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**THE CENTRAL HISTAMINERGIC NEURONS
IN VITRO, AND THEIR NEUROPROTECTIVE
ROLE IN EXCITOTOXIC CELL DEATH IN
THE POSTNATAL RAT HIPPOCAMPUS**

by

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To my family

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ABSTRACT

Histamine acts as a neurotransmitter in the central nervous system. Brain histamine is synthesized in neurons located to the posterior hypothalamus, from where these neurons send their projections to different parts of the brain. Released histamine participates in the regulation of several physiological functions such as arousal, attention and body homeostasis. Disturbances in the histaminergic system have been detected in diseases such as epilepsy, sleep disorders, anxiety, depression, Alzheimer's disease, and schizophrenia. The purpose of this thesis was to develop optimal culture conditions for the histaminergic neurons, to study their detailed morphology, and to find out their significance in the kainic acid (KA)-induced neuronal death in the immature rat hippocampus.

The morphology of the histaminergic neurons *in vitro* was comparable with the earlier findings. Histamine-containing vesicles were found in the axon but also in the cell body and dendrites suggesting a possibility for the somatodendritic release. Moreover, histamine was shown to be colocalized with the vesicular monoamine transporter 2 (VMAT2) suggesting that VMAT2 transports histamine to the subcellular storage vesicles. Furthermore, histamine was localized with γ -aminobutyric acid (GABA) in distinct storage vesicles and with neuropeptide galanin partly in the same storage vesicles suggesting different corelease mechanisms for GABA and galanin with histamine.

In the organotypic hippocampal slice cultures, KA-induced neuronal death was first detected 12 h after the treatment being restricted mainly to the CA3 subregion. Moreover, cell death was irreversible, since the 48 h recovery period did not save the cells, but instead increased the damage. Finally, neuronal death was suggested to be necrotic, since intracellular apoptotic pathways were not activated, and the morphological changes detected with the electron microscopy were characteristic for necrosis.

In the coculture system of the hippocampal and posterior hypothalamic slices, histaminergic neurons significantly decreased epileptiform burst activity and neuronal death in the hippocampal slices, this effect being mediated by histamine 1 (H1) and 3 (H3) receptors.

In conclusion, the histaminergic neurons were maintained successfully in the *in vitro* conditions exhibiting comparable morphological characteristics as detected earlier *in vivo*. Moreover, they developed functional innervations within the hippocampal slices in the coculture system. Finally, the KA-induced region-specific, irreversible and necrotic hippocampal pyramidal cell damage was significantly decreased by the histaminergic neurons through H1 and H3 receptors.

Key words: histamine, hippocampus, kainic acid, neuronal death, epilepsy

TIIVISTELMÄ

Histamiini on keskushermostossa toimiva välittäjäaine, jota syntetisoidaan hypothalamuksen takaosan neuroneissa. Histaminergisten neuronien aksonit ulottuvat kaikkialle aivoihin, ja hermosoluista vapautuva histamiini osallistuu mm. uni-valverytmin, ruokahalun, neste- sekä hormonitasapainon säätelyyn. Aivojen histaminergisen järjestelmän toimintahäiriöt liittyvät useisiin sairauksiin kuten epilepsia, unihäiriöt, ahdistuneisuus, masennus, Alzheimerin tauti sekä skitsofrenia. Tämän työn tarkoituksena oli kehittää optimaaliset viljelyolosuhteet histaminergisille neuroneille, tutkia niiden morfologiaa sekä selvittää histaminergisten neuronien suojavaikutusta kainaatti-indusoidussa eksitotoksisessa hermosolukuolemassa kehittyvän rotan hippokampuksessa.

Viljelmien histaminergisten neuronien morfologia vastasi aiemmissä tutkimuksissa tehtyjä havaintoja. Immunohistokemialliset värjäykset osoittivat, että histamiini oli varastoituneena solunsisäisiin varastovesikkeleihin, jotka paikallistettiin aksonien lisäksi myös solun soomaosaan ja dendriitteihin, mikä saattaa viitata histamiinin somatodendriittiseen vapautumiseen. Histamiini esiintyi osittain samoissa vesikkelirakenteissa sitä kuljettavan monoamiinikuljettaja-2:n kanssa. Histaminergisissä neuroneissa havaittiin histamiinin lisäksi γ -aminovoihappoa (GABA), joka oli varastoitunut eri vesikkeleihin kuin histamiini sekäuropeptidi galaniinia, joka oli osaksi samoissa vesikkeleissä histamiinin kanssa. GABA:n ja galaniinin erilainen varastoituminen histaminergisissä neuroneissa saattaa viitata eroihin välittäjäaineiden yhteisvapautumismekanismeissa.

Hippokampuksen leikeviljelmissä kainaatilla aiheutettu solukuolema havaittiin ensimmäisen kerran 12 h käsittelyn jälkeen, ja se rajoittui pääasiassa CA3 pyramidaalineuronien alueelle. Solukuolema oli palautumaton prosessi, sillä viljely normaalissa kasvatusliuoksessa ei pelastanut CA3 alueen neuroneita. Solukuoleman pääasiallinen mekanismi oli nekroosi, sillä apoptoottiset signaalinvälitysreitit eivät aktivoituneet ja elektronimikroskoopilla havaitut muutokset olivat tyypillisiä nekroosille.

Hippokampuksen ja hypothalamuksen kaksoisleikeviljelmämallissa osoitimme, että histaminergiset neuronit vähensivät merkittävästi epileptistyyppistä sähköistä aktiivisuutta ja solukuolemaa hippokampuksen pyramidaalineuroneissa. Tämä suojavaikutus tapahtui ainakin osittain histamiini 1 (H1)- ja 3 (H3)-reseptorivälitteisesti.

Tulokset osoittavat, että aivojen histaminergisiä neuroneita voidaan kasvattaa *in vitro* olosuhteissa, ja että niiden morfologiset erityispiirteet vastaavat *in vivo* kokeissa tutkittujen neuronien piirteitä. Leikeviljelmissä histaminergiset neuronit muodostivat toimivan hermotuksen hippokampuksen alueelle, missä ne vähensivät CA3 alueen hermosolujen eksitotoksista, nekroottista solukuolemaa H1- ja H3-reseptorivälitteisesti.

Avainsanat: histamiini, hippokampus, kainaatti, hermosolukuolema, epilepsia

ABBREVIATIONS

AA	aracidonic acid
AC	adenylate cyclase
AD	afterdischarge
α -FMH	α -fluoromethylhistidine
AMPA	α -amino-3-hydroxy-5-methylisoxazole-4- propionic acid
cAMP	cyclic adenosine monophosphate
CBZ	carbamazepine
CNS	central nervous system
CREB	cAMP response element-binding protein
DAG	1,2-diasylglycerol
DAO	diamine oxidase
DG	dentage gyrus
DIV	days <i>in vitro</i>
GABA	γ -aminobutyric acid
GAD	glutamic acid decarboxylase
GBP	gabapentin
GDP	giant depolarizing potential
GPCR	G protein-coupled receptor
E	embryonic day
EC	enzyme commission
EDAC	1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide
EEG	electroencephalogram
EPSC	excitatory postsynaptic current
FJB	Fluoro-Jade B
H1	histamine 1 receptor
H2	histamine 2 receptor
H3	histamine 3 receptor
H4	histamine 4 receptor
HDC	histidine decarboxylase
HI	organotypic cultures consisting of the hippocampal slice alone
HI+HY(POST)	organotypic cultures consisting of the hippocampal slice together with the posterior hypothalamic slice
HI+HY (ANT)	organotypic cultures consisting of the hippocampal slice together with the anterior hypothalamic slice
HPLC	high performance liquid chromatography
I_{APH}	Ca^{2+} -dependent K^{+} current
I_{h}	hyperpolarization-activated cation current
IP3	inositol 1,4,5-trisphosphate

IPSP	inhibitory postsynaptic potentials
KA	kainic acid
KO	knock out
LDCV	large dense core vesicle
LH	lateral hypothalamus
LTP	long term potentiation
MAO-A	monoamine oxidase A
MAO-B	monoamine oxidase B
MAP2	microtubule-associated protein
MAPK	mitogen-activated protein kinase
MES	maximal electroshock
mRNA	messenger ribonucleic acid
NMDA	N-methyl-D-aspartate
P	postnatal day
PARP	poly (ADP-ribose) polymerase
PB	phosphate buffer
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with Triton-x
PCD	programmed cell death
PDS	paroxysmal depolarization shift
PFA	paraformaldehyde
PHE	phenytoin
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
PLA	phospholipase A
PLC	phospholipase C
PTZ	pentylentetrazol
mRNA	messenger ribonucleic acid
RT	room temperature
SCN	suprachiasmatic nucleus
SD	Sprague-Dawley
SNAT	sodium-coupled neutral amino acid transporter
SSV	small synaptic vesicle
TLE	temporal lobe epilepsy
TM	tuberomamillary nucleus
VMAT2	vesicular monoamine transporter 2
VLPO	ventrolateral preoptic area

LIST OF ORIGINAL PUBLICATIONS

- I Kukko-Lukjanov T-K, Panula P (2002). Primary explant culture system for hypothalamic histaminergic neurons. *Inflamm. Res.* 51 Suppl 1:S37-9.
- II Kukko-Lukjanov T-K, Panula P (2003). Subcellular distribution of histamine, GABA and galanin in tuberomamillary neurons *in vitro*. *J. Chem. Neuroanat.* 25(4):279-92.
- III Holopainen IE, Järvelä J, Lopez-Picon FR Pelliniemi LJ, Kukko-Lukjanov T-K (2004). Mechanisms of kainate-induced region-specific neuronal death in immature organotypic hippocampal slice cultures. *Neurochem Int.* 45(1):1-10.
- IV Kukko-Lukjanov T-K, Soini SL, Taira T, Michelsen KA, Panula P, Holopainen IE (2006). Histaminergic neurons protect the developing hippocampus from kainic acid-induced neuronal damage in the organotypic co-culture system. *J. Neurosci.* 26(4):1088-97.

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1. INTRODUCTION

Histamine belongs chemically to the group of monoamines together with dopamine, serotonin, adrenaline, and noradrenaline. Drugs affecting the monoaminergic neuron systems are well known in diseases such as depression, anxiety, schizophrenia, and Parkinson's disease. In the central nervous system (CNS), monoamines are considered as modulatory neurotransmitters acting mainly through the metabotropic monoamine receptors. These receptors activate intracellular signalling cascades, which in turn can modulate ion channel activity and regulate gene expression.

The monoaminergic neurons are generally restricted to the specific nuclei of the CNS, but their projections cover almost the entire brain. They are known to release their neurotransmitters mainly from axonal varicosities (volume transmission) but also from the cell soma and dendrites (somatodendritic release mechanism). Furthermore, monoaminergic neurons usually contain several other compounds such as neuropeptides, glutamate or γ -aminobutyric acid (GABA), which together have been shown to produce more effective response than only one compound (see review Lapish et al. 2007).

Even though the importance of monoamines in different diseases is well known, there are still many open questions about the basic functions of the monoaminergic neurons. For example, at the cellular level, the importance and mechanism of somatodendritic release and corelease of several transmitters is not yet fully understood. Furthermore, it is not known, in which circumstances different monoamine receptor subtypes are activated, and what is the net effect of several subtype-specific intracellular cascades. At the level of the entire brain, it is not known, how monoamines tune the activity of different brain regions, and how this tuning sets the limits for neuronal activity carried out by fast ionotropic receptors. Finally, monoaminergic receptors are thought to control homeostatic balance of the body, and therefore, it would be important to know, how these neurons recognize the imbalance in homeostasis, and whether or not they are able to normalize it.

The thesis consists of two parts, the main focus being the brain histaminergic neurons. In the first part, the culture system for histaminergic neurons was created, and the morphology, histamine storage and colocalization with other compounds in the histaminergic neurons were studied in this system. In the second part, the region-specificity, reversibility and nature of kainic acid (KA)-induced neuronal death was examined in organotypic slice culture of the developing hippocampus. Moreover, the coculture system of the hippocampus and posterior hypothalamus was created to study whether or not histaminergic neurons regulate KA-induced epileptiform activity and neuronal death in the developing rat hippocampus.

2. REVIEW OF LITERATURE

2.1. The central histaminergic neurons

2.1.1. Histamine – a monoamine

Histamine (4-imidazolyl-2-ethylamine) belongs chemically to the monoamines, which contain one amino group connected to an aromatic ring by a two carbon chain (Fig.1). The other monoamines and also neurotransmitters in this group are for example dopamine, serotonin, noradrenaline, and adrenaline.

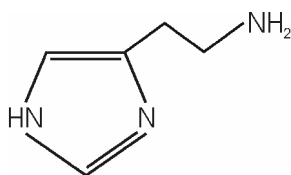


Figure 1. Molecular structure of histamine

Monoamines are synthesized from aromatic amino acids such as histidine, tyrosine, phenylalanine, and tryptophan. They mediate their action mainly by binding to the metabotropic monoamine receptors, which in turn activate a second-messenger cascade inside the cell. In the brain, cell bodies of the monoaminergic neurons are usually located to a restricted region, from where they spread their projections to other parts of the brain.

2.1.2. A brief history of finding histamine

Histamine was first synthesized in 1907 (Windaus and Vogt, 1907), and a few years later the British pharmacologist, Sir Henry Dale and his colleagues isolated histamine from the plant fungus ergot, and described for the first time the physiological role of histamine in muscle contraction and capillary dilation (Barger and Dale 1910, Dale and Laidlaw 1910). One year later they isolated histamine from the wall of the intestine demonstrating that histamine is actually an endogenous substance in the organism (Dale and Laidlaw 1911). In 1919, Dale and Laidlaw introduced the physiological role of histamine in the organism by administering histamine intravenously, which caused bronchoconstriction and shock-like syndromes (Dale and Laidlaw 1919). In 1920, histamine was shown to be a mediator of acid secretion in the stomach (Popielski 1920). During the 1930s, the search for antihistamines began at the Pasteur Institute, and in 1936 antihistamines proved to be effective in bronchospasm produced in guinea pigs by anaphylaxis or administration of histamine (Bovet and Staub, 1936).

Although histamine was known to be present in the brain tissue as early as 1919 (Abel and Kubota, 1919), its role as a neurotransmitter became evident only in the 1970s, when decrease in L-histidine decarboxylase (HDC) activity was discovered in many brain regions after the lesions of the lateral hypothalamic area (Garbarg et al. 1974). Ten years later, the brain histaminergic system was revealed by antibodies against histamine (Panula et al. 1984) and HDC (Watanabe et al. 1983).

Today, histamine is known to be localized in the gastric enterochromaffin-like cells (see reviews Chen et al. 1999), and in the basophils and mast cells both in the periphery and the brain (see reviews MacGlashan 2003, Bischoff 2007). The central histaminergic neurons, which synthesize histamine, are located to the tuberomammillary nucleus (see review Haas and Panula 2003). In addition, histamine containing neurons, which do not express HDC, have been found in the suprachiasmatic nucleus (Michelsen et al. 2005).

2.1.3. Organization of the brain histaminergic system

The central histaminergic neuron system can be found throughout the animal kingdom including mollusc (Elste et al. 1990, Soinila et al. 1990, Karhunen et al. 1990), insects (see review Nässel 1999), fish (Inagaki et al. 1991, Kaslin and Panula 2001), amphibians (Airaksinen and Panula 1990), reptiles (Inagaki et al. 1990a), and mammals such as rat (Watanabe et al. 1983, Panula et al. 1984), mouse (Airaksinen et al. 1992, Parmentier et al. 2002, Michelsen and Panula 2005), ground squirrel (Sallmen et al. 1999), sheep (Tillet et al. 1998), and human (Airaksinen et al. 1991, Jin et al. 2002, see review Yanai et al. 2007).

2.1.3.1. The tuberomammillary nucleus

In the brain, cell bodies of the neurons, which synthesize histamine, are localized in the posterior hypothalamus, and more specifically, in the tuberomammillary (TM) nucleus. The TM nucleus is not a single group of cells but more like scattered subgroups of neurons located to the posterior hypothalamic region. In the rat brain, these subgroups were first described as 600 neurons of medial subgroup of the TM (TMM), 1 500 neurons of the ventral subgroup of TM (TMV), and the diffuse part of the TM (TMdiff) signifying about 100 neurons diffusively scattered within the posterior hypothalamic area (Ericson et al. 1987). TMV was further divided into the rostral (TMVr) and caudal (TMVc) parts with relation to mammillary bodies. The total number of the TM histaminergic neurons estimated by Ericson and co-workers (1987) was 2200.

The classification of the subgroups was further developed to be equivalent to the nomenclature of other monoaminergic neurons, and according to this classification histaminergic neurons in rat are now divided to five clusters known as E1-E5 (Inagaki et al. 1990b, Zimatkin et al. 2006) (Fig. 2). The E1 and E2 groups are localized in the lateral region of the mammillary body, and the caudal E1 group is separated from the rostral E2 group by the lateral mammillary nucleus. The E3 and E4 groups are neuron clusters

in the ventromedial and dorsomedial regions of the mammillary body, respectively. The group E5 consists of diffusely distributed neurons between the E2 and E4 groups (Fig. 2).

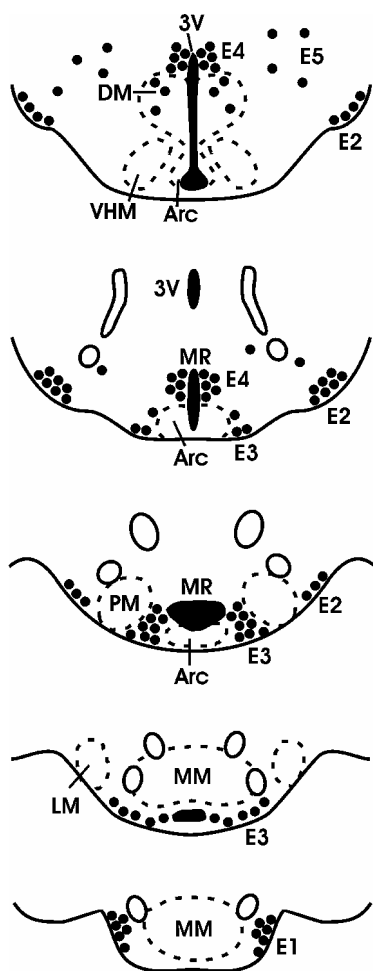


Figure 2. Series of schematic drawings of frontal sections through the posterior hypothalamic region of the rat illustrating the topographic localization of histaminergic neurons and subgroups E1-E5. Abbreviations: Arc=arcuate nucleus, DM=dorsomedial hypothalamic nucleus, LM=lateral mammillary nucleus, MM=medial mammillary nucleus, PM=pre-mammillary nucleus, 3V=third ventricle, VHM=ventromedial hypothalamic nucleus. (Modified from Wada et al. 1991, Zimatkin et al. 2006)

2.1.3.2. Histaminergic pathways

In the rat brain, histaminergic fibres are shown to form two ascending and one descending histaminergic pathways (Panula et al. 1989) (Fig. 3). The ventral ascending pathway leaves the TM nucleus ventrally innervating the major hypothalamic nuclei, nucleus of the diagonal band, septum and olfactory bulb (pathway 1 in Fig. 3), while the dorsal pathway leaves the TM nucleus dorsally and follows the lateral side of the third ventricle innervating thalamus, hippocampus, septum, and rostral forebrain structures (pathway 2 in Fig. 3). The descending pathway innervates the midbrain, brain stem, cerebellum and

spinal cord (pathway 3 in Fig. 3). Despite the prominent histaminergic pathways, there seems to be no selectivity between histaminergic subgroups and innervation of the certain brain areas (Ericson et al. 1987, Panula et al. 1989). On the contrary, histaminergic neurons of one subgroup may send fibres to different directions.

