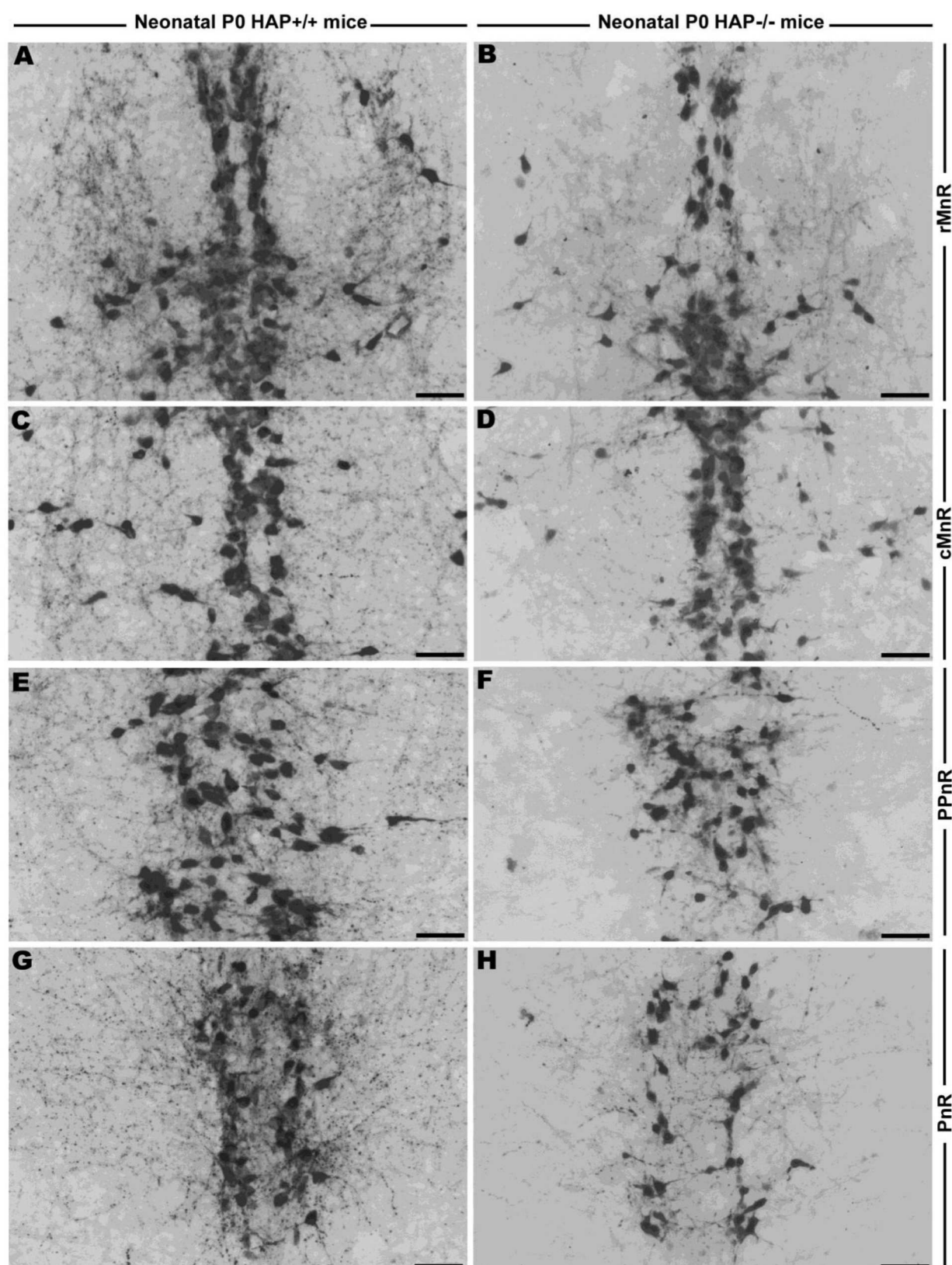


Figure 9. Comparative high magnification immunoperoxidase images of 5-HT immunoreactivity in the rostral cluster of raphe nuclei (caudal midbrain-rostral pons). (A–H) Photomicrographs showing serotonergic neuronal soma and fibers in the rMnR, cMnR, PPnR, and PnR of P0 wild (A, C, E, G) and HAP1–/– (B, D, F, H) pups respectively. Images signify representative differences in somatic morphology and fiber arborization with varicosities between the genotypes at neonatal age. Scale bar = 20 μ m in (A–H). For abbreviations, see “List of Glossary.”