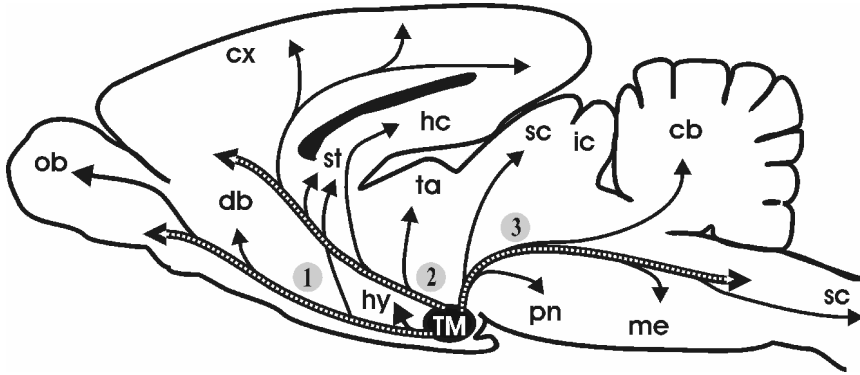


Figure 3. Schematic illustration of the histaminergic pathways in the rat brain. Abbreviations: cb=cerebellum, cx=cortex, db=diagonal band, hc=hippocampus, hy=hypothalamus, ic=inferior colliculus, me=medulla, ob=olfactory bulb, pn=pons, sc=superior colliculus, sp=spinal cord, st=striatum, ta=thalamus, TM=tuberomammillary nucleus (modified from Panula et al. 1989).

2.1.3.3. Afferent fibres

The best studied input to the TM histaminergic neurons comes from the ventrolateral preoptic nucleus (VLPO) located to the anterior hypothalamus (Ericson et al. 1991a, Sherin et al. 1998, Steininger et al. 2001), and from the hypocretin/orexin neurons located to the lateral hypothalamus (Bayer et al. 2001, Ishizuka et al. 2002). Together these cell groups constitute a key center for sleep regulation (see page 33).

The other prominent afferent inputs to the TM histaminergic neurons arise from prefrontal cortex (more specifically from the infralimbic cortex), lateral septum, diagonal band of Broca, and from brain stem adrenergic, noradrenergic, and serotonergic cell groups (Wouterlood et al. 1987, Wouterlood et al. 1988, Ericson et al. 1989, Ericson et al. 1991a). For example, adrenergic and noradrenergic neurons inhibit GABAergic inhibitory postsynaptic potential (IPSP) in the histaminergic neurons (Stevens et al. 2004), and serotonin has excitatory effect on tuberomammillary neurons by activation of $\text{Na}^+/\text{Ca}^{2+}$ -exchange (Eriksson et al. 2001).

2.1.3.4. Morphology of the histaminergic neurons

The morphology of histaminergic neurons has been described both *in vivo* and *in vitro* using both light and electron microscopy (Hayashi et al. 1984, Wouterlood et al. 1986, Ericson et al. 1987, Reiner et al. 1988, Airaksinen et al. 1991, Bajic et al.

2004, Zimatkin et al. 2006) (Table 1). The antibody against HDC shows that HDC is distributed diffusively throughout the perikarya, dendrites, and axon (Hayashi et al. 1984). The staining with histamine antibody shows that histamine is located to the vesicles, which are distributed throughout the cell (Michelsen and Panula 2002). The size of the histaminergic neurons varies from small (diameter 15-18 μm) to large (30-35 μm) (Table 1). In the diffuse subgroup E5, histaminergic neurons seem to be larger than in the subgroups E1-E4, in which small and intermediate cells predominate (Zimatkin et al. 2006). The histaminergic cell body has been described as oval or fusiform with two primary dendrites, or as multipolar cells with several neurites (Fig. 4, Table 1). Moreover, most of the neurons in the E1-E3 subgroups are round, while those in the E5 subgroup seem to be fusiform in shape (Zimatkin et al. 2006).

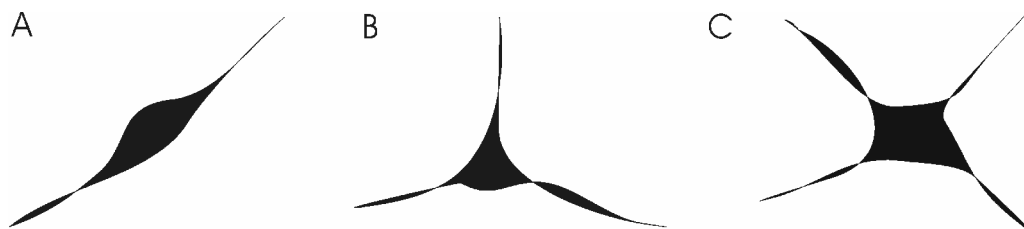


Figure 4. Different types of cell forms of histaminergic neurons. A, Oval or fusiform type. B and C, multipolar types of cells.

Dendrites of histaminergic neurons are usually long and constitute numerous branches (Hayashi et al. 1984, Ericson et al. 1987, Reiner et al. 1988) (Table 1). They also have enlargements or varicosities, which are full of histamine-containing vesicles (Hayashi et al. 1984, Airaksinen et al. 1987, Reiner et al. 1988). One histaminergic dendrite seems to be thick, while the others are thinner, and dendrites have occasionally spines (Wouterlood et al. 1986) (Table 1). Axons are usually very thin and mostly unmyelinated (Hayashi et al. 1984, Wouterlood et al. 1986). Histaminergic axons rarely form synaptic contacts with other cell types (Hayashi et al. 1984, Wouterlood et al. 1986).

Table 1. Common characteristics of the central histaminergic neurons. Abbreviations: DIV=days in vitro, EM=electron microscope, HDC=histidine decarboxylase, LM=light microscope, MAO-B=monoamine oxidase B.

Immuno marker	Animal	Form of the cell body	Size of the cell	Dendrites	Axon	Varicosities	Reference
HDC (LM, EM)	rat (adult)	round, fusiform, triangular	mostly large (25-30 μ m in diameter)	2-4 (mostly less than 7 μ m in diameter, many of them long, few spines, receive synaptic contacts	thickness of varicosities 0.5-2 μ m, branches, vesicles (48-55 nm), mostly unmyelinated (0.6 μ m in diameter), few synapses	in some dendrites	Hayashi et al. 1984
HDC (LM, EM)	rat (adult)	oval (large), fusiform (medium)	large (long axis 30-35 μ m, short axis 12-22 μ m), medium (long axis 15-20 μ m, short axis 10-15 μ m)	one thick (7-8 μ m), 1-2 thinner (3-5 μ m); few spines; receive synaptic contacts	presumed axon is thin, mostly arises from primary (thick) dendrite, varicosities, no synaptic contacts detected	in thin axons	Wouterlood et al. 1986
HDC (LM)	rat (adult)	round or oval (large), round or fusiform (small)	large (diameter > 25 μ m), medium (18-25 μ m), small (<18 μ m)	long (up to 300 μ m), branches rare, no spines	not specified	not studied	Ericson et al. 1987
HDC (LM)	cultures from P0 rat, 15-37 DIV	round, oblong, multipolar	not measured	2-3 primary dendrites, branches, long, no spines	presumed axons arises mostly from dendrite, varicosities	in neurites but not specified	Reiner et al. 1988
histamine (LM)	human (adult)	oval, multipolar (large), bipolar (small)	20-40 μ m, majority of the cells 25-30 μ m	in multipolar cells 3-6 thick primary dendrites, smaller cell bipolar	not specified	in neurites but not specified	Airaksinen et al. 1991
HDC (LM)	cultures from P3 rat, up to 90 DIV	fusiform, multipolar	5-32 μ m in diameter (mean 16.3 μ m)	not specified	not specified	not studied	Bajic et al. 2004
Histamine, MAO-B (LM)	rat (adult)	round, fusiform	16.2-58 μ m in diameter	not specified	not specified	not studied	Zimatkin et al. 2006

2.1.3.5. Other compounds in the histaminergic neurons

In addition to histamine and HDC, the tuberomammillary histaminergic neurons contain several other compounds including GABA (Ericson et al. 1991b, Airaksinen et al. 1992, Trottier et al. 2002), peptides galanin (Staines et al. 1986, Köhler et al. 1986, Airaksinen et al. 1992), substance P (Köhler et al. 1985, Airaksinen et al. 1992), thyrotropin releasing hormone (Airaksinen et al. 1992), heptapeptide met-enkephalin-arg-phe (Airaksinen et al. 1992), and enzymes glutamic acid decarboxylase (GAD) (Vincent et al. 1983, Takeda et al. 1984, Staines et al. 1986), more specifically GAD67 (Esclapez et al. 1993), GABA-transaminase (Nagai et al. 1983), and adenosine deaminase (Nagy et al. 1984, Bajic et al. 2004).

Some of the colocalized substances seem to be present in all histaminergic neurons, whereas the others are colocalized only in a subset of the histaminergic neurons. Moreover, the degree of coexistence seems to depend on species. For example, GABA or GAD is colocalized with histamine in most histamine or HDC immunoreactive neurons in the rat brain (Takeda et al. 1984, Airaksinen et al. 1992), whereas not more than 70-90 % of human HDC immunoreactive neurons are immunopositive to GAD67 (Trottier et al. 2002). Galanin immunoreactivity is found in 28 % of the rat histamine immunopositive neurons (Airaksinen et al. 1992), whereas galanin is not found in the human HDC immunopositive neurons (Trottier et al. 2002).

2.1.3.6. Electrophysiology of the histaminergic neurons

Histaminergic neurons fire spontaneously in a slow (2-5 Hz), regular, pacemaker fashion. They have broad action potentials (1.8 ms mid-amplitude duration), and deep (15-20 mV) long-lasting (100-200 ms) afterhyperpolarization (see review Stevens et al. 2001). Firing of the histaminergic neurons vary across the sleep-wake cycle being highest during the waking state, slowing during the slow-wave sleep, and stopping during the rapid eye movement (REM) sleep (Takahashi et al. 2006).

Histaminergic neurons are under tonic inhibition by histamine acting on histamine 3 (H3) receptors (see review Arrang et al. 2007). Therefore, application of the H3 receptor antagonist increases firing of the histaminergic neurons *in vitro*, whereas histamine itself reduces firing or has no effect (see review Brown et al. 2001). In addition, GABA inhibits firing of the histaminergic neurons by acting on both GABA_A and GABA_B receptors (Yang and Hatton 1997, Stevens et al. 1999). Other modulatory effects (mainly depolarizing) on the histaminergic neurons are induced by glutamate, acetylcholine, serotonin, and orexin (Uteshev et al. 1996, Eriksson et al. 2001a and b, see review Brown et al. 2001).

2.1.4. Metabolism and transport of histamine

2.1.4.1. Histidine transport and uptake to the brain

Histidine, a precursor of histamine, is an essential amino acid because of the imidazole ring, which can not be synthesized in the mammalian organism (see review Reeds 2000).

Therefore, histidine must be received from food proteins, and as part of a dipeptide or as a single amino acid, it is further absorbed from the small intestine to the circulation (Moriarty et al. 1984, Anagnostines et al. 1984).

In order to get to the brain, histidine must pass the blood brain barrier, in which at least three different amino acid transport systems are responsible for the histidine transport. The most important transport system is the large essential neutral amino acid transport system (system L1 or LAT1). The other systems, which also participate in histidine transport, are the facilitative transport system of cationic amino acids (system y⁺), and the facilitative transport system for glutamine (system n) (see review Hawkins et al. 2006) (Fig. 5).

Inside the brain, system A subtypes 1 (SNAT1=sodium-coupled neutral amino acid transporter 1) and 2 (SNAT2) are important in neuronal transport of histidine, while system N subtype 3 (SNAT3) is responsible for histidine transport to glia cells (see review MacKenzie and Erickson 2004). All these systems are not histidine specific, but more specific system, the brain peptide/histidine transporter has been cloned (Yamashita et al. 1997). This system is strongly expressed in several brain regions in neuronal and non-neuronal cells but also outside the CNS. Whether this system has any specific role in the histaminergic neurons is unknown.

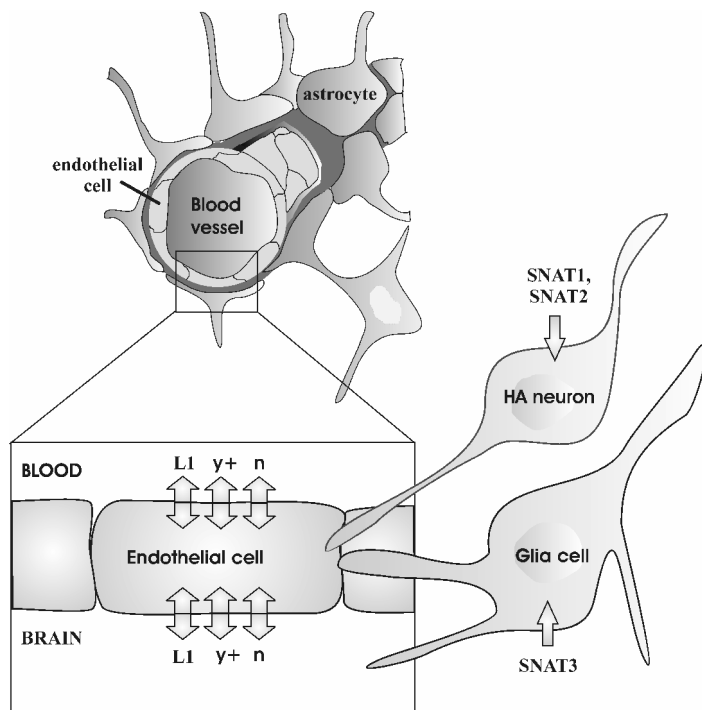


Figure 5. Uptake systems for histidine. Histidine travels to the brain in blood, from where endothelial cells of blood vessels take it up by system L1, y⁺ and n. In the brain, histidine is taken up by neurons and glia cells by SNAT1, 2 and 3. Abbreviations: L1=large essential neutral amino acid transport system, y⁺=facilitative transport system of cationic amino acids, n=facilitative transport system for glutamine (system n), SNAT=sodium-coupled neutral amino acid transporter. (Based on Mackenzie and Erickson 2004, Hawkins et al. 2006).

2.1.4.2. Histamine synthesis

In those cells, in which histamine operates as a signalling molecule, L-histidine is converted to histamine by HDC (EC 4.1.1.22.) in a single step decarboxylation reaction (Joseph et al. 1990, Bruneau et al. 1992) (Fig. 6). In the brain, HDC is found in neurons of the TM nucleus and in the brain mast cells (Martres et al. 1975, Watanabe et al. 1983). HDC activity is highest in the hypothalamus, in which the cell bodies of the histaminergic neurons are located (Watanabe et al. 1983). Its activity is also high in many other brain areas such as upper brain stem, amygdala, bed nucleus of the stria terminalis, hippocampus and cerebral cortex. These are the areas, in which the histaminergic projections are abundant. HDC activity in the hypothalamus, near the histaminergic cell bodies, has been detected in the cytoplasm, whereas outside the hypothalamus, HDC is mainly found in the cytoplasm of isolated nerve endings (Watanabe et al. 1983). Histamine synthesis can be specifically blocked by α -fluoromethylhistidine (α -FMH), which is a time- and concentration-dependent irreversible inhibitor of histamine synthesis (Garbarg et al. 1980, Watanabe et al. 1990).

2.1.4.3. Histamine storage

After synthesis, histamine is transported to the subcellular vesicles, in which it is protected from degradative enzymes, and becomes more concentrated before it is released. A protein responsible for histamine transport to the subcellular vesicles is a vesicular monoamine transporter 2 (VMAT2) (earlier termed synaptic vesicle transporter) (Erickson et al. 1995, Merickel and Edwards, 1995, Erickson et al. 1996). VMAT2 together with the vesicular monoamine transporter 1 (VMAT1) transports all monoamines to the storage vesicles without certain substrate specificity, in contrast to the plasma membrane transporters, which show high substrate specificity (see review Torres et al. 2003).

VMAT2 is an integral membrane protein having 12 transmembrane domains (TMD), of which TMD 5-8 and 9-12 seem to be required for the high affinity interactions of VMAT2 with monoamines (Peter et al. 1996). Particularly Pro-237 in TMD 5-8 is needed for the high affinity for histamine (Finn III and Edwards 1998). During the transport, VMAT2 uses the pH gradient across the vesicle membrane generated by vacuolar H⁺-ATP-ase to drive the uptake (see review Johnson 1987). In the cell soma, VMAT2 is located to the trans-Golgi network (Nirenberg et al. 1995, 1996, 1997). In the axon, VMAT2 is found in large dense core vesicles (LDCV) and small synaptic vesicles (SSV), and in dendrites, in tubulovesicular structures (Nirenberg et al. 1995, 1996, 1997).

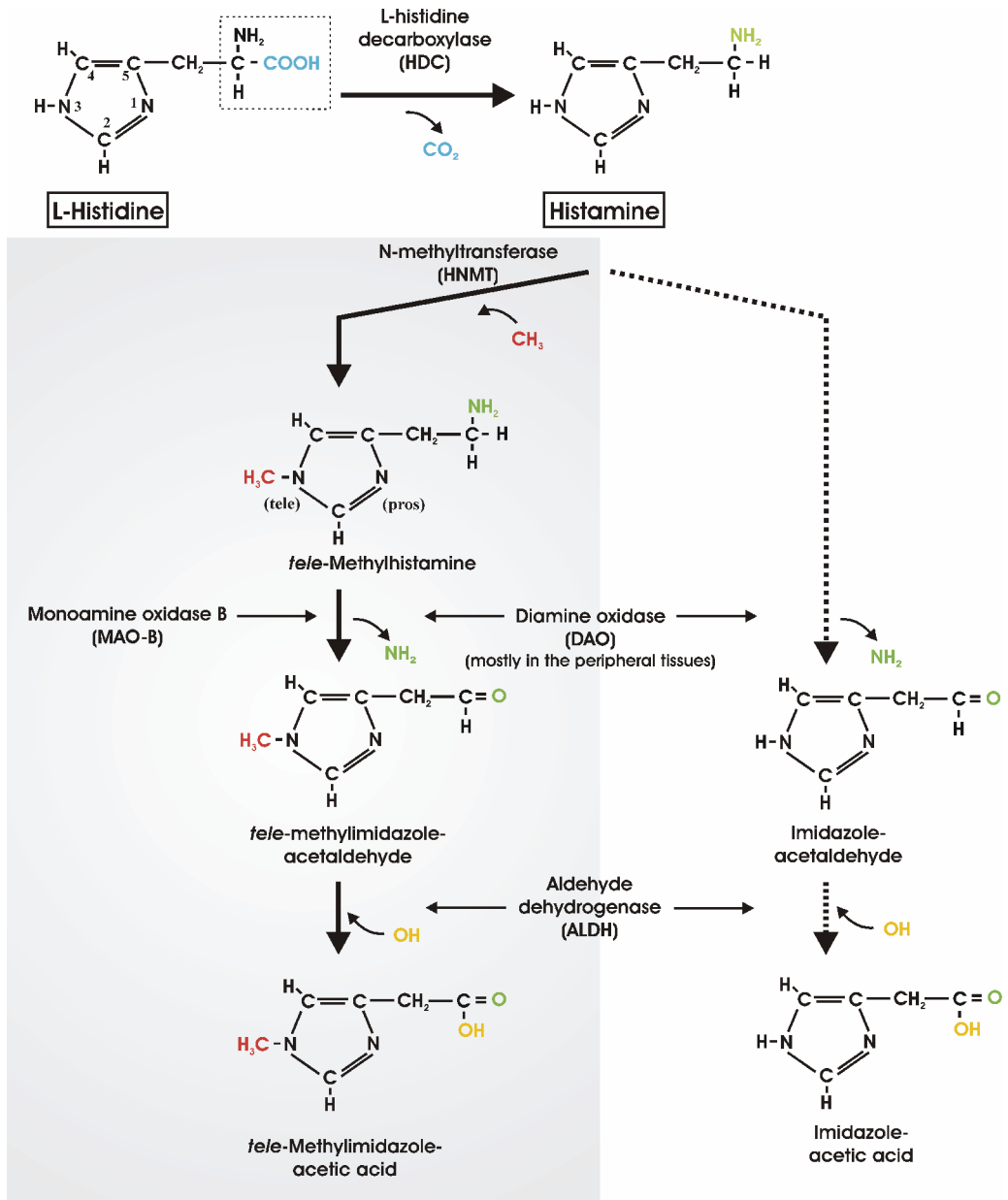


Figure 6. Histamine synthesis and inactivation. The imidazole nitrogen next to the side-chain residue is designed as “pros” and one further “tele”. Grey area indicates the main metabolic pathway occurring in the CNS. (Based on Martres et al. 1975, Hough and Domino 1979, Joseph et al. 1990, Lin et al. 1993, Ambroziak and Maslinski 1998, Kitanaka et al. 2001, Elmore et al. 2002).