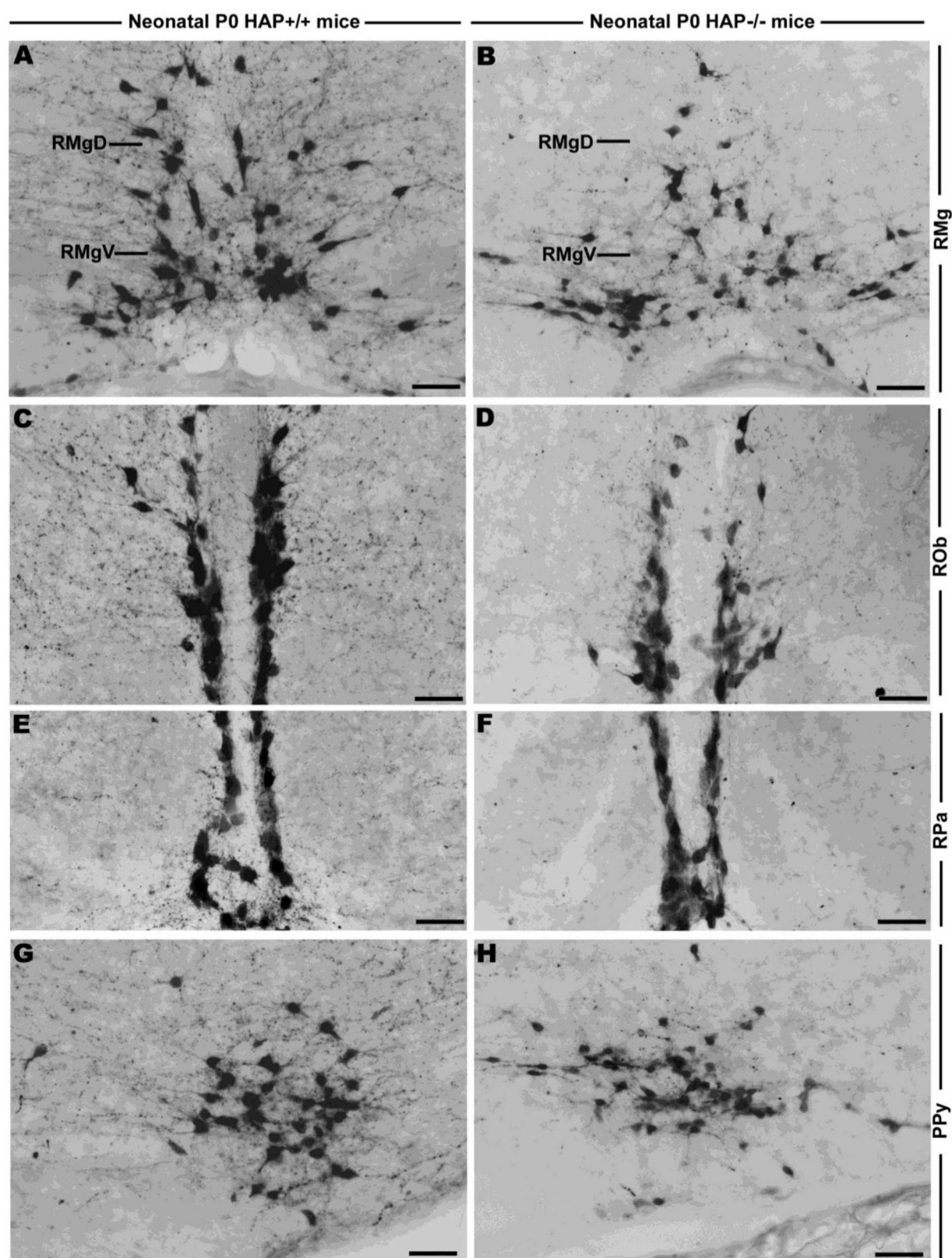


Figure 10. Comparative high magnification immunoperoxidase images of 5-HT immunoreactivity in the caudal cluster of raphe nuclei (caudal pons-medulla). (A–H) Photomicrographs showing serotonergic neuronal soma and fibers in the RMg, ROb, RPa, and PPy of P0 wild (A, C, E, G) and HAP1–/– (B, D, F, H) pups respectively. Images signify representative differences in somatic morphology and fiber arborization with varicosities between the genotypes at neonatal age. Scale bar = 20 μ m in (A–H). For abbreviations, see “List of Glossary.”

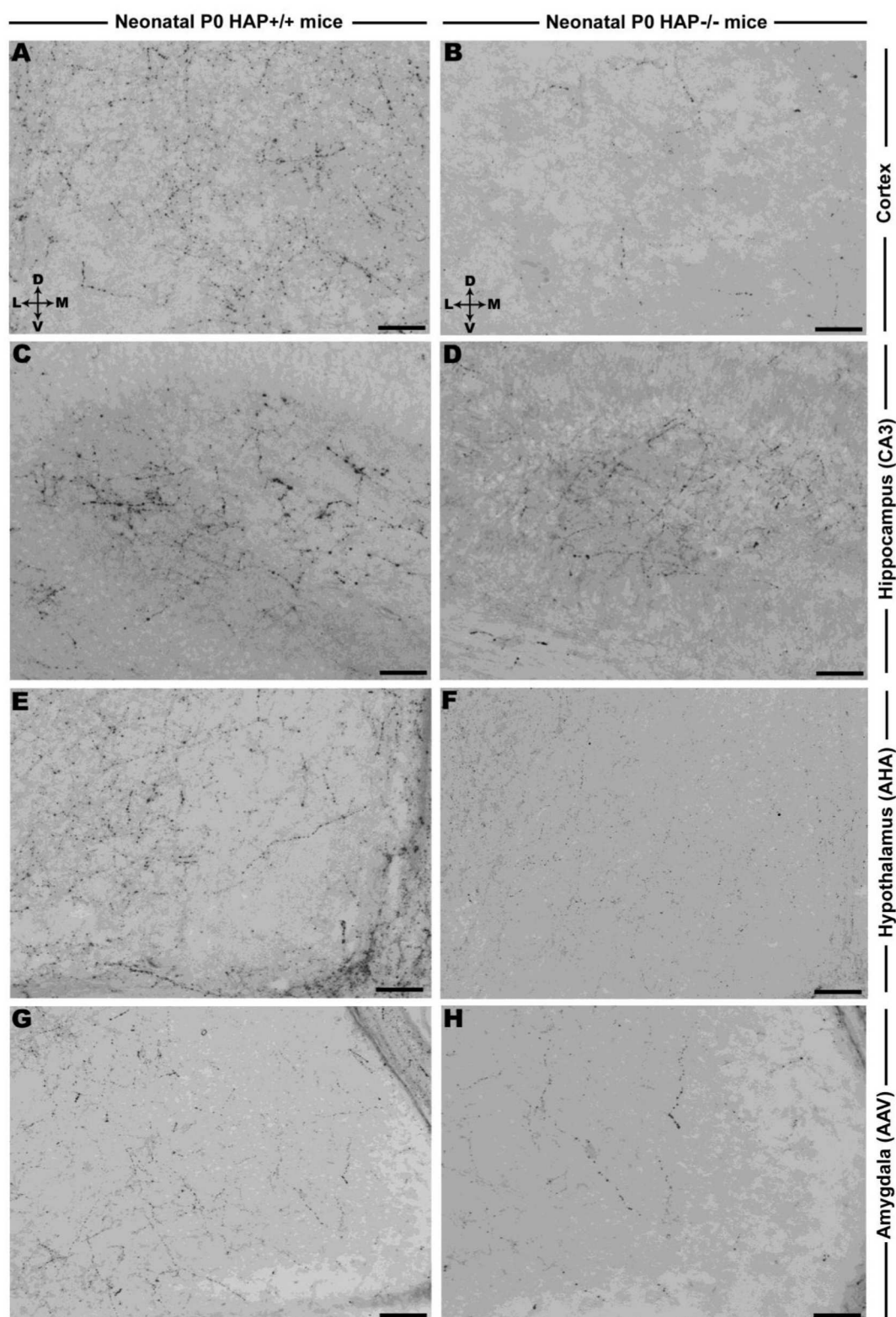


Figure 11. Comparative high-magnification immunoperoxidase images of 5-HT immunoreactive neuronal fibers arborization and varicosities in the cortex, hippocampus, hypothalamus, and amygdala of pups' forebrain. (A–H) Photomicrographs showing serotonergic neuronal axons and varicosities in the cortex, hippocampus (CA3; cornu ammonis 3), hypothalamus (AHA; anterior hypothalamic area, anterior), and amygdala (AAV; anterior amygdaloid area, ventral) of P0 wild (A, C, E, G) and HAP1–/– (B, D, F, H) pups respectively. Images signify representative differences in fiber arborization pattern and number of varicosities between the genotypes at neonatal age. Scale bar = 20 μ m in (A–H). For abbreviations, see “List of Glossary.”