2.1.4.4. Histamine release

Tonic histamine release follows a circadian rhythm, which parallels the change in firing rate over the sleep-wake cycle (Mochizuki et al. 1992). In addition, histamine release from the posterior hypothalamus follows much faster, ultradian rhythm, which correlates with the delta and theta bands in the electroencephalographic recordings (Prast et al. 1997).

Tritiated [^3H] histamine synthesized from [^3H] histidine is released from various brain regions by depolarization induced by K^+ ions or electrical stimulus (Verdiere et al. 1975, Arrang et al. 1983, van der Werf et al. 1987). Furthermore, release of [^3H] histamine from depolarized slices is completely inhibited in the absence of Ca^{2+} suggesting that histamine release results from opening of the voltage-sensitive Ca^{2+} channels (Verdiere et al. 1975, van der Werf et al. 1987). The release mechanism is suggested to be similar to those operating for other neurotransmitters.

Morphologically, histamine is suggested to be released mainly from axonal varicosities by the mechanism of volume transmission, since histaminergic neurons rarely form synapses (Hayashi et al. 1984, Wouterlood et al. 1986). However, somatodendritic release mechanism is also possible, since it has been shown to occur in other monoaminergic neurons (Kalivas and Duffy 1991, Heeringa and Abercrombie 1995, see review Adell and Artigas 2004, see review De-Miguel and Trueta 2005, Huang et al. 2007). Whether somatodendritic release takes place also in the histaminergic neurons remains to be studied.

2.1.4.5. Histamine reuptake

After release, monoamine neurotransmitters are usually recycled by taking them back to the cell by the substrate specific plasma membrane transporters (see review Torres et al. 2003). Histamine uptake system in neurons in the CNS has been a question mark, since no substrate specific system has been found so far. However, histamine uptake has been described in several other cell types such as in photo- and mechanoreceptors of arthropods (Stuart et al. 1996, Melzig et al. 1998, Morgan et al. 1999), murine hematopoietic progenitor cells (Corbel et al. 1995), vertebrate astrocytes, and cerebral endothelial cells of the capillaries (see review Huszti 2003). Moreover, studies with HDC knock out (KO) mice have shown that even if the animals can not synthesize histamine, they still have histamine in several tissues such as in brain, skin, stomach and spleen (Ohtsu et al. 2002). In addition, Michelsen and co-workers (2005) described histamine immunoreactivity without HDC immunoreactivity in mouse and rat suprachiasmatic nucleus indicating that these cells might have a specific uptake system for histamine. Recently, by using *in vitro* uptake techniques histamine uptake system has been detected in synaptosomes of the rat brain (Sakurai et al. 2006). These studies indicate that histamine might have a substrate specific uptake system, but further studies are needed to specify whether this

system is specific for only histamine or if it can take up several transmitters as organic cation transporters do (Amphoux et al. 2006).

2.1.4.6. Histamine inactivation

In general, three main types of enzymes including methyltransferase, amine oxidase and aldehyde hydrogenase, are involved in monoamine degradation process (see review Medina et al. 2003). Histamine catabolism in most tissues, including the brain, begins with methylation reaction, in which histamine N-methyltransferase (EC 2.1.1.8.) catalyses the transfer of a methyl group from S-adenosyl-L-methionine to histamine producing N-tele-methylhistamine (Reilly and Schayer 1971, Kitanaka et al. 2001) (Fig. 6). In the CNS and gastric enterocromaffin cells, methylated tele-methylhistamine can be further oxidized by MAO-B (EC 1.4.3.4.) (Hough and Domino 1979, Lin et al. 1993, Okauchi et al. 2004). In many peripheral tissues and to some extent also in the CNS, tele-methylhistamine and histamine can be oxidized by diamine oxidase (previously known as histaminase, EC 1.4.3.6.) (Jimenez Diaz et al. 1950, Elmore et al. 2002).

Oxidized compounds, imidazoleacetaldehyde and tele-methylimidazoleacetaldehyde, are further hydrolyzed by aldehyde dehydrogenase (1.2.1.3.) producing the end products imidazoleacetic acid and tele-methyl imidazoleacetic acid, respectively (Gitomer and Tipton 1983, Ambroziak and Maslinski 1998, Ambroziak and Pietruszko 1991).

2.1.5. Histamine receptors

Released histamine mediates its response mainly through four histamine activated guanosine nucleotide-binding protein-coupled receptors (GPCR). In addition, histamine has been shown to activate chloride channels and N-methyl-D-aspartate (NMDA) receptors (see reviews Hill et al. 1997, Haas and Panula 2003).

GPCRs are among the oldest signal transduction proteins being present in yeast, plants, protozoa, and metazoan (see review Fredriksson and Schiöth 2005). These receptors are activated by a highly diverse set of signals including light, odorants, cations, amines, amino acids, peptides, large proteins, lipids and sugars (see review Deupi and Kobilka 2007).

GPCRs have seven transmembrane domains, which are connected by three intracellular and three extracellular loops. The amino terminus is located in the extracellular space, whereas the carboxyl terminus shows an intracellular location (see review Kobilka 2007). The G protein, which is linked to the transmembrane receptor, consists of α , β and γ subunits. The classification of GPCRs is based on the amino acid identities and functional specialization of their G protein α ($G\alpha$) subunits (see review Simon et al. 1991). After ligand binding, the transmembrane receptor interacts with the G protein inducing a conformational change, which causes guanosine triphosphate (GTP) to displace guanosine diphosphate (GDP). The activated G protein then binds either to an ion channel changing its activity or to an effector enzyme, which in turn may change activity of an ion channel or other GPCRs (indirectly), activate intracellular enzyme

cascades, or regulate gene expression. Distribution and responses of H1, H2, H3 and H4 receptors are reviewed separately.

2.1.5.1. H1 receptor

By using H1 specific radioligands, H1 receptors have been shown to be expressed in various tissues including mammalian brain, smooth muscle in airways, gastrointestinal tract, genitourinary system, cardiovascular system, adrenal medulla, endothelial cells and lymphocytes (see review Hill et al. 1997). In the rat brain, [³H]mepyramine labelling indicates high densities of H1 receptors in bed nucleus stria terminalis, hippocampus, hypothalamus, amygdala, pons, and medulla (Palacios et al. 1981), which mostly agrees with the H1 receptor messenger ribonucleic acid (mRNA) expression pattern (Lintunen et al. 1998). In the hippocampus, high densities of H1 receptor are found in the polymorphic and molecular layer of the CA3 region, and in the polymorphic layer of hilus of the dentate gyrus (DG) (Palacios et al. 1981).

In the CNS, H1 receptor activation is mainly connected to the formation of inositol 1,4,5-trisphosphate (IP3), and elevation of intracellular Ca²⁺ (Daum et al. 1984, Carswell and Young 1986, Claro et al. 1986, Sarri et al. 1995) (Fig.6). H1 receptor belongs to the G_q subfamily of GPCRs (based on the classification of the G α subunit). The members of this subfamily are regulators of phosphatidylinositol-specific phospholipase C (PLC) β isoform, and include subunits G α_q , G α_{11} , G α_{14} , G $\alpha_{15/16}$ (Ho and Wong 2002).

In more detail, H1 receptor mediated activation of PLC leads to the formation of two second messengers, inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG) (Fig.7). The main physiological consequence of IP3 production, as previously mentioned, is the elevation of intracellular Ca²⁺, whereas DAG activates protein kinase C (PKC). Consequences of elevated intracellular Ca²⁺ are diverse: histamine-induced opening of the Ca²⁺-activated non-specific cation channel causes depolarization in vasopressin neurones in the supraoptic nucleus (Smith and Armstrong 1996), whereas opening of the Ca²⁺-dependent K⁺ channels causes hyperpolarization of the C6 glia cells (Weiger et al. 1997). Moreover, H1 receptor-mediated block of a leak potassium conductance results in depolarization in the thalamus (McCormick and Williamson 1991), striatum (Munakata and Akaike 1994), cortex (Reiner and Kamondi 1994), and suprachiasmatic nucleus (Li and Hatton 1996, Li et al. 1999). This has been shown to occur both Ca²⁺-dependently and independently. Alternative pathways (dotted lines in Fig. 7) such as the formation of a Ca²⁺/calmodulin complex and nitric oxide synthetase (NOS) activation, phospholipase A (PLA)-mediated formation of prostacyclin and thromboxane A₂, and modulation of adenylate cyclase (AC) activity have also been shown to be related to H1 receptor activation (Baenziger et al. 1980, Resink et al. 1987, Leurs et al 1994, Hishinuma and Ogura 2000, Li et al. 2003). H1 receptor mediated responses through PLC-DAG-PKC pathway are not well understood but the pathway might be connected to the enhancement of the NMDA receptor activity (Chen and Huang 1992, Payne and Neuman 1997).

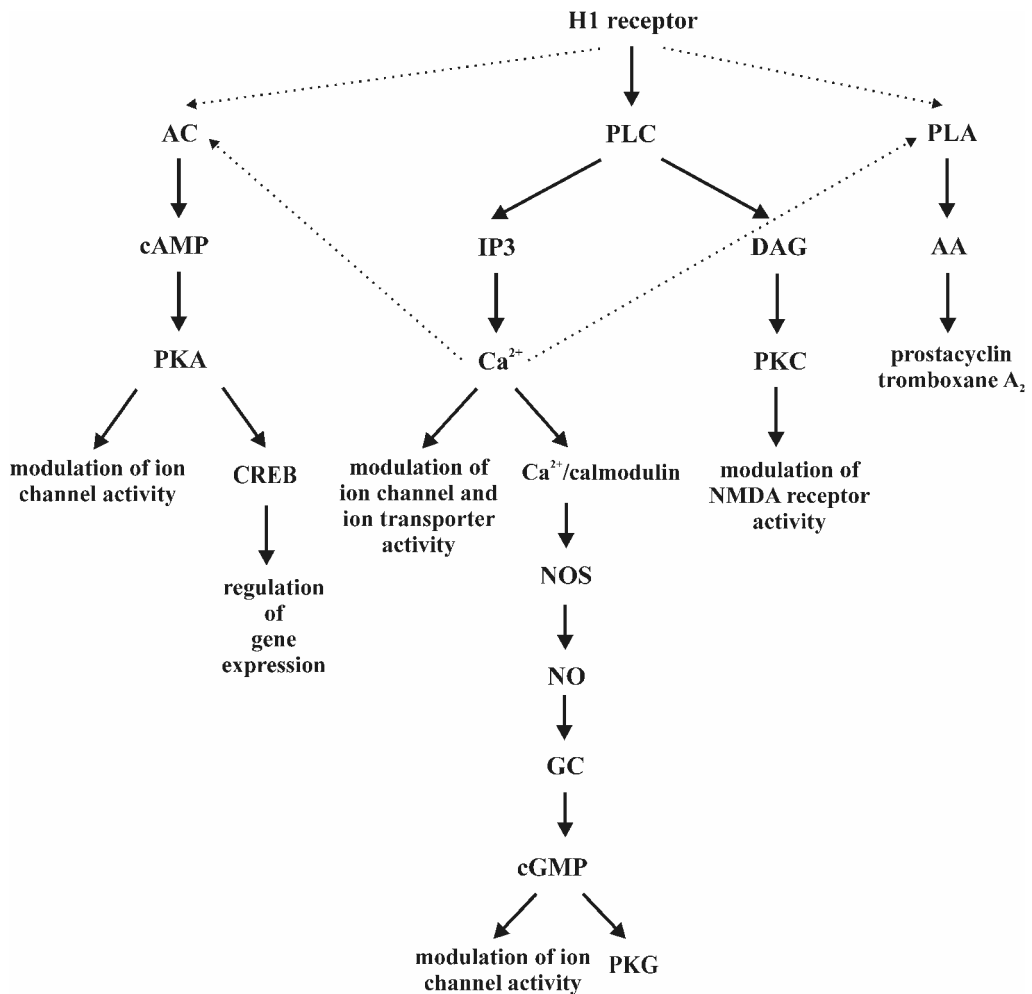


Figure 7. H1 receptor-mediated intracellular pathways. Dotted lines indicate the alternative H1 receptor-mediated pathways in the case of AC or PLA activation. Abbreviations: AA=aracidonic acid, AC=adenylate cyclase, cAMP=cyclic adenosine monophosphate, cGMP=cyclic guanosine monophosphate, CREB=cAMP response element-binding protein, DAG=1,2-diasylglyserol, GC=guanylate cyclase, IP3=inositol 1,4,5-trisphosphate, NO=nitric oxide, NOS= nitric oxide synthetase, PKA=protein kinase A, PKC=protein kinase C, PKG=protein kinase G, PLA=phospholipase A and PLC=phospholipase C. (Based on Daum et al. 1984, Sarri et al. 1995, Smith and Armstrong 1996, Weiger et al. 1997, McCormick and Williamson 1991, Bredt and Snyder 1990, Resink et al. 1987, Leurs et al 1994, Payne and Neuman 1997).

2.1.5.2. H2 receptor

H2 receptor-mediated responses have been detected in the brain, gastric mucosa, cardiac tissue, adipose tissue, airways, uterine, and immune system (see review Hill et al. 1997). [¹²⁵I]-iodopotentidine binding and *in situ* hybridization studies in the guinea pig brain show high H2 receptor densities in superficial cerebral cortex (layers I-III), hippocampus,

basal ganglia, amygdala, bed nucleus stria terminalis, superior colliculus, substantia nigra, ventral tegmental area, and inferior olive (Vizuete et al. 1997). In the rat brain, *in situ* hybridization studies show the most prominent expression of H2 receptor mRNA in hippocampus, piriform cortex, subrachiasmatic nucleus, and cerebellum (Karlstedt et al. 2001). In the hippocampus, highest levels of [¹²⁵I]-iodopotentidine binding were detected in the CA1 region, and the pre- and parasubiculum (Vizuete et al. 1997). Moderate expression of H2 receptors was found in the DG, and low expression in the CA2, CA3 regions, and in the subiculum. *In situ* hybridization studies in the hippocampal region show high H2 receptor mRNA expression in the pyramidal cells of CA1, CA2 and CA3 regions, granular and polymorphic layers of the DG, and in subicular complex both in guinea pig (Vizuete et al. 1997) and rat brain (Karlstedt et al. 2001).

The main H2 receptor signalling pathway is positively coupled to the AC converting adenosine 5'-triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), which acts as a second messenger molecule in the CNS (Palacios et al. 1978, Al-Gadi et al. 1985, Ozawa and Segawa 1988, Agullo et al. 1990) (Fig. 8). G proteins of this subfamily include subunits $G\alpha_s$, $G\alpha_{s(\text{long})}$, $G\alpha_{s(\text{short})}$, $G\alpha_{olf}$ and $XLG\alpha_s$ and they all stimulate AC (Ho and Wong 2002). AC initiates cAMP-protein kinase A (PKA) pathway activation, which may either modulate ion channel activity or regulate gene expression through the cAMP response element-binding (CREB).

Electrophysiological data indicate that histamine activates cAMP-PKA pathway through H2 receptors leading to the suppression of the Ca^{2+} -dependent K^+ current (I_{AHP}), and increase in neuronal excitability (Haas 1984). The second ion current, which has been shown to be regulated by H2 receptor, is the hyperpolarization-activated cation current (I_h) (McCormick and Williamson 1991), which is a slow inward current consisting of Na^+ and K^+ ions, and is turned on by hyperpolarization of the cell. H2 receptor leads to the enhancement in I_h by changing the voltage sensitivity of I_h to more positive, which results in a strong reduction in the response of the neuron to hyperpolarizing current pulses, while the ability of depolarizing current pulses to generate action potentials is slightly enhanced. The third ion current related to H2 receptor activation and PKA phosphorylation modulates an outward current through the $K_{v3.2}$ containing potassium channels (Atzori et al. 2000). $K_{v3.2}$ is a potassium channel subunit, predominantly expressed in the inhibitory interneurons permitting high frequency firing in the hippocampus (Atzori et al. 2000, Lien et al. 2002). H2 receptor mediated modulation of $K_{v3.2}$ subunit occurs through phosphorylation of $K_{v3.2}$ by PKA, which lowers the maximum firing frequency of the inhibitory neurons, which in turn reduces high-frequency population oscillations in the principal cell layer (Atzori et al 2000).

In addition to elevation of cAMP levels via G_s -proteins, H2 receptors have been described to activate PLC via different G proteins leading to increase in both IP3 and intracellular Ca^{2+} levels (Delvalle et al. 1992, Seifert et al. 1992, Kuhn et al. 1996, Koizumi and Ohkawara 1999) (dotted lines in Fig. 8).

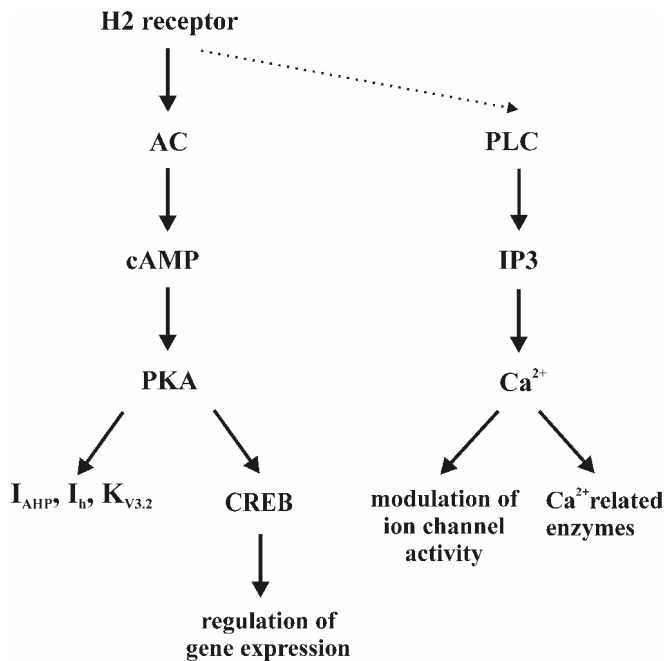


Figure 8. H2 receptor mediated intracellular pathways. Dotted lines indicate the alternative H2 receptor mediated pathways in case of AC or PLA activation. Abbreviations: AA=aracidonic acid, AC=adenylate cyclase, cAMP=cyclic adenosine monophosphate, CREB=cAMP response element-binding protein, IP3=inositol 1,4,5-trisphosphate, PKA=protein kinase A, and PLC=phospholipase C. (Based on Palacios et al. 1978, Haas 1984, Al-Gadi et al. 1985, Ozawa and Segawa 1988, McCormick and Williamson 1991, Atzori et al. 2000)

2.1.5.3. H3 receptor

In the radioligand binding studies, H3 receptors have been detected in the CNS and several peripheral tissues including intestine, pancreas and lung (see review Hill et al. 1997). Functional evidence suggests that H3 receptors are also expressed in sympathetic autonomic nerves and vascular endothelial cells (Hill et al. 1997). H3 receptor mRNA expression studies indicate that H3 receptor may also be expressed in several embryonic tissues including the liver and adipose tissue (Heron et al. 2001, Karlstedt et al. 2003).

In the brain, high or moderate densities of H3 receptor have been shown in cerebral cortex, hippocampus, amygdala, nucleus accumbens, striatum, olfactory tubercles, and substantia nigra (Pollard et al. 1993). The distribution correlates well with the histaminergic innervation suggesting autoregulation of histamine release in these regions. However, mismatches between the histaminergic innervation and the H3 receptor distribution have been detected in the hypothalamus, in which the histaminergic innervation is more abundant than the H3 receptor expression (Pollard et al. 1993). Moreover, in the thalamus, the H3 receptor expression is denser than histaminergic innervation. The high receptor expression and low histaminergic innervation might indicate inhibition of the release of other neurotransmitters through the heteroreceptor-mediated action. In the hippocampus, H3 receptor binding is most abundant in the DG and subiculum (Pollard et al. 1993).

The H3 receptor inhibits formation of cAMP, which indicates that it belongs to the G protein subfamily known as inhibitory G α subunits. These G proteins are negative regulators of AC having three Gai subtypes (Gai₁, Gai₂, Gai₃), and in some cells Gai_z and G α_o , (Ho and Wong 2002).

H3 autoreceptors are located in the histaminergic cell body, dendrites and axon (see review Haas and Panula 2003). Through this autoreceptor function, histamine is able to inhibit its own synthesis and release (see review Arrang et al. 2007). Autoreceptor mediated responses are regulated via the cAMP-PKA pathway (Morisset et al. 2000, Gomez-Ramirez et al. 2002). Finally, H3 autoreceptors show constitutive activity, which means that the intracellular cascades related to H3 receptor can be activated without receptor activation by a specific agonist (see review Arrang et al. 2007).

H3 receptors are also located to the other cell types and this heteroreceptor function of the H3 receptor is related to inhibition of synthesis and release of glutamate (Brown and Haas 1999, Garduno-Torres et al. 2007), GABA (Garcia et al. 1997, Arias-Montano et al. 2001), acetylcholine (Arrang et al. 1995), noradrenalin (Schlicker et al. 1992, Yamasaki et al. 2001, Seyedi et al. 2005), dopamine (Schlicker et al. 1993, Molina-Hernandez et al. 2000, Sanchez-Lemus et al. 2004), and serotonin (Schlicker et al. 1988, Schworer et al. 1994).

The other intracellular pathways related to H3 receptor include release of aracidonic acid (AA) (Morisset et al. 2000, Rouleau et al. 2002), the mitogen-activated protein kinase (MAPK) pathway (Drutel. et al. 2001), and negative coupling to PLC (Cherifi et al. 1992).

2.1.5.4. H4 receptor

H4 receptor is expressed at high levels in blood mononuclear cells and in such tissues, which contain high concentrations of blood cells (Liu et al. 2001, Zhu et al. 2001). For example, bone marrow and lung express H4 receptors. In the brain, the *in situ* hybridization studies show H4 receptor mRNA expression in the hippocampus (Zhu et al. 2001).

Similarly to the H3 receptor, the H4 receptor belongs to the family of the G_{i/o} coupled receptors. This has been verified by cloning the H4 receptor, and also with studies carried out in transfected cells, which show that H4 receptor inhibits cAMP accumulation (Nakamura et al. 2000, Oda et al. 2000, Liu et al. 2001).

Physiological significance of the H4 receptor is not yet fully understood. In the first place, it seems to be related to the immune system. H4 receptor antagonists have shown promising effects on down-regulating immune responses in various animal disease models including acute inflammation, hapten-mediated colitis, and allergic airway inflammation (see review Zhang et al. 2007).

2.1.5.5. Ionotropic receptors regulated by histamine

In the insect eye and lobster olfactory receptor neurons, histamine directly activates a chloride channel (Hardie 1989, McClintock et al. 1989, Gengs et al. 2002, Witte et al. 2002). Whether these channels are also activated by GABA remains to be studied. In the

vertebrate nervous system, oxytocin neurons of the supraoptic nucleus respond to single TM stimuli with fast inhibitory postsynaptic potentials (IPSPs), whose kinetics resemble those of GABA_A receptors (Hatton and Yang 2001). Recently, Saras and co-workers (2008) reported that histamine can potentiate (and possibly even gate) GABA_A receptor responses in *Xenopus* oocytes.

Histamine also enhances NMDA receptor-mediated currents in acutely isolated and cultured hippocampal pyramidal cells (Bekkers 1993, Vorobjev et al. 1993). This effect could not be blocked by any of the H1, H2 or H3 antagonists suggesting that histamine acts primarily on the polyamine site of the NMDA receptor. However, the enhancing effect shown in cell cultures has been difficult to repeat in acute hippocampal slices (Saybasili et al. 1995, Bekkers et al. 1996). One explanation for that might be the pH dependence of this phenomenon (Saybasili et al. 1995).

2.1.6. Postnatal development of the brain histaminergic system

In the rat brain, histamine content is usually low at birth but increases rapidly until postnatal day 4 (P4) (Pearce and Schanberg 1969) (Fig. 10) or P10 (Hough et al. 1982). In study of Martres and co-workers (1975), the brain histamine content is at the maximal level already at P1. After rapid increase, the brain histamine content decreases to the adult levels during the first 2-3 weeks. On the contrary, the HDC activity is low at P5 increasing very slowly until P16, thereafter its rapid increase lasts until P30, when the adult level of the HDC activity is reached (Martres et al. 1975, Ryu et al. 1995). The discrepancy between the high histamine content and the low HDC activity during development is not fully understood, but one explanation can be histamine storage in mast cells, which has been discussed in several studies (Martres et al. 1975, Hough et al. 1982). Indeed, high histamine levels seem to be distinct from neuronal histamine since the synaptosomal fraction of histamine increases from P5 until P30 (Martres et al. 1975). This indicates that levels of the neuronal histamine increase with the same pattern as the HDC activity, which in turn correlates well with the maturation of the histaminergic cells, fibres, and histamine receptors.

The histamine-immunoreactive neurons can be detected at the earliest at the embryonic day 13 (E13) (Auvinen and Panula 1988). During the first postnatal week, the histaminergic neurons occur in the three magnocellular nuclei, and by P14, the histamine-positive neurons show adult-like distribution (Auvinen and Panula 1988).

The first histaminergic fibers can be detected around the histaminergic cell bodies at E15 (Auvinen and Panula 1988). At P1, densely packed fibres are seen in the hypothalamus, olfactory bulb and nucleus, septum and supraoptic nucleus. Fewer fibers are detected for example in the thalamus, cortex and amygdala. In the hippocampus, the histaminergic innervation can be detected only in the most temporal parts. By P14, the histaminergic innervation increases throughout the cerebral cortex having a very high or high density also in supraoptic and olfactory nucleus, diagonal band of Broca,

hypothalamus, septum, bed nucleus stria terminalis, amygdala, substantia nigra, and vestibular nucleus (Auvinen and Panula 1988). Temporal parts of the hippocampus receive moderate density of histaminergic innervation.

The H1 receptor binding is first detected in the hypothalamus at P2 (Ryu et al. 1995). From P2 until P9 is the period of slow increase in the H1 receptor expression in the hypothalamus, cortex, striatum, hippocampus, amygdala, thalamus and substantia nigra. From P9 to P16, the H1 receptor expression rapidly increases to the adult levels in the hypothalamus, hippocampus and amygdala (Fig. 9), whereas in the other studied brain areas the H1 receptor densities remain quite low. Moreover, although the histamine-induced phosphoinositide hydrolysis is present already in the newborn rat, the potency of histamine to induce the H1 receptor-specific second messenger activation gradually decreases with the age (Claro et al. 1987).

The H3 receptor binding is not detectable until P9 (Ryu et al. 1995). At P9, the densities of H3 receptor are higher in the substantia nigra than in other brain regions. The H3 receptor binding is gradually increased reaching the adult levels by P16 in the substantia nigra, and in other brain regions such as the cortex, striatum, thalamus, hypothalamus, hippocampus and nucleus accumbens by P23 (Fig. 9).

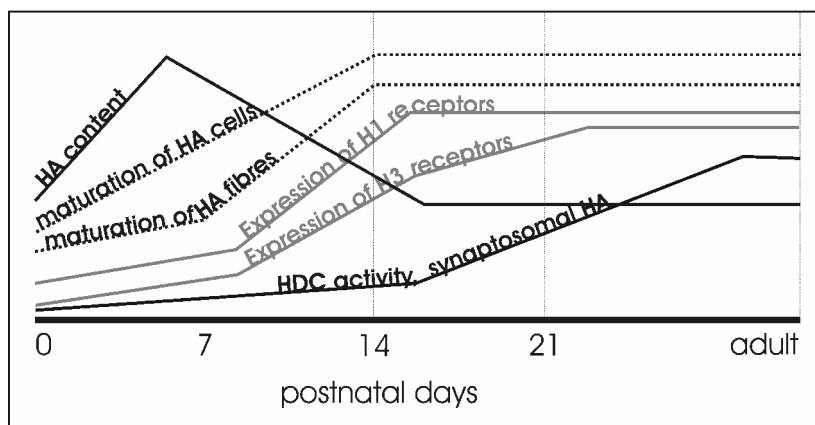


Figure 9. Schematic picture of the development of the central histaminergic system. Abbreviations: H1 receptor=histamine 1 receptor, H3 receptor=histamine 3 receptor, HA=histamine, HDC=histidine decarboxylase. (Based on Pearce and Schanberg 1969, Martres et al. 1975, Auvinen and Panula 1988, Ryu et al. 1995).

2.1.7. Physiological significance of the central histaminergic system

In several studies, histaminergic neurons have been shown to be involved in the regulation of brain functions related to arousal, sleep, attention, learning and memory. Furthermore, location of the histaminergic neuron system in the posterior hypothalamus makes it a powerful modulator in several hypothalamic nuclei. In this way the histaminergic system

can participate in the regulation of for example body temperature, blood pressure, liquid and food consumption, and hormonal release.

2.1.7.1. Arousal and sleep regulation

The sedative effect of the first generation antihistamines already indicated that histamine is involved in the regulation of alertness (Lin et al. 1988, Saitou et al. 1999, Kaneko et al. 2000). In the control of sleep-wake rhythmicity, the suprachiasmatic nucleus (SCN) is suggested to play a key role, since the circadian clock genes of the SNC are switched on and off according to the endogenous rhythmicity (see reviews Buijs and Kalsbeek 2001, Mignot et al. 2002) (Fig. 10). Furthermore, neurons of the SCN control directly the wake-promoting orexin neurons of the lateral hypothalamus (LH), and sleep promoting GABAergic/galaninergic of the ventrolateral preoptic area (VLPO) (see review Mignot et al. 2002). Hypocretin/orexin containing neurons in turn increase histamine release, and induce wakefulness (Bayer et al. 2001, Eriksson et al. 2001, Huang et al. 2001, Ishizuka et al. 2002), while sleep-promoting VLPO neurons have inhibitory effect on the histaminergic neurons (Yang and Hatton 1997, Shering et al. 1998, Stevens et al. 1999, see review Mignot et al. 2002) (Fig. 10). Histamine together with the other monoamines controls thalamocortical activity causing desynchronization during wakefulness and synchronization during sleep (see reviews Steriade 1996, Pace-Schott and Hobson 2002).

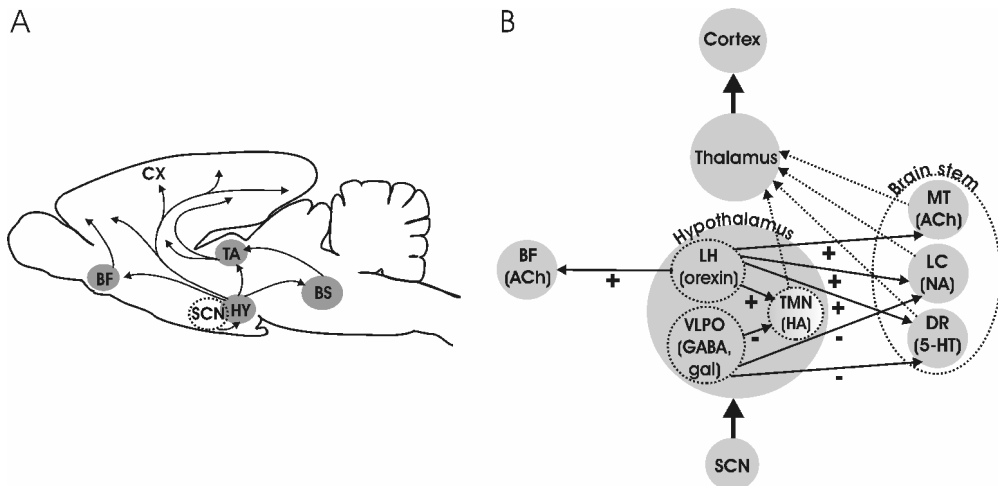


Figure 10. Tuberomammillary histaminergic (HA) neurons participate in sleep regulation. A, A schematic picture of the rat brain, and the approximate location of the brain regions, which are involved in sleep regulation. Suprachiasmatic nucleus (SCN) controls diurnal activity of different cell groups in the hypothalamus. B, A schematic picture of the relationships between different brain regions which are involved in the sleep-wake regulation. Wake-promoting orexin neurons in the lateral hypothalamus (LH) activate histaminergic (HA) neurons in the tuberomammillary nucleus (TM), noradrenergic (NA) neurons in the locus coeruleus (LC), serotonergic (5-HT) neurons in the dorsal raphe (DR), and cholinergic (ACh) neurons in the basal forebrain (BF) and mesopontine tegmentum (MT). Sleep promoting GABAergic/galaninergic (gal) in the ventrolateral preoptic area (VLPO) in turn inhibit histaminergic, noradrenergic and serotonergic neurons. Abbreviations: BS=brain stem, CX=cortex, HY=hypothalamus, TA=thalamus. (Modified from Mignot et al. 2002).

2.1.7.2. Attention, learning and memory

A well focused attention is an essential condition for learning and memory processes. A recent theory about attention suggests that attention is controlled by a set of independent, anatomically separate networks which control differentially intrinsic alertness, phasic alertness, orientation, and execution (which are separated functions of attention) (Posner et al. 2006, see review Raz and Buhle 2006, Fan et al. 2007). According to this definition, histamine is involved at least in the intrinsic alertness, as it was discussed in the previous chapter. Otherwise, role of histamine in modulation of attention has been studied in animal models of the passive avoidance and five-choice reaction time test, in which H3 receptor antagonists (i.p.), which increase brain histamine levels, enhance attention (Ligneau et al. 1998).

Spatial learning and memory, in which the hippocampus plays a major role, are improved after the bilateral lesion of the TM nucleus (Frisch et al. 1998). In accordance with that, HDC KO animals display improvement in the water maze performance (Dere et al. 2003). Furthermore, the H1 receptor antagonist ameliorates spatial learning in old rats indicating that histamine mediated effects occur through H1 receptors (Hasenöhrle et al. 1999). Also in the contextual fear conditioning, which measures learning skills primarily based on activation of the amygdala, injection the H3 receptor agonist to the basolateral amygdala improves learning and memory (Cangioli et al. 2002), while and the H3 antagonist induces an amnesic effect, which is suggested to be caused by decrease in release of acetyl choline mediated by H2 receptor (Passani et al. 2001).

On the contrary to spatial learning, object recognition and social memory are impaired after the α -FMH and the H3 receptor agonist treatment and in the HDC KO mice, which all decrease brain histamine levels (Prast et al. 1996, Giovannini et al. 1999, Dere et al. 2003), whereas an injection of histidine, histamine or the H3 receptor antagonist induces the opposite effect (Prast et al. 1996, Giovannini et al. 1999).

In conclusion, different learning protocols seem to provide different results, which might be connected to the brain region related to certain learning protocol and type of histamine receptors, which are expressed in that brain region. In addition, when H3 receptor function is modulated by agonists or antagonists, the constitutive activity of H3 receptor may effect on results.

2.1.7.3. Histaminergic control of the body homeostasis

Many nuclei controlling body homeostasis reside in the hypothalamus. Therefore, location of the histaminergic neurons to the posterior hypothalamus and dense histaminergic innervation in the surrounding area make histamine a powerful modulator of the adjacent hypothalamic nuclei. Physiological responses such as cardiovascular functions, fluid balance, lactation, and food intake are controlled by histamine.

Histaminergic neurons modulate cardiovascular responses directly through histamine receptors located to the vascular or cardiac cells or indirectly by activating sympatho-

adrenal axis and/or regulating the release of vasopressin (Bealer and Abell 1995, Armour 1996). Electrical stimulation of the TM nucleus or administration of histamine to the paraventricular nucleus of a conscious rat has been shown to increase the mean arterial blood pressure, which is suggested to occur through histamine-mediated activation of the noradrenergic neurons (see Table 2) (Akins and Bealer 1993, Bealer and Abell 1995). Heart rate can be modulated by electrical stimulation of the TM nucleus or histamine administration to the paraventricular nucleus, both inducing tachycardia (Akins and Bealer 1993, Bealer and Abell 1995), while intracerebroventricular injection of histamine results in bradycardia (Klein and Gertner 1981, Poulakos and Gertner 1989).

Fluid balance of the body is controlled primarily by the regulation of vasopressin release. In general, increased levels of vasopressin in the organism increase water resorption from the kidney, which leads to the concentration of urea increasing the amount of water in the organism (see review Antunes-Rodrigues et al. 2004). In the rat, histamine increases release of vasopressin through both H1 and H2 receptors, and partly by activating the noradrenergic system (Kjaer et al. 1998, Knigge et al. 1999, Radács et al. 2006). Furthermore, dehydration has been shown to activate histaminergic neurons, which in turn induces release of vasopressin via both the H1 and H2 receptors (Kjaer et al. 1994, Kjaer et al. 2000).

Release of oxytocin and consequently oxytocin-mediated physiological responses in pregnancy, parturition, lactation and sexual behaviour are also modulated by the central histaminergic system. Histamine administered intracerebroventricularly or to the paraventricular nucleus induces increase in oxytocin mRNA levels and release of oxytocin through both H1 and H2 receptors (see Table 2) (Kjaer et al. 1998, Bealer and Crowley 1999, Knigge et al. 1999, Radacs 2006). This is in accordance with the natural physiological responses during pregnancy and lactation, as the expression of HDC mRNA is increased at the end of pregnancy and during lactation (Luckman and Larsen 1997). Furthermore, inhibition of histamine synthesis or blocking of H1 receptors cause decrease in suckling-induced oxytocin release and delay in the birth of pups, respectively (Schagen et al. 1996, Luckman and Larsen 1997, Bealer and Crowley 2001).

Hypothalamus is involved in the control of feeding behaviour. Destruction of the hypothalamic ventromedial, paraventricular and dorsomedial nuclei results in an abnormal increase in appetite and food intake, while lesions of the lateral hypothalamus reduces food intake (see review Horvath and Diano 2004). An intracerebroventricular injection of histidine, histamine or H3 receptor antagonist, which all increase the brain histamine levels, depress feeding (Sheiner et al. 1985, Itowi et al. 1988, Masaki et al. 2003, Malmjöf et al. 2006, Malmjöf et al. 2007). In addition, H1 receptors seem to be involved in the feeding behaviour since their activation depresses feeding (Lecklin et al. 1998), whereas H1 receptor antagonists elicit it (Sakata et al. 1988, Mercer et al. 1994). Table 2 summarises the main physiological responses related to the histaminergic modulation of body homeostasis.