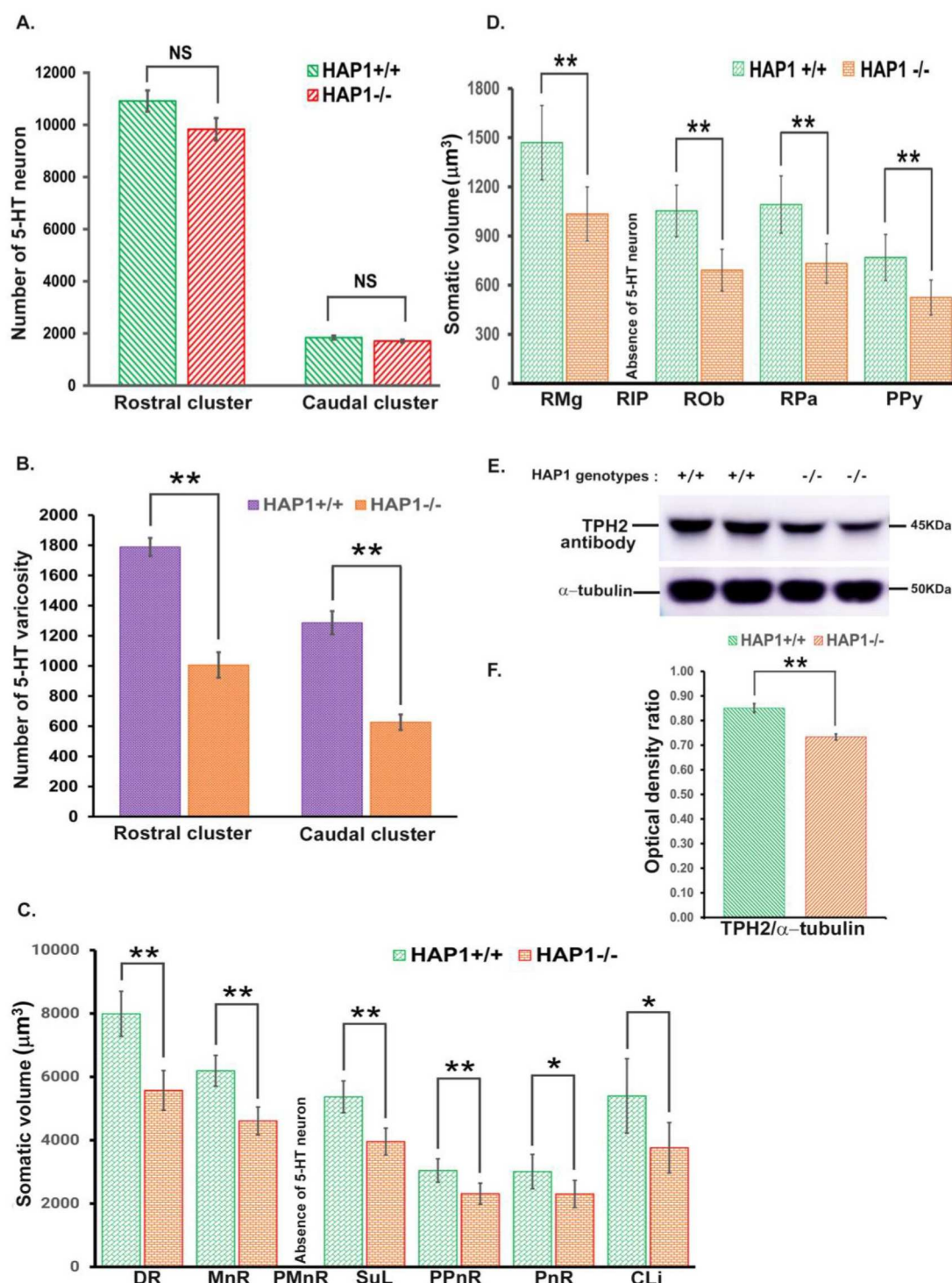


Figure 12. Morphological disparities of serotonergic neurons across the rostral and caudal clusters of raphe nuclei and the alteration of tryptophan hydroxylase 2 (TPH2) protein expression. (A) Number of 5-HT neuronal soma was not significantly differ ($p > 0.05$) between HAP1+/+ and HAP1-/- pups in both clusters of raphe nuclei (B) Number of 5-HT fiber varicosities per unit length of serotonergic axon were significantly reduced ($p < 0.01$) in HAP1-/- pups compared with wild-type controls in both raphe clusters (C, D) The somatic volume of 5-HT neurons was significantly decreased in each raphe nuclei (excluding PMnR & RIP) of HAP1-/- pups ($p < 0.01$; < 0.05), showing somal atrophy relative to its wild littermates (E) Western blot analysis showing TPH2 and α -tubulin immunoreactive (ir) bands in brain lysate of P0 wild pups. HAP1-/- neonates exhibited significantly reduced TPH2 protein expression in comparison to wild pups (F) Densitometric quantification of TPH2-ir bands normalized to α -tubulin (n = 6 per genotype) demonstrated a significant reduction ($p < 0.01$) in TPH2 protein levels in HAP1-/- neonates relative to HAP1+/+ littermates. For abbreviations, see “List of Glossary.”

4.3.6. Reduced expression of tryptophan hydroxylase 2 in HAP1^{-/-} pups

The protein level of TPH2 (~45 kDa), the rate-limiting enzyme in serotonin production, was significantly reduced in HAP1^{-/-} pups compared with wild-type controls, based on the Western blot study of brain lysate. The relative intensity of TPH2 immunoreactivity in the brain was quantitatively analyzed using the protein band ratio of TPH2 to α -tubulin (Figure 12D), which generated findings indicative of at least three different studies. According to our study findings, the TPH2/ α -tubulin band ratio was much lower in HAP1^{-/-} mice (Figure 12E), suggesting that serotonergic synthesis ability may be downregulated by genetic depletion of HAP1. This biochemical change is congruent with the reported decrease in serotonergic varicosity density in HAP1^{-/-} brains and can be regarded as an explanation for lower synthesis or release of serotonin.

4.4. Discussion

Our findings have consequences for the anatomo-functional parsing of serotonergic raphe cell groups and, for the first time, demonstrate the role of HAP1 on 5-HT raphe neurons. All of these findings show that HAP1 is extensively co-expressed with serotonergic neurons throughout the raphe nuclei and is crucial for preserving the morphological integrity of the 5-HT system. Serotonergic fiber arborization was substantially altered by HAP1 deficiency, which correlated with reduced axonal varicosities, altered neuronal morphology and reduced TPH2 expression. All of these results point to HAP1 as a neuroprotective regulator that is crucial for the biochemical and structural maintenance of serotonergic neural pathways in the early postnatal period.

4.4.1. Developmental implications and neonatal lethality

Our HAP1^{-/-} neonates failed to survive past 24 hours post-delivery despite appearing morphologically normal at birth, which is in line with previous research demonstrating that HAP1-KO mice die during the early postnatal period due to deprivation in feeding (Dragatsis et al., 2004). The impaired serotonergic function in brain circuits controlling autonomic and homeostatic functions such as feeding, respiration, and thermoregulation may be involved in this postnatal mortality. In general, serotonergic projections from the raphe nuclei significantly influenced these activities (Hornung, 2003). Therefore, at this crucial period of life, the structural abnormalities of serotonergic neurons in HAP1-deficient newborns may be a factor in the lethal physiological instability.

4.4.2. HAP1 regulates the formation and maturation of serotonergic circuits

The serotonergic system arises from the raphe nuclei that exhibit unique migration patterns (Lidov and Molliver, 1982a; Wallace and Lauder, 1983). The initial contributing of the raphe nuclei is based on the ultimately cluster of newborn 5-HT neurons in different disparate regions of the

midbrain, pons and medulla. Serotonergic neurons are first specified between embryonic days 9.5-12 in mice. At least until the end of the third postnatal week, these serotonergic neurons go through a variety of postnatal maturation events, such as cell migration, dendritic growth, autoregulatory feedback, formation of highly collateralized axonal pathways, and synaptogenesis (Lidov and Molliver, 1982a). Our results show that HAP1 expression may coincide temporally with these maturation events, suggesting that it may help with vesicular transport, cytoskeletal stabilization, or axonal guidance during the assemblage of serotonergic networks.

HAP1^{-/-} neonates have shown somal atrophy, reduced synaptic varicosities and shortened, disorganized 5-HT fibers, indicating that HAP1 may promote axon elongation, branching, and terminal (synaptic bouton) formation of 5-HT neurons. These are thought to be the essential characteristics for generating precise serotonergic connections. According to (Djalali et al., 2005), this phenotype is similar to developmental impairments observed for other neurotrophic and cytoskeletal regulators, including BDNF and S-100 β , which both indirectly affect serotonergic projection patterns *in vitro*. Intriguingly, HAP1 has been reported to stabilize BDNF and its receptor TrkB (Xiang et al., 2014). The reciprocal residing interaction additionally strengthens the evidence for a likely functional relationship between 5-HT neurons and HAP1. Thus, HAP1 is probably a potential intracellular mediator of the process coupling serotonergic differentiation to trophic signaling cascades.

4.4.3. Impaired serotonergic neuronal process integrity in HAP1 deficient pups

The serotonergic axons arborize in the functionally diverse parts of the brain with remarkable morphological heterogeneity. Rodent anterograde tracing investigations showed that MnR projections had large, sparse varicosities, whereas DR axons have fine beaded varicosities (Morrison et al., 1982; Campbell et al., 1987; Halasy et al., 1992; Hornung and Celio, 1992; Acsády et al., 1993; Smiley and Goldman-Rakic, 1996; Lambe et al., 2000; Wilson et al., 2018). These morphological patterns were corresponding to our observations in HAP1^{+/+} mice. These characteristics pointed to the well-developed axonal structure and evenly distributed synaptic boutons that are characteristic of the serotonergic maturation in the early postnatal development. These typical axonal beads are reduced in HAP1^{-/-} mice, implying that there is disrupted structural maintenance or accumulation of synaptic vesicles' protein in the absence of HAP1. In agreement with earlier ultrastructural descriptions, we also observed robust varicose basket formations around neurons in the CLiW, rMnR, cMnR, PPnR, and PnR in HAP1^{+/+} mice (Kosofsky and Molliver, 1987; Freund et al., 1990; Hornung et al., 1990; Hornung and Celio, 1992; Smiley and Goldman-Rakic, 1996; Trottier et al., 1996; Wilson et al., 2018). On the other hand, the fibers of HAP1^{-/-} mice were sparse, disrupted, indicating that the terminal fields maturation was impaired. Our study also demonstrated reduced

serotonergic fiber arborization of forebrain structures such as the cortex, hippocampus, hypothalamus, and amygdala in HAP1^{-/-} mice in comparison to HAP1^{+/+} mice. This decrease in fiber density is probably associated with the deterioration of intracellular transport and inefficient cargo delivery to axonal terminals. It has been previously demonstrated that HAP1 deficiency disturbs the localization of synaptosome protein complexes, as well as neuronal process integrity, especially orexin neurons (Lin et al., 2010). These data, together with our results, show that the function of HAP1 in promoting axonal transport and synaptic stability is also observed in serotonergic cells, affecting their morphology and connectivity.