Table 2. Examples of physiological responses, which are modulated by the central histaminergic neurons.

Physiological system involved	Functional effect of histamine	Reference
Cardiovascular system	Rise of blood pressure and increase/decrease of heart rate	Klein and Gertner 1981, Poulakos and Gertner 1989, Akins and Bealer 1993, Bealer and Abell 1995
Fluid balance	Enhanced release of vasopressin (<i>in vivo and in vitro</i>) → concentration of urea → increase in amount of water in organism	Kjaer et al. 1998, Knigge et al. 1999, Radács et al. 2006
	Dehydration induces activation of histaminergic neurons, which in turn induce release of vasopressin (<i>in vivo</i>)	Kjaer et al. 1994, Kjaer et al. 2002
Pregnancy, parturition, lactation, sexual behavior	Enhanced release of oxytocin (<i>in vivo and in vitro</i>)	Kjaer et al. 1998, Bealer and Crowley 1999, Knigge et al. 1999, Radács et al. 2006
Food intake	Suppressed food intake	Sheiner et al. 1985, Itowi et al. 1988, Meade and Denbow 2001, Masaki et al. 2003, Malmjöf et al. 2005, Malmjöf et al. 2007

2.1.8. Histaminergic system and epilepsy

The brain histamine levels have been shown to be significantly lower in epilepsy-prone Krushinski-Molodkina rats, when compared to the epilepsy-resistant Wistar rats (Onodera et al. 1992). This is in accordance with the clinical study, in which histamine concentration measured from the cerebrospinal fluid was significantly lower in children with febrile seizures, when compared to the group of febrile children without seizures (Kiviranta et al. 1995). However, in normal rats, systemic KA administration increases brain histamine levels and number of histamine-immunoreactive fibres in several brain regions, which might be a compensatory effect of histamine on KA-induced changes in the brain (Lintunen et al. 2005).

Increased histamine levels or electrical stimulation of the posterior hypothalamus have been shown to inhibit seizures in several earlier studies, whereas lesion of the TM nucleus or inhibition of histamine synthesis shows the opposite effect. For example, in the pentylenetetrazol (PTZ)-induced seizures, injection of L-histidine and metoprine (inhibitor of HNMT) increase brain histamine levels and seizure threshold in mouse (Scherkl et al. 1991). In addition, histamine significantly and dose-dependently decreases frequency of seizures in the PTZ-induced seizure model in mouse (Yokoyama et al. 1994a). In the PTZ kindling model in rat, L-histidine prolongs latency for onset of myoclonic jerks and generalized clonic seizures (Chen et al. 2002). Finally, deep brain

stimulation of the posterior hypothalamus during the PTZ-induced seizures in rat activates the histaminergic system and cause desynchronization of cortical electroencephalogram (EEG) decreasing the seizure-like activity (Nishida et al. 2007).

In the amygdaloid kindling in rat, L-histidine, histamine and metoprine increase brain histamine levels and prolong latency to the onset of bilateral forelimb clonus (Wada et al. 1996). In addition, they cause significant inhibition of seizure stage and decrease duration of afterdischarge (AD) (Kamei et al. 1998, Kakinoki et al. 1998, Okuma et al. 2001, Kamei et al. 2001). In electrically-induced seizures (100 Hz, 30mA, 0.1 s), histamine significantly and dose-dependently decrease duration of tonic, clonic, and convulsive coma phase in rat (Yokoyama et al. 1994a). Moreover, in vestibular stimulation-induced seizures in mouse, L-histidine and metoprine induce delay in onset of seizure episode (Yawata et al. 2004). Finally, in transauricular kindled seizures in rat, L-histidine dose-dependently inhibits tonic hindlimb extension (Li et al. 2005).

On the contrary, lesion of the TM E2 nucleus or intracerebroventricular injection of α -FMH attenuates postictal seizure protection during intermittent maximal electroshock (MES) (Jin et al. 2007), which normally produces a progressive decrease in seizure severity with the motor pattern scores and durations of tonic fore- and hindlimb extension (Tortella et al. 1985). Furthermore, HDC-deficient mice show significantly accelerated development of amygdaloid and PTZ kindled seizures when compared to their respective wild-type mice (Chen et al. 2003, Hirai et al. 2004). In addition, AD duration and generalized seizure duration were prolonged in HDC KO mice (Hirai et al. 2004).

H3 receptor is an important regulator of seizures because of its autoreceptor function. H3 receptor antagonists, which increase histamine synthesis and release in the brain, inhibit also epileptiform activity. H3 receptor antagonists, thioperamide and clobenpropit, significantly and dose-dependently decrease duration of tonic, clonic and convulsive coma phases in seizures induced by MES in mouse (Yokoyama et al. 1993 and 1994b, Fisher and Goot 1998, Harada et al 2004a). In PTZ-induced seizures in mouse, thioperamide significantly and dose-dependently prolongs latency and reduces the incidence of clonic generalized seizures (Vohora et al. 2000). In addition, clobenpropit dose-dependently inhibits seizure stages and prolongs latency for myoclonic jerks and clonic generalized seizures after PTZ kindling in rat (Zhang et al. 2003). In amygdaloid kindling model in rat, thioperamide and clobenpropit cause significant and dose-dependent inhibition of seizure stage and decrease in duration of AD (Kakinoki et al 1998, Kamei et al. 2001, Harada et al. 2004b). Also other H3 antagonists such as AQ-0145, iodophenpropit, VUF 4929, VUF 5514, and VUF 5515 have been shown to be effective in inhibiting seizures (Murakami et al. 1995, Kamei et al. 2001, Harada et al. 2004a).

Histamine is suggested to produce partly its anticonvulsive effect through H1 receptor. When H1 receptor is blocked by H1 receptor antagonists mepyramine and diphenhydramine (intravenous injection, 10 mg/kg), seizures can be induced without any other convulsive compound indicating that H1 antagonists are strongly proconvulsive

(Kamei et al. 2000). In addition, in several seizure models, H1 antagonists have been shown to eliminate the anticonvulsive effect of L-histidine, histamine, or H3 receptor antagonist (Yokoyama et al. 1993, 1994a and b, Murakami et al. 1995, Zhang et al. 2003, Kamei et al. 1998, Kakinoki et al. 1998).

In amygdala kindling in rat, H1 receptor antagonists, mepyramine (pyrilamine) and ketotifen accelerate the rate of electrical stimulation to develop fully convulsive seizures (Yokoyama et al. 1996). In addition, mepyramine, ketotifen and diphenhydramine result in ictal activity in EEG and behavioural seizures in amygdala kindled rats at doses, which cause no or negligible seizures in sham rats (Fujii et al. 2003). In seizures induced by MES, ketotifen significantly diminish electroconvulsive threshold (Swiader et al. 2001). In addition, in 3-week-old rats, ketotifen and diphenhydramine significantly and dose-dependently increase duration of ictal activity in EEG and tonic phase of behavioural seizures induced by MES (Ishikawa et al. 2007). Furthermore, in seizures induced by vestibular stimulation, mepyramine and diphenhydramine significantly and dose-dependently decrease the number of tosses required to develop convulsive seizures, and increase duration of seizures (Sturman et al. 2001). On the contrary, most of the second generation antihistamines such as terfenadine, astemizole, epinastine, and loratadine, which do not penetrate the blood brain barrier, show no proconvulsant effect (Yokoyama et al. 1993, 1994a, 1996, Kamei et al. 2001).

H1 receptor KO mice show accelerated development of PTZ-induced seizures when compared to their respective wild type mice (Chen et al. 2003). The development of amygdaloid kindled seizures was significantly accelerated in H1 receptor KO mice when compared to the wild-type mice (Hirai et al. 2004). In addition, duration of AD and generalized seizure were prolonged in H1 receptor KO mice (Hirai et al. 2004).

H1 receptor agonist 2-thiazolylethylamine in turn decreases seizure susceptibility to PTZ and electrically induced seizures in mouse (Yokoyama et al. 1994a). In addition, 2-methylhistamine and 2-thiazolylethylamine show dose-dependent suppressive effect on seizure stage and duration of AD in amygdaloid kindled seizures in rat (Kamei et al. 2001). Finally, systemic KA administration induces increased expression of H1 receptor mRNA in the caudate-putamen and dentate gyrus, which may be seizure-induced compensatory effect (Lintunen et al. 1998).

In different seizure models, H2 receptor antagonists show no effect on several measured seizure parameters (Scherkl et al. 1991, Yokoyama et al. 1993, 1994a and b, Kakinoki et al. 1998, Seeley et al. 1999, Kamei 2001, Chen et al. 2002). Four H2 receptor antagonists administered intracerebrally induced convulsions with ED₅₀ values for convulsive occurrence: cimetidine 997 nmol, ranitidine 662 nmol, famotidine 23.4 nmol, and nizatidine 404 nmol (Shimokawa et al. 1996). However, the concentrations of the H2 antagonists in the brain in this study were considerable higher than those penetrating to the CNS when H2 antagonists are used clinically.

H2 receptor agonist drimaprit and amthamine have dose-dependently reduced the incidence of leptazol- and picrotoxin-induced seizures (respectively) in mouse (Seeley et al. 1999, Seeley and Sturman 2001).

Histaminergic system seems to potentiate the effect of the antiepileptic drugs. In the MES-induced seizures in mouse and transauricular kindled seizures in rat, L-histidine significantly enhanced the protective effect of carbamazepine (CBZ) and phenytoin (PHE) (Kaminski et al 2004). On the other hand, gabapentin (GBP) and PHE increased histamine levels in several brain regions in both MES- and PTZ-induced seizures in mouse (Vohora and Pillai 2001). Furthermore, H3 receptor antagonist thioperamide at the subeffective dose together with GBP (also at subeffective dose), prolong latency to myoclonic jerks, clonic generalized seizures, and duration of tonic and clonic phases (Vohora et al. 2001). The duration of the tonic phase was also reduced with PHE used together with thioperamide. Finally, the protective effect of clobenpropit have been observed in model of amygdaloid kindled seizures in rat, in which clobenpropit inhibited the seizure stage and decreased duration of AD at subeffective doses with diazepam, valproate, and muscimol (Ishikawa et al. 2000).

In conclusion, increased brain histamine levels, which can be induced by increasing levels of L-histidine or histamine directly, inhibiting histamine metabolism by metoprine, or increasing histamine synthesis and release by H3 receptor antagonist, have been shown to be anticonvulsive. In addition, histamine-mediated anticonvulsive effect seems to be mediated mainly through H1 receptor.

2.2. Kainic acid-induced epileptiform activity and cell damage in the hippocampus

2.2.1. The hippocampus

2.2.1.1. Structure of the hippocampus

The rat hippocampus is an elongated C- or banana-shaped subcortical structure with its long axis (named as septo-temporal axis) extending from the midline of the brain near the septal nuclei over and behind the thalamus into the temporal lobe (Fig. 11 A, B). The hippocampus can be defined as part of the limbic system together with the amygdala and limbic association cortex or as an independent functional system called the hippocampal formation consisting of the hippocampus proper with its three subdivisions, CA1, CA2 and CA3, and the dentate gyrus (DG), subiculum, presubiculum, parasubiculum, and entorhinal cortex (Amaral and Lavenex 2007).

The hippocampus can be cut perpendicular to the septo-temporal axis (Fig. 11 B), and each section is organized in a lamellar fashion signifying that small strips of the hippocampus may operate as independent functional units, in which different subregions are connected to each other with unidirectional connections (Fig. 12).

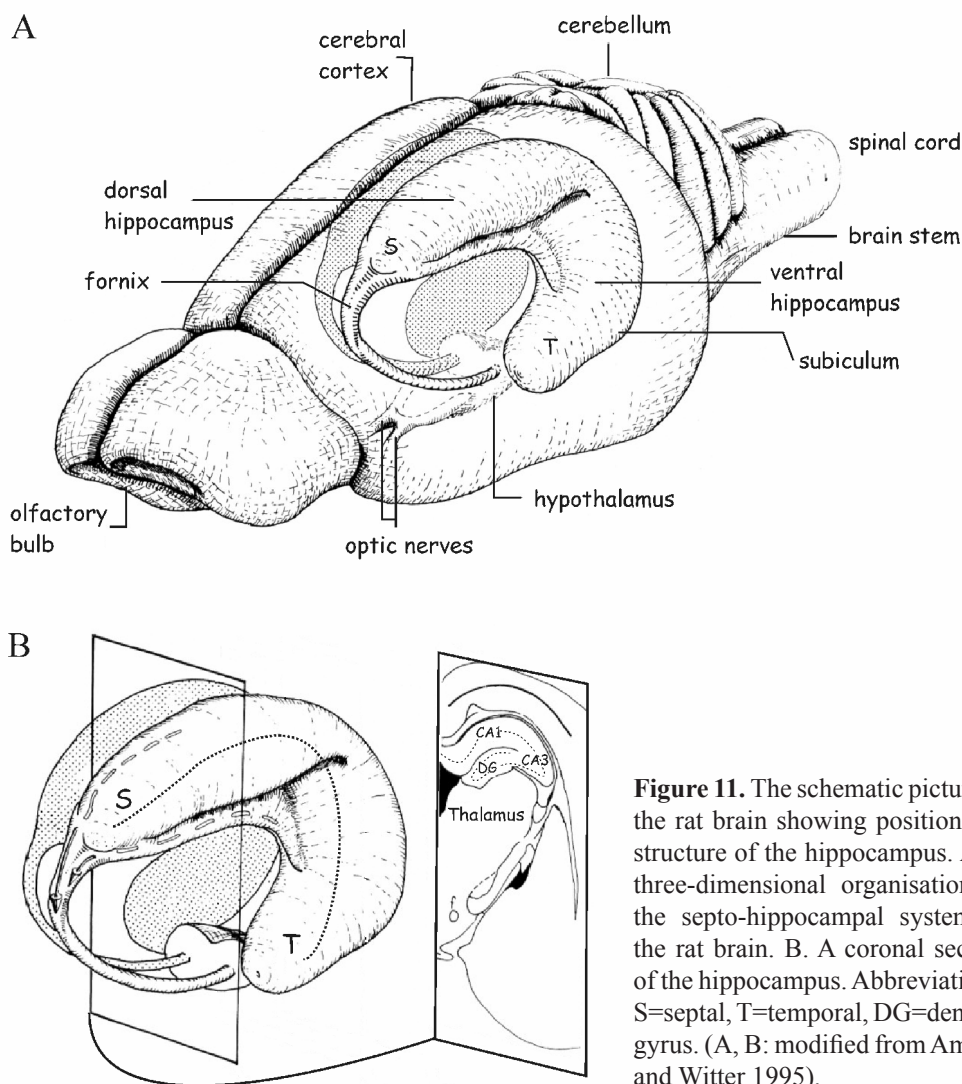


Figure 11. The schematic picture of the rat brain showing position and structure of the hippocampus. A. A three-dimensional organisation of the septo-hippocampal system in the rat brain. B. A coronal section of the hippocampus. Abbreviations: S=septal, T=temporal, DG=dentate gyrus. (A, B: modified from Amaral and Witter 1995).

In a simplified structural organization, cells in the entorhinal cortex send their axons (perforant path) to the dendritic area of the DG granular cells (molecular layer), their cell bodies located to the granular cell layer of the DG (Fig. 12 B, C) (Amaral and Lavenex 2007). Granular cells in turn project their axons (mossy fibers) through the polymorphic layer to the CA3 layer *stratum lucidum*, which is a narrow zone next to the CA3 pyramidal cell layer (12 B, C). The CA3 pyramidal cell bodies are localized in the pyramidal cell layer directing their apical dendritic tree to the *stratum radiatum* layer towards the hippocampal fissure, and their basal dendritic tree to the *stratum oriens* (12 B, C). CA3 pyramidal cells extend their axons (Schaffer collaterals) first to the *stratum oriens* of the CA3 region, from where they turn back crossing the pyramidal cell layer continuing to

the apical dendritic layer of the CA1 pyramidal neurons, *stratum radiatum*. Axons from the CA1 neurons project further through the *stratum oriens* of the CA1 region to the subiculum, from where axons project to the presubiculum and parasubiculum, which in turn are connected back to the entorhinal cortex (Fig. 12 B, C).

The hippocampal formation is connected to the neocortex through the entorhinal cortex and to subcortical regions such as amygdala through the external capsule, to the anterior thalamus through the thalamic radiations and supracallosal stria, and to the septum, hypothalamus, and brain stem through the fimbria-fornix (Amaral and Lavenex 2007).

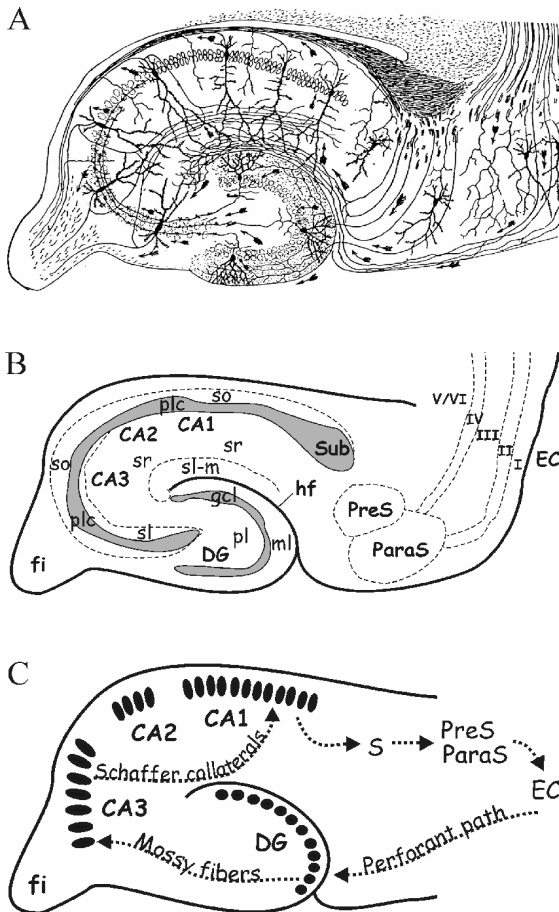


Figure 12. Lamellar structure of the hippocampus. A. Drawing of the neural circuitry of the rodent hippocampal section. B. Layers of different subregions in the hippocampal section. C. Schematic picture of the intrinsic pathways between different subregions. Abbreviations: DG= dentate gyrus, EC=entorhinal cortex, fi=fimbria, gcl=granule cell layer of the DG, hf=hippocampal fissure, ml=molecular layer of the DG, ParaS=parasubiculum, plc=pyramidal cell layer of the hippocampus, pl=polymorphic layer of the dentate gyrus, PreS=presubiculum, sl=stratum lucidum of the CA3, sr=stratum radiatum, sl-m=stratum lacunosum moleculare. Roman numerals indicate different cortical layers. (A: modified from Cajal 1911, B, C: based on Amaral and Lavenex, 2007).

2.2.1.2. Main cell types of the hippocampus

The main cell types in the hippocampus are the principal neurons including granular and pyramidal cells, and several types of interneurons (Amaral and Lavenex 2007). All principal cells are regarded as excitatory neurons releasing neurotransmitter glutamic acid, which depolarizes the membrane potential through ionotropic α -amino-3-hydroxy-5-methylisoxazole-4- propionic acid (AMPA), KA and NMDA receptors, and has slower

modulatory effect through the metabotropic glutamate receptors (see reviews Dingledine et al. 1999, Nicoletti et al. 2007).