4.4.4. Molecular mechanisms: intracellular trafficking, neurotrophic signaling, and cytoskeletal control

HAP1 is a multifunctional trafficking adaptor, which regulates intracellular trafficking and mediates receptor stabilization. It controls BDNF-TrkB endocytosis, and HAP1 knockout mice are characterized by a lack of TrkB internalization and ERK, Akt and PLCγ-1 downstream signaling that inhibits neurite outgrowth, and enhances postnatal death in mice (Lim et al., 2018; Wu et al., 2024). Since endocytosis of BDNF/TrkB complex is clathrin dependent (Zheng et al., 2008) and BDNF is a trophic factor in neuronal arborization and survival (Bathina and Das, 2015), this pathway may be the cause of the low levels of serotonergic fiber networks in mice with HAP1 deficits. When it comes to receptor stabilization, HAP1 has been reported to stabilize the GABA_A receptor, BDNF, and its receptor TrkB (Kittler et al., 2004; Yang et al., 2011; Xiang et al., 2014). HAP1 also interacts with Bcr on microtubules to regulate neuronal differentiation via ERK and p38 signaling (Huang et al., 2015). The interface of HAP1 to each of these partners hence influences signaling pathways that are important in neuronal morphogenesis (Lim et al., 2018). In addition, HAP1 also interacts with TSC1 and regulates the mTORC1 activity, which is connected to cytoskeletal remodeling needed for axonal differentiation (Mejia et al., 2013). Correspondingly, the mTOR protein kinase is essential for the activation of 5-HT₇ receptor to stimulate neurite elongation (Speranza et al., 2015). HAP1 deficiency thus impairs the microtubule organization and vesicular transport (Rong et al., 2006; Wu and Zhou, 2009), which probably results in the inhibition of serotonergic fiber development. This hypothesis is consistent with the previous literature that indicates that mutant mHTT blocks HAP1 functions (Li et al., 1995b) and BDNF transport in neuronal cells (Gauthier et al., 2004), leading to neuronal impairment. In addition, HAP1 deficiency affects the trafficking of proteins relevant for neurotransmission, which is also the case in HD pathology (Gunawardena et al., 2003; Morfini et al., 2009). These pathways have led to the identification of HAP1 as an intracellular coordinator of vesicular dynamics, axonal transport, and neuronal survival.

4.4.5. Regulation of serotonin synthesis: TPH2 expression and HAP1 dependence

TPH2 is the rate-limiting enzyme of the process of central 5-HT synthesis, and a low level of its expression can be an indication of impaired intracellular homeostasis or a compensating mechanism for the loss of axonal connections (Walther et al., 2003; Zhang et al., 2004; Lam and Heisler, 2007). This study has evidence that TPH2 protein levels in HAP1^{-/-} neonates' brain lysate is reduced considerably relative to littermates of wild-type, indicating that HAP1 is required to maintain 5-HT metabolic stability. TPH2 expression commences at approximately E11.25, reaches its greatest intensity at birth, and is augmented in raphe nuclei (Gutekunst et al., 1998; Gutknecht et al., 2009). Hence, HAP1 interference at this stage probably disrupts the stabilization or transcriptional regulation of TPH2. The genetically engineered animals (TPH2 knockout (-/-) mice) with a deficiency of 5-HT in the brain are able to be born and survive but exhibit growth retardation and enhanced mortality during the early stages of life in addition to abnormal autonomic regulation as denoted by irregularities in sleep, respiration, body temperature, blood pressure, and heart rate (Alenina et al., 2009). This observation is in agreement with a decline in growth rate in HAP1^{+/-} mice, indicating that disrupted 5-HT synthesis could result in impaired vital autonomic and metabolic processes that are required for supporting postnatal adaptation. All of these parallels emphasize that the dysfunction of the serotonergic system might occur due to the impaired synthesis of 5-HT or when HAP1-mediated intracellular regulation is disrupted. Long-term vulnerability to disruptions in homeostatic regulation and neurodegenerative processes stability may result from this.

4.4.6. Stress Axis and glucocorticoid regulation by HAP1 and serotonergic vulnerability

The HAP1 selective enrichment in the hypothalamus (Fujinaga et al., 2004, 2009) is indicative of the possibility that HAP1 performed a particular role within the hypothalamus capable of controlling metabolism and stress response. HAP1 co-localizes with GR in the neurons of the hypothalamus, and genetic loss of HAP1 reduces stability of GR, which regulates the feedback of the hypothalamus-pituitary-adrenal (HPA) axis (Chen et al., 2020). Additionally, the stress axis and 5-HT system are closely connected, where hypothalamus controls 5-HT neurons in a push-pull mechanism (Zhou et al., 2017). The expression of TPH2 is also stress sensitive or responds to stressful events or stressors (Boutrel et al., 1999; Müller et al., 2006; Gardner et al., 2009; Rahman and Thomas, 2009), presumably because stress-induced alteration in glucocorticoid levels. Although TPH2 expression regulation by glucocorticoids is species-specific and could be tissue-specific. Based on this, the activation of the HPA axis increases the levels of glucocorticoids that consequently affect the TPH2 gene expression and the 5-HT neurotransmission in mice (Clark and Russo, 1997; Heydendael and Jacobson, 2009). It has been demonstrated that the stress response is substantially affected by the level of GR expression and its stability (Chen et al., 2020). Thus, the stabilizing

association between HAP1 and GR represents a molecular connection between serotonergic homeostasis and stress signaling characteristics. Since HAP1 plays a role in intracellular signaling and receptor transport, it may also indirectly influence TPH2 expression by stabilizing GR expression. This would provide a plausible link between the dysregulation of the stress response and the reduced production of 5-HT in early-stage HAP1-deficient mice. There is a close connection between the circadian system and the stress system as well. In consonance with the earlier noted association between rhythmic TPH protein level and 5-HT synthesis in raphe neurons projecting to the primary circadian pacemaker (suprachiasmatic nucleus of hypothalamus, SCN), it vehemently indicates that the shift in TPH2 gene expression is closely related to the biological clock and the adaptive response to stress (Malek et al., 2004). This circadian system indirectly and directly regulates the occurrence of Alzheimer's disease (AD). In AD mice with intact circadian rhythms, circadian oscillations promoted the removal of A β plaques; however, the removal of A β plaques was inhibited when circadian rhythms were disrupted (Zhu et al., 2018; Lee et al., 2020). In this way, downregulation of TPH2 in HAP1-deficient mice may play a role in the extended stress-associated neuropathology.

4.4.7. Plausible neurobiological and clinical implications of HAP1 on 5-HT neurons in the raphe nuclei

Serotonin is a neurotransmitter and a developmental trophic factor, which controls the differentiation, migration, synaptogenesis, and synaptic plasticity of neurons (Azmitia et al., 1996; Brummelte et al., 2017). These mechanisms are required to generate the topographic precision of 5-HT projections, which have diverse functions in motor, limbic, and sensory regions. Serotonergic axon terminals reach every part of the brain that will usually receive serotonergic innervation before the end of pregnancy, and during the first three weeks after birth, they actively sprout to innervate their targets abundantly (Lidov and Molliver, 1982b; Wallace and Lauder, 1983). It has been found that when this serotonergic circuitry is perturbed during development, there are long-term cognitive and affective impairments (Doraiswamy, 2003; Gudelsky and Yamamoto, 2003). Therefore, early developmental stress or disturbance in HAP1 $^{-/-}$ mice has the potential to alter brain architecture and may repair much later in life. Consequently, surviving mutants (HAP1 +/-) usually exhibit slow growth (Dragatsis et al., 2004). An accumulating amount of data demonstrates that early-life stress and genetic predisposition interact in an epigenetic manner, including DNA methylation and histone modification, to influence serotonergic plasticity (Liu et al., 1997; Weaver et al., 2004; Weaver, 2007, 2009). Moreover, a distinctive feature of the 5-HT system is the possibility to recover and re-innervate the brain regions following traumatic brain damage and chronic stress (Jin et al., 2016; Kajstura et al., 2018; Zahrai et al., 2020). In line

with this resilience, partial loss of HAP1 (HAP1 +/-) failed to generate any noticeable phenotypic changes in their adulthood.

The reduction in varicosity number suggests that HAP1 supports synaptic stabilization dynamics, mechanisms that may ultimately determine serotonergic connectivity strength and plasticity. The deficiency of HAP1 in mice might result in the reduction of the number of different proteins in the axon terminal enriched with synaptosome, which might have an effect on the interaction of 5-HT neurons. In support, synaptic vesicle-associated protein (SVP38) and BDNF levels are decreased in synaptosome-enriched fractions from HAP1-deficient mouse brain lysate (Lin et al., 2010), and HAP1 is required for TrkB internalization and BDNF-dependent survival signaling (Lim et al., 2018). This type of disruption is comparable to known pathways in neurodegenerative diseases, including Parkinson's disease (PD) and AD (Kang et al., 2017). Also, neurodevelopmental disorders that are co-occurring with 5-HT dysfunction are the results of defective axonal transport of presynaptic cargos or proteins, including autism spectrum disorders, attention-deficit/hyperactivity disorder, disruption of learning and motor function, developmental epilepsies, and schizophrenia (Thapar et al., 2017; Sydnor et al., 2021; Xiong and Sheng, 2024). HAP1-expressing neurons are known to be abundant in the amygdala and locus coeruleus (Fujinaga et al., 2004; Islam et al., 2022). The pathophysiology of autism has been connected to this amygdala (Baron-Cohen et al., 2000). Therefore, serotonergic projections from the dorsal raphe and noradrenergic projections from the locus coeruleus modulate the function of the central and medial group of the amygdala, which is involved in processing social information, emotional interpretation, anxiety, and fear (Baron-Cohen et al., 2000; Amaral et al., 2003; Vasudeva et al., 2011). Furthermore, HAP1 interacts with Abelson helper integration site 1 (Ahi1) to regulate the development of the brainstem and cerebellum in mice (Sheng et al., 2008). Significantly lower Ahi1 levels were also reported in HAP1^{-/-} mice. In Ahi1KO mice, the aberrant early brain development and neurological symptoms of the autosomal recessive Joubert syndrome may be caused by a disruption in HAP1-mediated intracellular trafficking (Sheng et al., 2008). Additionally, our research has shown that the brain lysate of HAP1^{-/-} mice has substantially reduced TPH2 protein expression. Similarly, the male Ahi1 KO mice showed considerably lower TPH2 protein expression in the brainstem and hippocampus than that of control animals, while Ahi1 KO had no effect on SERT expression. Ahi1 therefore regulates the Ahi1/GR/ER β /TPH2 pathway in the brain for serotonin synthesis (Wang et al., 2022), where HAP1 may have indirect effects. It may also be involved in the regulation of sex differences in depressive behaviors (Wang et al., 2022). Besides, HAP1 is important for neuronal differentiation and GR stability in the hypothalamus during early development (Li et al., 2003). Notably, the mRNA expression of TPH2

is sensitive to glucocorticoids (Clark and Russo, 1997), and reduced GR expression is a hallmark of neuropsychiatric and neurodegenerative diseases, including PD, AD, and HD (Oakley and Cidlowski, 1993; Karanth et al., 1997; Uys et al., 2006; Vyas and Maatouk, 2013; Boero et al., 2018). Evidence suggests that HAP1 is also necessary in regulating the embryonic development process, and the re-establishment of HAP1 function in neuronal cells at various stages of development may help to avert premature death (Dragatsis et al., 2004). In P0 HAP1^{-/-} mice, the dual loss of structural integrity and metabolic capacity in 5-HT neurons may recapitulate early features of these diseases. Consistent with this, HAP1^{-/-} neonates showed reduced TPH2 protein levels, reminiscent of SIDS cases with decreased medullary TPH2 and 5-HT (Duncan, 2010). Additionally, the relevance of the HAP1 protection hypothesis is underscored by evidence that degeneration of 5-HT terminals contributes to the emergence of both motor and non-motor symptoms in PD (Halliday et al., 1990; Paulus and Jellinger, 1991; Kish et al., 2008). 5-HT activity is generally lower in PD patients, serving as a compensatory strategy for the lower dopaminergic activity (Leentjens et al., 1998) in which raphe degeneration and Lewy pathology emerge in Braak stage II (Braak et al., 2003). The fact that neurodegeneration of the raphe nuclei can possibly be the second cause of the failure of striatal 5-HT to be innervated in PD (D'Amato et al., 1987; Braak et al., 2003). Likewise, our current study revealed the decreased varicosity numbers and disrupted fiber arborization in the forebrain structures. It has been known that the abnormalities of intracellular trafficking usually cause protein mislocalization and the accumulation of undegraded proteins in neurons (Inoshita and Imai, 2015). Given that HAP1 is crucial for vesicular and membrane receptor transport (Lin et al., 2010). These defects likely stem from impaired intracellular trafficking, which supports the hypothesis of HAP1 protection as a pathway that connects serotonergic vulnerability and neurodegeneration. Moreover, sleep disruption and tau aggregation are facilitated by serotonergic depletion (John et al., 1991; Bodner et al., 1995), linking 5-HT dysfunction to PD, AD, and tau pathology (Yang and Schmitt, 2001; Doraiswamy, 2003; Gudelsky and Yamamoto, 2003; Kovacs et al., 2003). Therefore, our results imply that HAP1 ablation in raphe neurons may represent a molecular connection between serotonergic dysregulation and vulnerability to neurodevelopmental and neurodegenerative diseases.