The activity of the hippocampal principal neurons is tightly controlled by the inhibitory interneurons, which represent only 10% of the total neuronal population of the hippocampal formation, but despite of that, they form one of the most diverse cell populations having highly divergent anatomical and functional properties (Amaral and Lavenex 2007). The common feature for all interneurons is that they release GABA as a neurotransmitter. GABA is an inhibitory neurotransmitter, and its fast responses are mediated through GABA_A ionotropic receptors, opening of which allows influx of Cl⁻ ions into the cell causing increased membrane conductance (shunting inhibition) and hyperpolarization of the cell (see review Farrant and Kaila 2007), which in turn decreases the probability for action potential generation. The slow modulatory responses of GABA are mediated by metabotropic GABA receptors (Billinton et al. 2001).

One of the most important roles of the inhibitory interneurons is synchronization of the principal cell activity, which means that interneurons enforce a large group of principal neurons to fire action potentials simultaneously. Moreover, the rhythmic oscillation of the specific brain region seems to be related to certain behaviour, for example theta rhythm (6-12 Hz) recorded from the hippocampus correlates with the rat's movement through a space (see review Buzsaki 2005).

2.2.1.3. Function of the hippocampus

The function of the hippocampus has been studied intensively, and there is a strong evidence that the hippocampus is primarily involved in the formation of declarative memory (memory for everyday facts and events) in humans (see review Eichenbaum 2004), and in spatial navigation and formation of spatial memory both in animals and humans (Ekström et al. 2003, see review Eichenbaum 2004, McNaughton et al. 2006). In addition, the hippocampus has been shown to have a modulatory function on amygdala in fear conditioning, which is type of a learning related to formation of anxiety-related behavior in humans (see review Maren and Quirk 2004).

A lot of information about the human hippocampus in declarative memory has been received from patients, who have had hippocampal lesion connected to problems with memory functions. Probably the most famous study, which showed the obvious link between the temporal lobe region and memory, is the case of patient H.M., who went through bilateral temporal lobectomy in order to control intractable seizures (Scoville and Milner 1957). After surgery, H.M. unexpectedly showed persistent memory deficits including inability to recall stories and drawings that he has heard or seen earlier. In addition, he had difficulties in making associations. However, he did not have any other neurological dysfunctions, and he still had good general intelligence, normal perception, abstract thinking, reasoning ability, and motivation. In the case of H.M., also several other brain regions in addition to the hippocampus were removed (Corkin et al. 1997).

During the last decades, more restricted surgical operations of the hippocampus have confirmed that the hippocampus is definitely related to memory functions (Jones-Gotman 1986, Goldstein et al. 1989, Frisk and Milner 1990, Helmstaedter 1997, Gleissner et al. 2002, Martin et al. 2002, Crane and Milner 2005). In addition, hippocampal atrophy together with memory impairment have been observed in several illnesses such as severe temporal lobe epilepsy (Baxendale et al. 1998, Grunwald et al. 1998, Jokeit et al. 1999, Bergin et al. 2000, Pauli et al. 2000, Viskontas et al. 2000), viral infection (Yoneda et al. 1994, Kapur and Brooks 1999), depression (von Gunten and Ron 2004, Hickie et al. 2005), and Alzheimer's disease (Laakso et al. 1998, Garrido et al. 2002, Gilboa et al. 2005). All these studies indicate that hippocampus is outstandingly important for human declarative memory. Moreover, different sides of the hippocampus are specialized on different tasks, since the left hippocampus seems to be more related to coding verbal information (Frisk and Milner 1990, Helmstaedter 1997, Gleissner et al. 2002, Martin et al. 2002), whereas the right hippocampus is more specialized on spatial information, abstract designs and object location (Jones-Gotman 1986, Goldstein et al. 1989, Crane and Milner 2005). Finally, memory formation and storage seem to have a temporal pattern, in which the hippocampus participates in the memory formation only temporarily, and the long-term memory is stored in the neocortex becoming gradually independent of the medial temporal lobe structures (see review Frankland and Bontempi, 2005).

2.2.2. Kainic acid

Originally kainic acid (KA) was isolated from the seaweed *Digenea simplex* in Japan in 1953 (Murakami and Takemoto 1953). Two decades later, Shinozaki and Konishi (1970) described for the first time the potent excitatory action of KA in the mammalian nervous system. In the early 1980's, Robinson and Deadwyler (1981) showed that local application of KA on CA3 apical dendritic area of the hippocampus induces concentration-dependent increase in membrane depolarization, and spontaneous burst firing. Furthermore, at that time it was also demonstrated that the CA3 subregion of the hippocampus is especially vulnerable to KA-induced neuronal death (Nadler et al. 1978). KA, albeit showing a clear preference for KA receptors, can also bind and activate AMPA receptors (Lerma et al. 1993). Although KA receptors activate different cell types in a complicated manner in the CA3 region, AMPA receptors are mainly responsible for maintaining the recurrent activity between CA3 pyramidal neurons during the epileptiform activity (Traub et al. 1996, Strowbridge 1999).

KA has been used in epilepsy research for two reasons. First, it induces seizures and mimics the synchronized activity involved in epilepsy, and second, it produces neuronal death within the hippocampus, which is suggested to mimic the pathology of temporal lobe epilepsy.

2.2.2.1. KA and AMPA receptors in the hippocampus

KA and AMPA receptors belong to the group of ionotropic glutamate receptors. They respond to endogenous neurotransmitter glutamic acid (or glutamate) acting mainly as cation-selective ion channels (see review Dingledine et al. 1999). Moreover, AMPA and KA receptors display rapid opening and closing kinetics. Amplitude of the KA receptor-mediated excitatory postsynaptic current (EPSC) is usually less than 10 % of the AMPA receptor-mediated EPSC (Castillo et al. 1997, Frerking et al. 1998), and the KA receptor-mediated EPSC has considerably slower rise and decay times when compared to AMPA receptor-mediated responses (Castillo et al. 1997, Vignes and Collingridge 1997). Because of the fashion of KA receptor-mediated postsynaptic response, it has been suggested that the KA receptor-mediated EPSCs primarily promote temporal summation (Frerking and Ohliger-Frerking 2002, Ito 2004, see review Pinheiro and Mulle 2006). KA receptors are also located presynaptically in axons where they modulate transmitter release (see reviews Lerma 2001).

Both KA and AMPA receptors consist of four subunits. In KA receptors, GluR5-GluR7 are the low affinity subunits, whereas KA1 and KA2 are the high-affinity subunits (see review Pinheiro and Mulle 2006). In the formation of functional receptors, KA1 and KA2 subunits can form only heteromeric receptors together with the low-affinity subunits, whereas the low-affinity subunits can form also functional homomeric receptors (see reviews Lerma 2001, Pinheiro and Mulle 2006). AMPA receptors in turn consist of GluR1-GluR4 subunits, and they seem to be mainly heteromers comprised of GluR2 plus GluR1 or GluR3 subunits at the hippocampal CA3-CA1 synapse as well as in cultured hippocampal neurons of the CA1/CA2 region (Craig et al. 1993, Wenthold et al 1996).

In the CA3 region of the hippocampus, AMPA receptors mediate fast excitatory synaptic transmission and are important for maintaining recurrent activity between CA3 pyramidal cells (Traub et al. 1996, Strowbridge 1999, see review Dingledine et al. 1999). KA receptor-mediated pre- and postsynaptic responses at the CA3 mossy fiber synapses depend on both stimulus frequency and KA concentration (Castillo et al. 1997, Kamiya and Ozawa 2000, Schmitz et al. 2000, Lauri et al. 2001, Kamiya et al. 2002, Lauri et al. 2003). Moreover, KA receptors of the CA3 region play an important role in modulation of gamma oscillation (20-80 Hz), in which GluR5 subunit-containing KA receptors in axons of interneurons and GluR6-containing KA receptors in the somatodendritic region of both interneurons and pyramidal cells form a complicated functional interplay (Fisahn et al. 2004).

In conclusion, it is suggested that fast glutamatergic synaptic transmission in the hippocampus is primarily mediated by AMPA receptors (and NMDA receptors when Mg^{2+} block is alleviated), with the contribution of KA receptors, which is restricted to special conditions such as the high-frequency firing, during which KA receptors modify responses pre- and postsynaptically both in principal cells and in interneurons.

2.2.2.2. Mechanisms of KA-induced epileptiform activity

Clinically, epilepsy is a diverse disorder arising from the numerous underlying cellular and molecular mechanisms as well as from the spatial and temporal characteristics of seizure. The first step in the induction of epileptiform activity is a generation of paroxysmal depolarization shift (PDS) followed by a brief burst discharges, which correlate with the interictal spikes detected in EEG (see review McCormick and Contreras 2001). PDSs and subsequent intracellular bursts can be experimentally generated by several manipulations such as stimulating the ionotropic glutamate receptors, blocking the GABA_A receptor-mediated inhibition, induction of rapid kindling through repetitive local electrical stimulation, increasing extracellular K⁺ concentration, and reduction of Mg²⁺ concentration in the extracellular fluid. These manipulations are suggested to induce imbalance between depolarizing and hyperpolarizing influences in the large interconnected network of neurons. The transition from the generation of single PDSs during interictal spikes to full seizures has been associated with the gradual loss of the burst after-hyperpolarisation and the progressive appearance of repetitive bursts activity during more and more prolonged after-depolarization. In many of these models (reduction of GABA_A receptor-mediated inhibition, increase in K⁺ concentration, and repetitive stimulation), the initial burst of epileptiform action potential activity is mediated both by the non-NMDA and NMDA receptors, since it can be blocked by antagonists of these receptors. However, repetitive bursts of action potentials that follow this initial burst are sensitive to the NMDA receptor antagonists alone (see review McCormick and Contreras 2001).

The CA3 neuronal network contains pyramidal neurons, which intrinsically generate bursts upon activation by a brief depolarization (Wong and Prince 1978, Hemond et al. 2008). Moreover, CA3 excitatory cells form a network, which is able to maintain recurrent activity within the CA3 region (Traub et al. 1996, Strowbridge 1999). These functional properties, which in normal physiological conditions allow CA3 region to perform varied complicated tasks, make the CA3 region also vulnerable to generation of epileptiform activity.

In the hippocampal slices, KA (0.05-0.1 μM) induce spontaneous burst activity, which originates in the CA3 region, from where it propagates to the CA1 region (Westbrook and Lothman 1983). KA (at these concentrations) does not change resting membrane potential or input resistance of the pyramidal cells, but increases excitability of CA3 and CA1 pyramidal cells, and lowers the threshold for stimulus intensity necessary for the activation of action potential (Westbrook and Lothman 1983). This in turn causes augmentation and synchronization of bursting in pyramidal cells, and prolongs EPSP without an increase in amplitude. This kind of activity pattern is probably mediated mainly by KA receptors. However, when KA concentration increases, KA activates also AMPA receptors, which are especially important in maintaining the recurrent activity between pyramidal cells within the CA3 region. Also other mechanisms such as voltage-

gated Ca^{2+} -channels, and NMDA receptors are involved in KA-induced epileptiform activity and excitotoxicity (see review McCormick and Contreras 2001).

Postsynaptic KA receptors at the mossy fiber synapses containing GluR6 subunit seem to be particularly important in seizure generation, since in GluR6 KO mice, higher concentrations of KA are required to generate seizures (Mulle et al. 1998). Moreover, overexpression of fully edited GluR6 subunit leads to increased seizure activity and spontaneous bursting *in vitro* (Telfeian et al. 2000). In addition, GluR5 subunits, which are mainly expressed in interneurons, but also presynaptically in the mossy fiber synapses, seem to play an important role in the KA- and pilocarpine-induced seizures (Khalilov et al. 2002, Smolders et al. 2002).

2.2.2.3. Mechanisms of excitotoxic cell death

KA induces excitotoxic cell death in several brain regions, the hippocampus being one of the most vulnerable structures (Sperk et al. 1983, Ben-Ari et al. 1980, Covolan and Mello 2000). The term excitotoxicity was first introduced by Olney and co-workers (1971, 1972) to refer it as an acute process, in which glutamate or one of its structural analogs induces nerve cell death in the CNS. Excitotoxic neurodegeneration can be caused by interference of normal neurotransmission as it is the case in epileptic seizures, or by brain insults i.e. hypoxia/ischemia or brain trauma.

Classification of excitotoxic cell death has been previously based primarily on morphological features, and has been divided into two categories: apoptosis and necrosis. Increasing number of different biochemical markers, which detect the intracellular cascades involved in cell death, has been questioned this classical division of cell death. Therefore, in the current classification, the more important criterion seems to be whether cell death is programmed or not. In many studies, the classical term apoptosis has been replaced by the term programmed cell death (PCD), which in turn has several subcategories such as morphological apoptosis, apoptotic PCD, necrotic PCD, caspase-dependent PCD, and caspase-independent PCD (see review Leist and Jäättelä 2001). Moreover, within this classification, several biochemical markers of PCD have been used to specify the routes by which cells die. For example, family of caspases, cytochrome c, apoptosis inducing factor (AIF), anti-apoptotic Bcl-2 protein, and pro-apoptotic Bax and BAD, and apoptotic-protease-activating factor 1 (Apaf-1) belong to the group of most studied markers of the PCD. There are not so many reliable markers for non-programmed or classical necrotic cell death, and therefore, when markers of the PCD are not detected, and morphological features resemble the classical necrosis, cell death is considered as necrotic.

The mechanisms of excitotoxic cell death have been extensively studied, and it seems that excess in glutamate release overactivates Ca^{2+} permeable glutamate channels and induces imbalance in the intracellular Ca^{2+} concentration. This in turn activates biochemical pathways leading to the PCD (see review Orrenius et al. 2003) or necrosis

(see review Syntichaki and Tavernarakis 2003) depending on the strength of the insult. In case of the programmed apoptosis by the mitochondrial pathway, increased Ca^{2+} levels can induce apoptosis either through caspase-dependent or caspase-independent pathways. In the caspase-dependent pathway, increased Ca^{2+} levels lead to formation of permeability transition pores in the mitochondrial membrane, and release of cytochrome c, whereas in the caspase-independent pathway, AIF or endonuclease G (Endo G) are released into the cytosol (Fig. 13). Furthermore, in the caspase-dependent pathway, cytochrome c forms an apoptosome together with Apaf-1, pro-caspase-9, and deoxyadenosine triphosphate (dATP). Pro-caspase-9 is metabolized to its activated form caspase-9, which in turn activates caspase-3. Caspase-3 begins the degradation phase of apoptosis, in which various caspase enzymes are activated resulting in characteristic changes in the plasma membrane (blebbing and exposure of phosphatidylserine on the cell surface, which is a signal that stimulates cell phagocytosis by macrophages/microglia). Finally, the nuclear chromatin becomes condensed and fragmented.

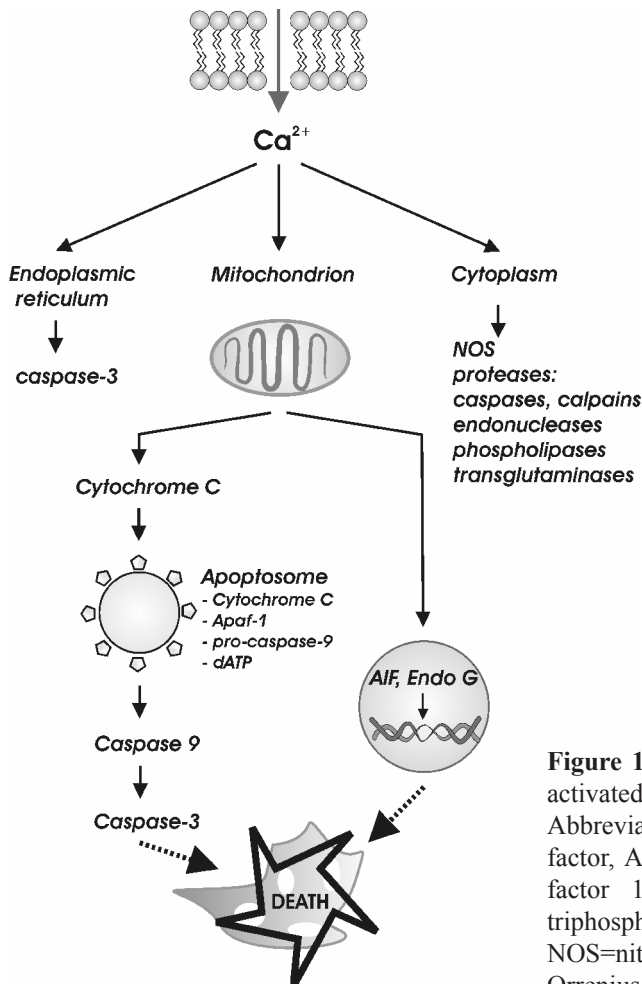


Figure 13. Mitochondrial apoptotic pathway activated by overflow of Ca^{2+} ions. Abbreviations: AIF=apoptosis inducing factor, Apaf-1= apoptotic-protease-activating factor 1 (Apaf-1), dATP=deoxyadenosine triphosphate, Endo G=endonuclease G, NOS=nitric oxide synthetase. (Based on Orrenius et al. 2003).

Although molecular mechanism of the classical necrosis is less clear than in apoptosis, some hypotheses have been suggested. Necrotic process is thought to be initiated, when conditions exceed the buffering capacity of the cell's protective systems (see review Syntichaki and Tavernakis 2003). For example, when the intracellular concentration of Ca^{2+} ions exceed the buffering capacity of the cell, Ca^{2+} ions activate calpains in the cytoplasm causing the lysosome rupture. Lysosomes contain over 80 types of hydrolytic enzymes including cathepsins, which degrade cellular structures and interfere with normal metabolism.

One commonly used biochemical marker for cell damage is poly(ADP-ribose) polymerase (PARP), which is a multifunctional protein involved mainly in deoxyribonucleic acid (DNA) repair after excitotoxic, oxidative, and nitrosative stress. In general, cell survives if PARP is able to repair DNA damage. Earlier PARP was thought to be involved only in apoptosis (Yu et al. 2002), but when mechanisms of PARP activation were studied in more detailed, it became evident that PARP is functional also in necrosis (see review Jagtap and Szabo 2005). The critical factor, which determines whether PARP activation leads to apoptosis or necrosis, is the cellular energetic depletion, which PARP partly causes, when trying to repair damaged DNA.

2.2.2.4. The KA-induced excitotoxicity in the developing hippocampus

Type and amount of KA-induced neuronal death is depends on how KA has been administered and what is the age of the studied animal. After an intrahippocampal injection, KA induces neuronal death already at P5 (Cook and Crutcher 1986), and intracerebroventricularly given KA (10 or 50 nmol) in the P7 rats leads to acute necrotic loss in the CA3 region, and at P14-P40 increasing amount of apoptotic neuronal death appears first in the CA3 region, and later also in the CA1 region (Humprey et al. 2002).

If KA is administered intraperitoneally, KA is able to induce seizures already at P1 (Sayin et al 2004), and indeed, during the first two postnatal weeks, animals are even more prone to KA-induced seizures than later in their life (Hauser 1994). However, intraperitoneally induced seizures by KA results in neuronal death only after the second postnatal week, approximately at P19 (Ben-Ari et al. 1984). The same effect can be seen with the other seizure-inducing drugs suggesting that seizures do not cause neuronal death in the developing brain during the first two weeks (Cavalheiro et al. 1987, Hirsch et al. 1992, Sperber et al. 1999, Haas et al. 2001). The reason for this remains largely unknown.

3. AIMS OF THE STUDY

Histaminergic neuron system is well characterized *in vivo*. However, a lot of detailed information is still needed to understand the function of these neurons in the normal physiological conditions as well as in various diseases.

The specific aims of this study were:

1. to set up an *in vitro* culture system for the histaminergic neurons
2. to study development and detailed morphology of the cultured histaminergic neurons, and to visualize storage and transport of histamine, as well as the subcellular localization of GABA and galanin in the histaminergic neurons
3. to study the mechanisms of KA-induced neuronal damage in the slice culture system of the immature rat hippocampus
4. to develop a culture system mimicking the immature brain structures, and to study the effect of histaminergic neuron system (separately from other monoaminergic system) on regulation of KA-induced epileptiform activity and neuronal death
5. to find out whether or not histaminergic neurons have a neuroprotective effect in the KA-induced neuronal damage in the coculture system of the immature hippocampus and rat posterior hypothalamus

4. MATERIALS AND METHODS

4.1. Animals used in different culture systems (I-IV)

The 18-day-old embryos of Sprague–Dawley (SD) rats were used for the explant culture system of the histaminergic neurons. The appropriate permits were obtained from the Committee for Animal Experiments of the Abo Akademi University according to the ethical guidelines of the European Convention in Strasbourg (1986).

Hippocampal slice cultures were prepared from the hippocampi of 6–7 day-old (P6–P7) Wistar rats, and the cocultures of hippocampal and hypothalamic slice cultures were prepared from the P4 SD rats. All treatments of these animals were according to the European Community Council directives 86/609/EEC, and had the approval of the Animal Use and Care Committee of the University of Turku. All efforts were made to minimize the pain, discomfort, and the number of experimental animals.

4.2. The primary culture systems (I-IV)

4.1.1. *The explant culture system (I, II)*

The posterior hypothalamic regions of the 18-day-old embryos of the SD rats were dissected, cut into smaller pieces and placed on the coverslips coated with poly-L-lysine (10 µg/ml, Sigma) or laminin (10 µg/ml, Sigma). The culture medium consisted of Dulbecco's modified essential medium, 20% fetal calf serum, 10% horse serum, 1% Glutamax, glucose (5.5 g/l) (Sigma), and 1% antibiotics (penicillin and streptomycin). The medium was then changed every third or fourth day for up to 13 DIV. The incubation conditions were +37 °C temperature, 5% CO₂, and 80-90% humidity.

4.1.2. *The organotypic hippocampal slice culture system (III)*

Hippocampal slice cultures were prepared from the hippocampi of P6–P7 Wistar rats using the method of Stoppini et al. (1991). Hippocampi were dissected in cold Gey's balanced salt solution (Gibco) supplemented with glucose (6.5 mg/ml). Slices (400 µm) were cut perpendicular to the septotemporal axis of the hippocampus using the McIlwain tissue chopper, and placed on top of semipermeable membrane inserts (Millipore) in a six-well plate containing culture medium with the following composition: 50% of minimum essential medium, 25% Hanks's balanced salt solution, 25% heat-inactivated horse serum, 25 mM HEPES, supplemented with 0.5 ml GlutaMaxII (Gibco) and 6.5 mg/ml glucose. Slices were cultured in an incubator (37 °C, 5% CO₂) for 7 DIV with medium change twice a week. After 7 DIV, slices were treated with KA (5 µM) (Sigma) for 6, 12, 24, and 48 h and stained with Fluoro-Jade B (FJB). For the Western blotting studies slices were treated with KA (5 µM) for 4, 8, and 24 h. After these time points, a

subset of inserts was further cultured in normal culture medium (without KA) for 48 h prior to immunoblotting and immunocytochemistry.

4.1.3. The coculture system

The double slice culture system consisted of hypothalamic and hippocampal slices, taken from the P4 rats, and cultured together. The hippocampal slices were cut as previously described, and the hypothalamus was cut into 8 slices. Histaminergic neurons were usually found in the first four slices of the hypothalamus (considered as posterior part of the hypothalamus). The first coculture system consisted of the hippocampal slice, which was cultured together with the posterior hypothalamic slice (HI+HY, POST). The hypothalamic slices 6-8, which were taken from the anterior part of the hypothalamus, were used as control slices, since they did not contain histaminergic neurons (revealed by the immunostaining). They were used in the second coculture system consisting of the hippocampal slice cultured together with the anterior hypothalamic slice (HI+HY, ANT). Otherwise the cutting solution, culture medium, and the main culture protocol was the same as previously described for the hippocampal slices (HI).

4.2. Immunocytochemistry (I-IV)

4.2.1. Immunostainings in the explant culture systems (I, II)

After 4-13 DIV, the explants were washed with phosphate buffered saline (PBS), and fixed either with 4% 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide (EDAC) (Sigma) and 0.5% paraformaldehyde (PFA) for histamine and GABA staining or with 4% PFA in staining of VMAT2 and galanin. Fixatives were diluted in phosphate buffer (PB), and the explants were fixed for 2-4 h at +4 °C, and washed with PBS containing 0.1% saponin (Sigma). The primary and secondary antibodies were diluted as described in Table 3. The antibody combinations in double-stainings were histamine/VMAT2, histamine/microtubule associated protein 2 (MAP2), VMAT2/GABA and VMAT2/galanin. The explants were incubated overnight at +4 °C, washed with PBS+saponin, and incubated with the secondary antibody for 4-6 h at +4°C. After a wash with PBS+saponin the second similar immunostaining followed. Finally, coverslips with the explant cultures were mounted on slides using 80% glycerol.

Table 3. Primary and secondary antibodies used in the immunostainings. Abbreviations: GABA= γ aminobutyric acid, MAP-2=microtubule associated protein 2, VMAT2=vesicular monoamine transporter 2.

<i>Primary antibody</i>	<i>Dilution</i>	<i>Origin</i>	<i>Secondary antibody</i>	<i>Dilution</i>	<i>Study</i>
Rabbit anti-histamine	1:10 000	P. Panula	Alexa 568 goat anti-rabbit	1:1000	I, II, IV
Mouse anti-MAP2	1:5000	Sigma	Alexa 488 goat anti-mouse	1:1000	II
Guinea pig anti-VMAT2	1:10 000	Euro-Diagnostica, SWE	Alexa 488 goat anti-guinea pig	1:1000	II
Rabbit anti-GABA	1:2000-1:5000	P. Panula	Alexa 568 goat anti-rabbit	1:1000	II
Rabbit anti-galanin	1:1000-1:2500	Peninsula Laboratories, USA	Alexa 568 goat anti-rabbit	1:1000	II
Rabbit anti-caspase-3	1:3500	Cell signalling, Technology Inc., Beverly, MA	Goat anti-rabbit IgG1	1:4000	III

4.2.2. Immunostainings in the slice culture systems (III, IV)

Immunocytochemical detection of histamine and active caspase-3 was carried out in slices after 7 DIV. During the entire staining procedure, cultured slices were attached to semipermeable membrane inserts. For caspase-3 staining, hippocampal slices were first washed with PBS, fixed with 4% PFA for 1 h at room temperature (RT), and then washed with PBS containing 2.5% Triton-X-100 (PBS-T). Fixation for histamine was the same as described in explant cultures. Slices were then incubated with the primary antibody, histamine or caspase-3 (see Table 3) overnight at +4 °C, washed with PBS-T, and thereafter incubated with the secondary antibody, Alexa 568 for caspase-3 and Alexa 488 for histamine (Molecular Probes) (see table 3). Finally, slices were washed with PBS, and mounted with 80% glycerol on gelatin-coated glasses. Negative controls for the immunostaining included omission of the primary antibody, and incubation with the primary antibody together with 10% normal goat serum. We used the apoptotic cells from HELA-cell line as a positive control in caspase-3 staining.

4.3. Western blotting (III)

Western blotting was performed to determine whether caspase-3 and PARP proteins are cleaved in response to KA treatment as a consequence of an apoptotic cell death in the hippocampal slices. One control group, and the 4, 8, and 24 h KA-treated (5 μ M) groups of slices from two different culture batches (n=93–96 slices in both batches of each time group) were collected in ice-cold homogenization buffer containing 50 mM Tris–HCl, 1% SDS, 2 mM EDTA, 1 mM PMSF, and 0.7 mM dithiothreitol, homogenized. Homogenates were boiled immediately and then centrifuged at 12,000 rpm for 30 min at 4 °C. Supernatants were collected, frozen, and stored at –80 °C until used. Protein

concentration of the samples was measured using Lowry-based Biorad DC Protein assay (Biorad). Fifty micrograms (μg) of protein were applied to each lane for SDS-PAGE and separated by electrophoresis with a 10% acrylamide minigel, and transferred to a polyvinylidene fluoride immobilon-P (Millipore) membrane. Membranes were incubated at 4 °C overnight with the primary antibodies polyclonal anti-cleaved caspase-3 (see table 3), and monoclonal anti-PARP (1:3500) (Sigma), which detects the 116 kDa protein corresponding to PARP, and its 85 kDa apoptosis-induced cleavage product. Thereafter, samples were incubated for 1 h at RT with the HRP-conjugated secondary antibodies, goat anti-mouse IgG1 (γ 1 chain specific, 1:3500) (SouthernBiotech) to detect PARP, and goat anti-rabbit IgG (1:4000) (Sigma) to detect the active caspase-3. The signal was obtained using chemiluminescence ECL system and Hyperfilm ECL (Amersham). The optical signals were quantified with Image J 1.20s (NIH), and the results are given as arbitrary units (a.u.) per mg of protein.

4.4. Fluoro-Jade B staining and verification of neuronal damage (III-IV)

Neuronal cell death was examined in cultural hippocampal slices using Fluoro-Jade B (FJB), which is an anionic fluorescein having excitation peaks are 362 and 390nm and emission peak 550nm. Slices were first washed with PBS, fixed with 4% PFA (Sigma), and moved to 0.06% potassium permanganate (KMnO_4) solution for 2-5 min. After KMnO_4 –treatment, slices were washed with water and transferred to 0.001% FJB solution for 30 min, washed with water, removed from membranes to gelatin-coated glasses, dried overnight at RT, immersed in xylene and coverslipped.

For our scoring analyses, the area of stained neurons (i.e., degenerating neurons) was measured from maximum projections using ImageJ software (<http://rsb.info.nih.gov/ij>). The following scoring system was used to evaluate the extent of the damage: 0 = no FJB-stained neurons (regarded as normal), 1 = 1–100 $\times 10^3 \mu\text{m}^2$, 2 = 101–200 $\times 10^3 \mu\text{m}^2$, 3 = 201–300 $\times 10^3 \mu\text{m}^2$, and 4 = >300 $\times 10^3 \mu\text{m}^2$.

FJB-stained neurons were counted throughout the entire thickness of the slice within the 250- μm^2 area of the central part of the CA3a/b pyramidal cell layer. For the counting, 10 slices were blinded selected from three different groups: 1) control HI cultured in normal medium; 2) HI with KA for 12 h; and 3) HI+HY, POST treated with KA for 12 h. In each slice, pyramidal neurons were counted in confocal optical sections of the 250 μm^2 region through the z-axis with Adobe Photoshop.

4.5. Thionin staining (IV)

Slices were removed from semipermeable membranes to gelatin-coated glass slides and dried. For thionin staining, slices were rehydrated, stained in 0.1% thionin for 15-20 s, washed, dehydrated in alcohol series, cleared in xylene, and coverslipped. Digital camera

Olympus U-TV1 X was used to capture pictures using Olympus BX60 microscope, and pictures were further processed using Adobe Photoshop and Corel Draw.

4.6. Confocal microscopy (I-IV)

Histamine and FJB stainings were examined with a Leica TCS SP confocal microscopy system (Leica) equipped with an Argon-Krypton laser (Omnichrome). The excitation wavelength for FJB and Alexa 488 was 488 nm and for Alexa 568 568 nm. The fluorophores were chosen so that light absorption of the fluorophore was maximal at the given excitation wavelength, and emission wavelengths of the two fluorophores were as far apart as possible. The emission wavelength filter settings were for FJB and Alexa 488 500-550 nm, and for Alexa 568 600-700 nm. The images were acquired at 0.5 or 2 μm steps and analyzed with Leica TCS NT/SP Scanware software. The most used algorithm was maximum projection, which determines the maximum of all intensity values in a stack of sections. The picture of the whole hippocampus was reconstructed by connecting 10–15 maximum projections of different areas of the hippocampus. All figures were produced and edited with Adobe Photoshop and Corel Draw software.

4.7. Electron microscopy (III)

Slices were fixed with freshly prepared 5% glutaraldehyde (Electron Microscopy Sciences), the CA3a/b region was isolated by knife cuts, and the specimen was further prepared according to standard procedures as previously described (Fröjman et al., 1992). For the light microscopy, orientation sections from the CA3a/b region were cut at 1 μm thickness and stained with toluidine blue. The ultrathin sections (70 nm) were stained with 5% uranyl acetate and 5% lead citrate in Ultrastainer (Leica), and examined in a JEM-1200EX (JEOL) transmission electron microscope.

4.8. Pharmacological studies (IV)

4.8.1. Alpha-fluoromethylhistidine

To find out an optimal concentration and time for the inhibition of histamine synthesis by α -FMH, HI+HY, POST was incubated with different concentrations of α -FMH (10 nM, 100 nM and 1 μM) for 6 h, 12 h and 24 h. In addition, to find out whether histamine, which disappeared from histaminergic fibers after α -FMH treatment, would reappear in fibers during the next 12 h (the duration of KA treatment), a subset of cultures was further incubated in normal medium for 12 h. Immunostaining with the anti-histamine antibody was used to verify location of histamine in cell bodies and fibers. FJB staining was performed to study whether or not KA-induced neuronal degeneration was changed in the absence of endogenous histamine in histaminergic fibers innervating the hippocampus.

4.8.2. Histamine

To study the effect of extracellularly applied histamine on neuronal survival in HI, different concentrations of histamine (1 nM, 10 nM, 100 nM, 1 μ M, 10 μ M and 100 μ M, Sigma) were added to hippocampal slices after 7 DIV. After the 30-min incubation with histamine, KA (5 μ M) was added, and slices were further incubated with both histamine and KA for 12 h. Neuronal degeneration was detected with FJB staining.

4.8.3. H1 and H3 receptor antagonists

The effect of the histamine H1 and H3 receptors on KA-induced neuronal damage was studied in HI+HY, POST, which was incubated together with different concentrations of the H1 receptor antagonists triprolidine (2 nM, 20 nM, 200 nM and 2 μ M), and mepyramine (1 nM, 10 nM, 100 nM and 1 μ M). The significance of H3 receptor was studied by treating HI+HY, POST with the H3 receptor antagonists clobenpropit (10 nM, 100 nM, 1 μ M, and 5 μ M), and thioperamide (1 nM, 10 nM, 100 nM and 1 μ M). KA (5 μ M) was added 30 min after the antagonists, and cultures were further incubated for 12 h. Neuronal degeneration was detected with FJB staining.

4.9. Electrophysiology (IV)

HI and HI+HY, POST were cultured for 7 DIV and then incubated with 5 μ M KA for 6 h. Control HI had no KA treatment. An insert with a slice was briefly washed and transferred to a dish containing an artificial cerebrospinal fluid (aCSF) with the following composition (mM); 124.0 NaCl, 26.0 NaHCO₃, 10.0 D-glucose, 4.5 KCl, 1.2 NaH₂PO₄, 1.5 MgCl₂, and 2.0 CaCl₂. After equilibration (35 °C, max 1 h), the slice was transferred to the recording chamber (capacity 6 ml) mounted to a Leica DM IRB microscope. Slices were superfused with oxygenated (95% O₂ and 5% CO₂) aCSF during the entire experiment.

Extracellular field recordings were carried out in the CA1 pyramidal layer with glass microelectrodes (<1 M Ω , tip diameter ~30 μ m) filled with 0.15 M NaCl. After stabilization, spontaneous activity was recorded in the CA1 pyramidal cell layer using Axoclamp 2B amplifier (Axon Instruments Inc.), data were stored, and analyzed using the pClamp software. Digitization was performed using the 12-bit A/D interface Digidata 1200 (Axon Instruments Inc.). The burst was defined as continuous electrical activity lasting > 3 s.

4.10. HPLC (IV)

To exclude the possibility that external histamine in the culture medium could have an effect on survival of neurons, histamine content was determined in both horse serum and culture medium using high performance liquid chromatograph (HPLC) equipped with a fluorometric detector. Briefly, samples were diluted in 10 volumes of 2% perchloric

acid, and centrifuged at 15 000 g for 15 min. Histamine content was analyzed from the supernatants using HPCL with post-column derivatization and fluorimetric detection according to the method of Yamatodani (Yamatodani et al., 1985). The detection limit was 10 pmol/g of the original sample.

4.11. Statistical analysis (I-IV)

The overall group differences in score and neuron numbers after the FJB staining were assessed with one-way analysis of variance (ANOVA) with Tukey-Kramer Multiple Comparison Test as a post hoc test. The nonparametric one-way ANOVA with Kruskal–Wallis Test was used to analyze the group differences (acute and recovery groups) in the immunoblots. In the electrophysiological recordings, the statistical significance was determined using nonparametric Mann–Whitney test. Prism program (GraphPad Software) was used in all statistical analysis, and the level of significance was set at $p < 0.05$.

5. RESULTS

5.1. Development of a culture system for the central histaminergic neurons (I)

In the primary culture system of the embryonic hypothalamic tissue, the histaminergic neurons attached and extended their neurites faster on the laminin as a coating molecule when compared to poly-L-lysine. Moreover, high serum content (30 %) was required to maintain the histaminergic neurons in the culture conditions and to promote migration of histaminergic neurites. Finally, at the beginning of the culture period, the culture medium was added with small amounts within several hours in order to avoid the detachment of the explants.

5.2. Detailed morphology of the histaminergic neurons in the explant culture system (I, II)

The most typical shapes of histaminergic neurons were ovoid, rounded or triangular, of which ovoid was the most common type with 73 % of the 133 examined neurons during the entire culture period from day 4 to 13. The size of the cell body was measured so that the length, width and thickness were measured separately. The average length, width and thickness of 212 neurons were 19.2 μm (range 12.3–28.4 μm), 12.5 μm (range 9.4–15.3 μm) and 11.7 μm (7.6–15.8 μm), respectively. These parameters were not changed during the culture period from 4 to 12 DIV.

MAP2 was used as a marker for dendrites and cell bodies, while the unstained structures were supposed to be axons. Most of the histaminergic neurons (79 %) possessed two or three MAP2-positive dendrites, but in some histaminergic neurons, the number of dendrites was even five or six indicating that there might be different subtypes of histaminergic neurons. Histamine-positive but MAP2-negative axons were usually thin emanating from cell body or from the dendritic structure, but also thicker axons were detected. Both axons and dendrites had enlargements, which usually contained large amount of histamine-containing vesicles.

5.3. Histamine storage, transport and co-localization of GABA and galanin in histaminergic neurons (I, II)

5.3.1. Histamine storage and transport

Histamine-immunofluorescence was detected in granular structures in the TM neurons. This granular immunofluorescence was distributed evenly in the neurons including the cell soma, dendrites and axons.

To study whether VMAT2 is codistributed with histamine, histaminergic neurons were double-stained with the histamine and VMAT2 antibodies. VMAT2 was found

in almost all histaminergic neurons indicating that VMAT2 transports histamine to the subcellular vesicles. In the histaminergic cells, three types of immunoreactive granules were seen: those, which were both histamine- and VMAT2-positive and those, which were either histamine- or VMAT2-positive. Similar to histamine-positive granular deposits, the VMAT2-positive deposits were also evenly distributed in the cells. Since VMAT2 was found in almost all histaminergic neurons and only few VMAT2-positive neurons in the cultures were histamine negative, VMAT2 was subsequently used as an indicator of the tuberomamillary histaminergic neurons in such cases when antibodies were not compatible with the histamine antibody in double-stainings.

5.3.2. Co-localization of histamine with GABA and galanin

VMAT2 and GABA were distributed throughout the neuron. Moreover, GABA was located in small granular deposits distinct from those containing VMAT2. Galanin was also located in the VMAT2-positive neurons, their morphology being identical with the tuberomamillary neurons. Neurons, which were both galanin- and VMAT2-immunoreactive, were few in number. Moreover, galanin-immunoreactive structures were mainly located in neuronal somata but some immunopositive deposits were also seen in neurites. The colocalization of VMAT2 and galanin was found in some vesicles, but also vesicles which were immunopositive only to histamine or galanin, were found.