Finally, HAP1 transfection protects neurons from proteasome-inhibition-induced apoptosis in cultured cells (Fujinaga et al., 2011), and proteasome inhibitor (MG132) efficiency declines with age (Zhao et al., 2016). With HAP1 mRNA expression also decreasing in aged brains (Page et al., 1998), altered HAP1 availability may weaken proteasome inhibition system over time. Future work should determine whether HAP1 expression in raphe nuclei undergoes temporal

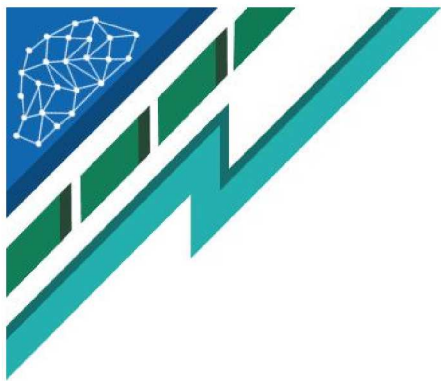
regulation across development and aging, and whether its loss accelerates susceptibility to serotonergic dysfunction and circuit degeneration.

4.4.8. Limitations and future directions

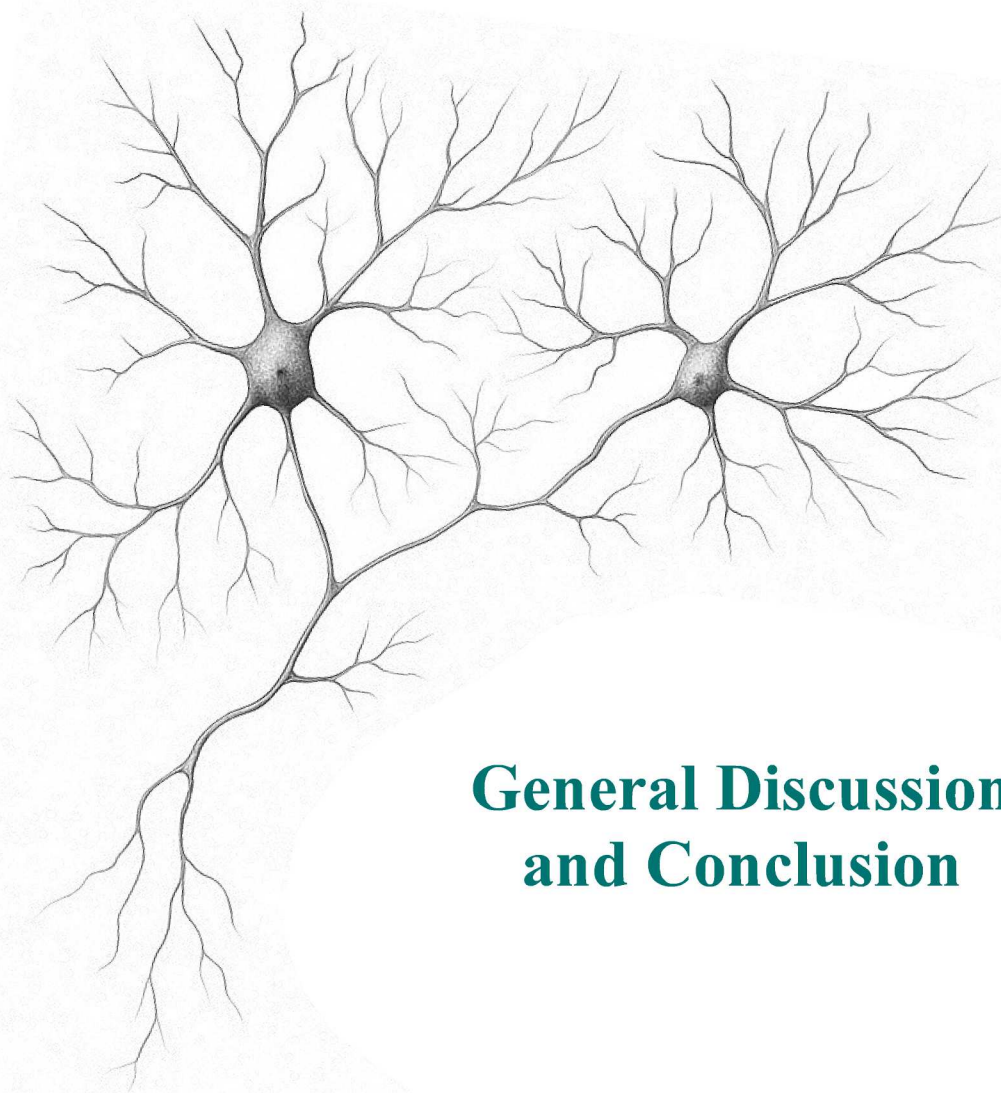
The precise significance of HAP1 in regulating serotonergic neurons remains to be determined completely. For example, it is not known how HAP1 regulates the axonal guidance cues, cytoskeletal structure, or synaptic plasticity in 5-HT neurons. Future studies using cell-type-specific perturbation and live imaging will determine whether HAP1 directly regulates cytoskeletal dynamics and/or interacts with molecular motors required for serotonergic axon targeting. Moreover, the determination of whether specifically restoring HAP1 expression after birth may reinstate serotonergic architecture as well as the fiber arborization phenotype would provide additional evidence of a critical developmental period when HAP1 has its influence. In addition, it will be important to study the physiological and behavioral consequences of HAP1 deficiency in order to correlate genetic and anatomical defects with functional outcome. Therefore, future research should focus on more methodically defining the molecular and transcriptional heterogeneity of HAP1 and 5-HT neurons. Mapping the molecular composition of different HAP1 and 5-HT cell groups within the raphe by genome-wide transcriptomic and proteomic studies or single-cell RNA sequencing combined with functional and spatial profiling could be a promising approach. A more thorough knowledge of how HAP1 interacts with serotonergic molecular architecture and controls serotonergic development, connection, and plasticity will be attributable due to such integrative methods.

4.5. Conclusion

HAP1 is considered as a key stabilizing element to maintain the integrity of neuronal functions by interacting with key pathogenic factors in multiple neurodegenerative disorders. Our findings defined the basic cytoarchitectonic framework for serotonergic signaling in the developing mouse brainstem, which reveals the postnatal serotonergic domains delineating a continuous yet regionally differentiated network of raphe nuclei. Loss of HAP1 leads to severe morphological abnormalities, disrupts the development of varicosity, and downregulates TPH2, thereby weakening the serotonergic network. These findings unambiguously demonstrate that HAP1 is a neuroprotective regulator that is important for the structural and biochemical integrity of the serotonergic system in neonatal mouse brain. Thus, the understanding of how HAP1 integrates trafficking, trophic signaling, and stress axis control will help to explain the development of the serotonergic system and possibly find shared pathways underpinning neurodevelopmental and neurodegenerative diseases.



Chapter 5



**General Discussion
and Conclusion**



5.1. General Discussion

The current study investigated the pathophysiological function of HAP1 that acts as a neuroprotective interactor and supports the structural and functional integrity of serotonergic neurons in the rostral and caudal clusters of brainstem raphe nuclei. Grounded on the "HAP1 protection hypothesis," which states that HAP1 enriched regions were particularly resistant to neurodegenerative stress, we explored if this idea is equally applicable to the raphe system. Our results support this hypothesis and show that HAP1 is highly integrated in both the morphology and early development of 5-HT neurons.

The first hypothesis was supported by the extensive HAP1 immunoreactivity across the rostral and caudal raphe clusters in adult mice. Nearly all serotonergic neurons co-expressed HAP1 strengthening the notion that 5-HT neurons depend on HAP1-associated intracellular processes. The cytoarchitectural relationship of HAP1 with 5-HT neurons put HAP1 as a stabilizing factor in axonal trafficking, receptor complexes maintenance, and cytoskeletal regulation. It has been shown that, long-range axonal trajectories and extensive terminal arborization characteristic of raphe projections ([Donovan et al., 2019](#)). Therefore, it is plausible that HAP1 may provide the intracellular integrity of 5-HT neurons for maintaining homeostasis and resisting cellular stress.

In the second hypothesis, the study using HAP1 knockout neonates further reinforce the abovementioned interpretation. Although HAP1^{-/-} pups were morphologically normal at birth, they died within 24 hours; in line with previous models for the essential role of HAP1 in postnatal survival ([Dragatsis et al., 2004](#)). At the cellular level, a deletion of HAP1 resulted in a number of pronounced abnormalities in serotonergic neurons. First, we found a significant decrease in the rate-limiting enzyme of the 5-HT biosynthesis, tryptophan hydroxylase-2 (TPH2). The loss of TPH2 expression suggests reduced metabolic capacity for serotonin production, which may have a direct impact on homeostasis such as respiration, feeding, and thermoregulation, which are processes dependent on raphe circuits in neonates ([Hornung, 2003](#)).

Second, HAP1 deficiency affected the structural morphology of 5-HT neurons, and decreased the somatic volume, disrupting axonal structure. The serotonergic projections of HAP1^{-/-} mice had sparse and poorly branched fibers with considerably reduced synaptic varicosities in several target regions, including the cortex, hippocampus, hypothalamus, and amygdala. Even HAP1^{+/-} mice showed moderate decreases in varicosity density, suggesting that HAP1 may affect the development of synapses. Given that 5-HT plays dual roles as a neurotransmitter and a trophic factor during early life ([Brummelte et al., 2017](#)), dysfunctions in axon growth and terminal specialization are likely to

have wide-reaching effects on circuit formation and early neural maturation.

These structural defects can be interpreted in the light of the molecular functions of HAP1 as known. HAP1 has interactions with dynactin, kinesin, Trk receptors, GABA_A receptors, and the GR, which are involved in the control of vesicle trafficking, recycling of receptors, and intracellular transport (Engelender, 1997; Li et al., 1998a; Gauthier et al., 2004; Kittler et al., 2004; McGuire et al., 2006; Rong et al., 2006; Chen et al., 2020). Loss of HAP1 disrupts these pathways and may result in a malfunction of neurons, mislocalization of proteins and blocked transport of vesicles and neurotrophic signaling. In 5-HT neurons, such deficits would be expected to lead to weakening of cytoskeletal support, modification of growth cone dynamics and reduced delivery of enzymes, synaptic proteins and trophic receptors required for axon extension and synaptic stabilization. The reduction in TPH2 observed in HAP1^{-/-} pups may therefore reflect impaired intracellular transport and altered GR-dependent transcriptional control since there is known to be GR-dependent control of TPH2 expression.

The abovementioned findings placed HAP1 as a scaffold of neurodevelopmental and neurodegenerative susceptibility. Serotonergic circuits exhibit early and progressive degeneration in a number of disorders, such as AD, PD, and HD (Yang and Schmitt, 2001; Doraiswamy, 2003; Gudelsky and Yamamoto, 2003; Kovacs et al., 2003; Pla et al., 2014). These conditions were the result of defective axonal transport, mitochondrial stress, synapse maintenance disruption and abnormal neurotrophic signaling (Kang et al., 2017) which were closely related to HAP1 function (Chen et al., 2022; Wu et al., 2024). Furthermore, previous study illustrates a possible involvement of serotonin in the shaping of neuronal proliferation, migration and synapse formation during embryogenesis and in the early postnatal period (Azmitia et al., 1996; Brummelte et al., 2017). Disruption of serotonin neuron structure or synthesis during this period may result in alterations in stress response and emotional and cognitive behavior. These may involve ASD, pathologies caused by stress at an early age, or developmental contributions to subsequent psychiatric pathology (Thapar et al., 2017; Sydnor et al., 2021; Xiong and Sheng, 2024). Our results thus raise the possibility of HAP1 to act as an intrinsic molecular factor that helps serotonergic neurons to withstand this pathogenic stress. Therefore, the abundant expression of HAP1 in the brainstem raphe nuclei could be a contributing factor to cellular resilience, and otherwise, HAP1 deletion or malfunction may increase vulnerability to stress or degeneration.

5.2. Final conclusion

The present findings show that HAP1 is fundamental for the maintenance of structural and biochemical integrity of raphe serotonergic neurons. HAP1 ubiquitous expression across the raphe nuclei, co-localization with almost all 5-HT neurons, and the severe morphological and synthetic defects found upon its absence suggest that HAP1 is a molecular scaffold essential for the development and homeostasis of serotonergic neurons. However, HAP1 participates in the cytoarchitectural development, fiber arborization, and stress response pathways, which are essential in maintaining 5-HT neuron integrity. These findings further generalize the HAP1 protection hypothesis to the serotonergic system and have identified HAP1 as a possible contributor to resilience or vulnerability in conditions where serotonin system function is impaired.

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 2022 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. **Afrin M**, Islam MN, Meher MM, Jahan MR, Nozaki K, Masumoto KH, Yanai A, Shinoda K. Neuroanatomical mapping of huntingtin-associated protein 1 across the rostral and caudal clusters of mouse raphe nuclei and its immunohistochemical relationships with serotonin. *Frontiers in Neuroanatomy*. 2025 Jul 22; 19:1625793. doi: 10.3389/fnana.2025.1625793. PMID: 40766770; PMCID: PMC12321798.

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

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