5.4. Mechanism of KA-induced neuronal death in cultured hippocampal slices (III)

In order to find out the time course of KA-induced neuronal death, organotypic hippocampal slices were treated with KA (5 μ M) for various time periods (6, 12, 24, and 48 h), and the recovery period (without KA) in the normal medium after the treatment was performed to find out whether the damaged neurons could be rescued. After the 6-h KA treatment, no cell death was detected in the cultured hippocampal slices. However, obviously cell death related cascades were switched on during that period, since neuronal death was detected in the CA3 region after the incubation of cultures in the normal medium for 48 h. Furthermore, longer KA treatment times (12, 24 and 48 h) induced time-dependent increase in neuronal death, which increased further after culturing slices in the normal culture medium for 48 h. Neuronal death was restricted mainly to the CA3 region, but occasionally damaged neurons were detected also in the DG, and CA1 regions.

In the subsequent experiments, the possible pathways involved in the KA-induced death process were defined. As hallmarks of apoptosis, expression levels of the active 17-kDa form of caspase-3 and the 84-kDa cleaved fragment of PARP were studied by Western blotting. Neither caspase-3 nor PARP levels altered from the control levels in the cultures treated with KA for 4, 8, and 24 h. Furthermore, no caspase-3 labelled cells were detected in the immunocytochemical study 24 h after KA treatment in the acute or

in the recovery (48 h) groups. Electron microscopic studies revealed the hallmarks of an early phase of necrosis such as irregular broken areas in the cytoplasm, and ruptures of plasmamembrane in the CA3 region. The damage was more advanced in the cell processes in the neuropile, in which broken cell debris in the interstitial space between the perikaryons in the cell processes was found. Otherwise KA-treated neurons had no signs of apoptotic pyknosis or chromatin condensation.

5.5. Decrease of KA-induced neuronal death in co-culture system with histaminergic neurons (IV)

The significance of the histaminergic neurons in the KA-induced neuronal death was examined by using an organotypic coculture system, in which the hippocampus was cultured together with the posterior hypothalamic slice containing histaminergic neurons (HI+HY, POST). Immunostainings in HI+HY, POST showed that histaminergic neurons survived well in the posterior hypothalamic slice, and histaminergic fibers grew into the hippocampal slice innervating the entire hippocampal slice in a diffuse manner. When the hippocampus was cultured together with the anterior hypothalamic slice devoid of histaminergic neurons (HI+HY, ANT), no histamine staining was detected.

No FJB-stained neurons were detected in control HI after 7 DIV in the normal medium indicating a good viability of slices in the culture system, whereas KA treatment (5 μ M, 12 h) resulted in neuronal damage in HI, the highest amount of degenerating neurons being in the CA3a/b subregion. In the scoring system, the area of FJB-stained neurons was measured (see page 53). Cell damage was significantly ($p < 0.001$) reduced in HI+HY, POST, when compared with the KA-treated HI. In HI+HY, ANT, the amount of FJB-stained neurons did not differ from the KA-treated HI ones. To confirm the results of scoring, number of the FJB-stained was counted focusing to the CA3 region. In accordance with the scoring results, the number of FJB-stained neurons was significantly ($p < 0.001$) decreased in KA-treated HI+HY, POST (17 ± 4 , mean \pm SEM) when compared with the KA-treated HI (74 ± 7 , mean \pm SEM) in the 250 μ m² area of the CA3a/b region.

The conventional thionin staining showed that the decrease in neuronal degeneration was not attributable to the disappearance of CA3a/b neurons after the 12 h KA treatment. However, after the 48 h KA (5 μ M) treatment with the 48 h recovery period, neurons in HI+HY, POST were more protected in the CA3a/b region than in HI with the same treatment.

5.5.1. Role of histamine levels on neuronal death

Alpha-FMH (100 nM, 24 h) inhibited histamine synthesis and led to practically total disappearance of histamine-labelled fibers within the hypothalamus and hippocampus. The duration of the α -FMH blocking effect in histamine synthesis was further studied in experiments, in which cocultures were cultured in normal medium for 12 h (recovery phase) after the initial α -FMH treatment (100 nM, 24 h). The 12 h recovery phase did not

lead to the reappearance of histamine-positive fibers in cultured posterior hypothalamic slices, which confirmed that histamine will not reappear in histaminergic fibers during the 12 h KA treatment. The scoring analysis of the FJB-stained neurons showed that neuronal damage significantly ($p < 0.001$) increased in HI+HY, POST treated with α -FMH (100 nM, 24 h) and followed by KA (5 μ M, 12 h) when compared with HI plus HY (POST) without the α -FMH treatment.

Extracellularly applied histamine resulted in significant neuroprotective effect only at the 1 nM histamine, whereas histamine concentrations from 10 nM to 100 μ M dose-dependently attenuated the neuroprotective effect of histamine in the CA3a/b region.

5.5.2. Role of histaminergic receptors in neuronal death

In order to find out the mechanisms, by which histamine-mediated neuroprotection may occur in the hippocampus, the H1 and H3 specific antagonists were used in HI+HY, POST. The H1 receptor antagonist triprolidine (2 nM - 2 μ M) and mepyramine (1 nM - 1 μ M) dose-dependently decreased the neuroprotection this effect being significant ($p < 0.05$) at low micromolar triprolidine (2 μ M) and mepyramine (1 μ M) concentrations. The H3 receptor-specific antagonist thioperamide (1 nM - 1 μ M) significantly ($p < 0.05$) increased the neuroprotective effect at 1 μ M concentration in HI+HY, POST, whereas clobenpropit (10 nM to 5 μ M) had no significant effect at any concentration.

5.5.3. Decrease in KA-induced epileptiform burst activity by the histaminergic neurons in the hippocampus

In control slices, typically only infrequent spontaneous activity was seen in the CA1 pyramidal cells. In HI treated with 5 μ M KA, the spontaneous activity was characterized by frequent ictal-type bursting. In HI+HY, POST, only occasional ictal-type bursts could be seen and the activity mainly consisted of interictal-type discharges. The occurrence of KA-induced spontaneous burst activity was significantly ($p < 0.001$) lower in HI+HY, POST (0.003 ± 0.0003 Hz) when compared with HI (0.024 ± 0.0027 Hz). Moreover, the interburst intervals were significantly ($p < 0.01$) longer in HI+HY, POST (117 ± 33 s) compared with HI (32 ± 4 s), indicating the different bursting pattern in these two culture types.

6. DISCUSSION

6.1. Methodological considerations

6.1.1. *The explant culture system for the histaminergic neurons*

In order to study the basic mechanisms of histamine synthesis, storage, and release, the primary culture system for the histaminergic neurons was developed. The first step in this process was to create a culture system, in which the histaminergic neurons would survive. The second step was to define whether the basic morphology of these neurons as well as the coexisting transmitters and transporters were comparable to those reported in the *in vivo* studies.

Previously, the histaminergic neurons have been shown to grow in the slice culture system (Reiner et al. 1988, Diewald et al. 1997). The main purpose of our study was to develop a culture system consisting of a few cell layers in a way that the histaminergic neurons would grow on the top of the glia cell layer. This kind of culture system would be more suitable for further studies with different immunomarkers by using the confocal microscopy system. After our study in 2004, a dissociated histaminergic neuron culture was published by a group, which has a long history in culturing monoaminergic neurons (Bajic et al. 2004). They cultured the histaminergic neurons on the glial feeder layer taken from the cerebral cortex of the 2-3-day-old rats. The feeder layers consisted of the protoplasmic astrocytes type 1 without the fibroblast-like, spindle-shaped cells. Moreover, they did not use fetal bovine or horse serum, but instead, they used rat serum, which was more suitable for maintaining rat neurons in culture conditions for a long period (Dichter, 1978). In addition, the culture medium was conditioned, which means that the medium was incubated overnight with glial feeder layers. According to the previous finding, during the conditioning period, glia cells take up extra glutamate, which is suggested to be toxic for monoaminergic neurons (Baughman et al. 1991). In this culture system the histaminergic neurons survived up to 2 months.

When comparing these two primary culture systems for the histaminergic neurons i.e. the one developed by us and that published by Bajic and coworkers (2004), both systems fulfil the main purpose: the histaminergic neurons survive and can be maintained successfully well in the *in vitro* conditions. Moreover, the neurons seem to morphologically correspond to those detected *in vivo*. In the Bajic's system, however, the culture conditions were defined in more detail, since they used only the certain glial cell type as the growth base for the histaminergic neurons, and moreover, they cultured cells in the conditioned culture medium. In our culture system, the critical issue was the coating molecules, from which laminin showed preference to poly-L-lysine. When using laminin, histaminergic neurons started to spread out from the explant and extend their axon and dendrites after day 4. The mechanism of laminin in enhancing cell survival in

our culture system is unknown. It is, however, generally known that different laminin molecules have multiple roles in the CNS. For example, they are important in Schwann cell segregation and growth, which in turn is important for the axon myelination (see review Colognato et al. 2005). In addition, laminin is known to regulate signalling of oligodendrocytes. Thus, laminin seems to play an important role in survival, growth and function of glial cells. Finally, in Bajic's study (2004), conditioned medium was used to decrease the toxic effect of extra glutamate on the histaminergic neurons, which were placed on the top of the glial feeder layer. In our study, conditioned medium was not used but the explant might have protected the histaminergic neurons during the critical early phase of the culture period.

Commercial fetal calf and horse serum was used in our culture system. It is possible that the rat serum used by Bajic and co-workers (2004) would be more suitable for cultures of the rat brain tissue. Interestingly, they used a low concentration (2 %) of serum, thus minimizing the amount of unknown molecules such as different growth factors in their culture medium. However, whether or not the rat serum was at equal quality in all experiments is not known.

Although the culture system used in our studies was suitable for culturing histaminergic neurons, numerous histaminergic neurons remained inside the explant, and could not be studied separately. Therefore, the culture system for the dissociated histaminergic neurons would probably give higher amount of neurons for the further studies. Moreover, the dissociated histaminergic neuron culture system might be better for maintaining the monoaminergic neurons for a long time.

6.1.2. The organotypic hippocampal culture system

In order to understand the pathological phenomena related to human epilepsy, different *in vivo* animal models are valuable tools, since in these models different systems are activated simultaneously thus resembling the situation during the seizure activity in humans. However, this advantage can also lead to difficulties due to the many variables that must be taken into account. In order to decrease variables without losing the functional network activity, the cultures of organotypic hippocampal slices have been widely used as an *in vitro* model to study physiological and pathological activity of the hippocampus. In this model, the hippocampus is cut through its septo-temporal axis, and each slice is considered as a functional unit having its intrinsic neuronal connections between different subregions. Therefore, both advantage and disadvantage of this model is that all the extrinsic connections from septum, thalamus, amygdala, hypothalamus, and brain stem are disconnected (see review Amaral and Lavenex 2007). Furthermore, also intrinsic three dimensional hippocampal connections such as innervation from CA3 region to the CA3 and CA1 regions, as well as the CA1 projections to the subiculum through the septo-temporal axis are lost (Amaral and Witter 1989, see review Amaral and Lavenex 2007). In addition, the connections of CA3 pyramidal cells with the neurons

located in the contralateral side of the hippocampus are cut. Therefore, we should know the limits of this model and be aware of the connections, which are lost. It is therefore of importance that new findings are always confirmed in the *in vivo* model.

Instead of using acute hippocampal slices, the organotypic culture system was found to be suitable for our studies, since we studied phenomena in time scale of a few days. Earlier studies have revealed that in these cultures synaptic organization, expression of receptors, and intrinsic hippocampal fiber pathways are developed corresponding to their *in vivo* counterparts (see review Frotscher et al. 1995, Holopainen and Lauren, 2003). However, the intrahippocampal connections are partly reorganized, and if the slices are cultured for longer periods, spontaneous activity resembling epileptiform activity can be detected in the *in vitro* conditions (Mohajerani and Cherubini, 2005, Lindroos et al. 2005).

6.1.3. The coculture system

The coculture system consisted of hippocampal slices, which were cultured together with the posterior or anterior hypothalamic slices in the semipermeable membranes. Previously, this kind of coculture system has been used with slightly different culture protocol (Reiner et al. 1988, Diewald et al. 1997). Morphology and physiological features of the histaminergic neurons in the coculture system confirms that the system is physiologically relevant (Diewald et al. 1997).

Our purpose was to create a system, in which we would induce physiologically relevant histamine release mimicking the function of the histaminergic neurons in the brain. Furthermore, our previous studies in the explant culture system suggested that histaminergic neurons contain also other neuroactive molecules such as GABA and galanin, which may be coreleased from the histaminergic neurons. Therefore, the net effect of histamine and other costored compounds during the KA treatment might only be achieved, when these molecules are released together from the histaminergic neuron. Interestingly, we actually showed that histamine released from the histaminergic neurons was neuroprotective, whereas commercial histamine, when added to the culture system, was protective only at low nanomolar concentrations. We therefore challenge the pharmacological experiments, in which compounds are added to the extracellular fluid and the phenomena are studied in the slices of one brain region.

In the coculture system, however, the histaminergic innervation in the hippocampus might differ from the *in vivo* conditions. Moreover, different hippocampal slices received partly quantitatively different innervation, since only four slices of the posterior hypothalamus could be used in the cocultures, and they contained different amounts of histaminergic neurons. In the *in vitro* system, the innervation was always diffuse having no primary subregion target in the hippocampus (Diewald et al. 1997), which is not fully in agreement with that of the *in vivo* conditions, in which the most prominent

histaminergic innervation occurred in the subiculum and DG, whereas the CA3 and CA1 regions received only weak innervation (Panula et al. 1989).

6.1.4. Immunostainings

Monoamines, which are relatively small molecules, are usually not detected directly by antibodies. Instead, antibodies are developed against larger peptides or proteins, for example enzymes, which are involved in the synthesis and degradation of monoamines. However, Panula and co-workers (1984, 1988) worked out a method, in which histamine was coupled to a carrier protein with EDAC, and by using EDAC also in the fixation protocol, the histamine-conjugate was possible to stain directly. By using the antibody against this histamine conjugate it is possible to detect reliably the localization of histamine in the cellular structures. However, the atypical fixation with EDAC produces problems when doublestained with another antibody. Because of that, VMAT2 was used as a marker of histaminergic cells, when colocalization of histamine with GABA and galanin was studied. Thus, there is a small possibility that some of the VMAT2-positive cells were not histaminergic but for example dopaminergic cells, which are located in the hypothalamic region (Lindvall and Stenevi 1978). However, the initial preparation of the explant from the posterior hypothalamus was done carefully in order to exclude the surrounding areas to the culture. Indeed, when the neurons were doublestained with antibodies against histamine and VMAT2, only a few cells were VMAT2-positive without being histamine-positive.

In order to clarify, whether the colocalization of different molecules was qualitatively reliable at the level of a subcellular vesicle several factors need to be considered. Firstly, whether the EDAC fixation (with 0.5% of PFA) compromised too much detection of VMAT2, for which the best fixation was 4% PFA. Indeed, when the amount of PFA was increased from 0.5% to 4%, as used in doublestaining of VMAT2 with galanin, also the amount of VMAT2-positive vesicles seemed to increase. Therefore, if the fixation were ideal for both histamine and VMAT2, there is a possibility, that all histamine-positive vesicles would also be VMAT2-positive. Secondly, the amount of antibody modified the results in a way, that at low concentrations, the amount of stained vesicles was low, whereas at high concentrations the amount of vesicles together with the unspecific staining increased. However, concentration of the antibody did not change the qualitative result of colocalization of the two compounds. Thirdly, different concentrations and incubation times of compounds used to increase penetration of the antibody did not change the results. Even though different variabilities of the stainings (EDAC-PFA-fixation, the amount of the antibody, and the compounds used to increase the penetration of the antibody) effected on the quantitative results, the fact that VMAT2 and GABA never coexisted in the same vesicular structures indicates that the antibodies really showed the reliable qualitative results.

6.1.5. FJB and estimation of the extend of neuronal damage

The exact mechanism of FJB staining is not known. It is suggested that the degenerating neurons express strongly basic molecule, which has high affinity for the strongly acidic FJB (Schmued et al. 1997). However, conventional histochemical staining methods such as hematoxylin-eosin and toluidine blue stainings, de Olmos' cupric-silver method, and propidium iodine uptake have shown similar results suggesting that FJB is a reliable marker for degenerating neurons (Schmued et al. 1997, Noraberg et al. 1999). However, although it is obvious that FJB-stained neurons are damaged, it is not clear whether or not they really die. There is a possibility that some of the cells may recover.

The conventional thionin staining showed that the attenuated neuronal degeneration in HI+HY, POST when compared to HI was not attributable to the disappearance of CA3a/b neurons after the 12 h KA treatment. This suggests that at the beginning of the degeneration process FJB-stained neurons were damaged but not yet dead. However, after the 48 h KA treatment with the 48 h recovery period, thionin staining showed that in HI+HY, POST neurons were more protected in the CA3a/b region than in HI with the same treatment indicating that these neurons do disappear when the insult is strong or long enough, and that histaminergic neurons are protective even during the longer insult.

6.2. Morphology and neurotransmitter storage related to function of the histaminergic neurons

Morphology of histaminergic neurons has previously been studied to characterize newly found neurons (Watanabe et al. 1983, Panula et al. 1984), to compare histaminergic neurons in different species (see review Nässel 1991, Kaslin and Panula 2001, Parmentier et al. 2002), and to understand the physiological properties of these neurons (Reiner et al. 1988, Diewald et al. 1997). In our study, morphology of the histaminergic neurons was examined to verify that the histaminergic neurons in our culture system were morphologically comparable to those *in vivo*, and that this kind of culture system would give reliable results in the following experiments.

6.2.1. Size and form of the histaminergic neurons

Size of the neuron is difficult to measure two-dimensionally, since shape of the neuron can be very complex, and it is not easy to define the axis, which gives the most reliable estimation of the size. The best way to measure the size would be to define the volume of the neuron by the three-dimensional reconstruction of thin slices and mathematical volume estimation. However, this method is rather laborious.

Previously, the size of the histaminergic cell body *in vivo* has been measured in the adult rats. Based on the size, the histaminergic neurons have been classified into two or three categories, in which cells are of small (< 18 μm), medium ($\sim 25 \mu\text{m}$) or large (> 30

μm) size (Wouterlood et al. 1986, Ericson et al. 1987). In two studies, the histaminergic neurons have been shown to be mostly large (25-40 μm) (Hayashi et al. 1984, Airaksinen et al. 1991). There is no *in vivo* information about the size of the histaminergic neurons during the postnatal development. The *in vitro* study of Bajic and coworkers (2004) demonstrated that when the cultures are started from the 3-day-old rat and cultured for 2 months, the soma diameter of the histaminergic neuron (only one value) varies from 5 to 30 μm . The average value of the soma diameter of adenosine deaminase-positive neurons (18.8 μm) and histamine-positive neurons (16.3 μm) in Bajic's work is close to our value for the long axis (19.2 μm).

Although we did not categorize the histaminergic neurons by size, our values of size of the cell soma are similar to those found in the *in vivo* studies in the rat (see table 1 in page 19) (Hayashi et al. 1984, Wouterlood et al. 1986, Ericson et al. 1987). Our highest value of the long axis was 28.4 μm and the lowest 12.3 μm , which is in agreement with the earlier *in vivo* studies. The value of the long axis in our study may indicate the existence of a subpopulation of large histaminergic neurons, and the smallest value the subpopulation of small histaminergic neurons. However, categorizing of the histaminergic neurons does not so far give any additional information, since no functional characteristics have been connected to the cell size.

Three most common cell shapes of the histaminergic neurons were now detected: oval, rounded, and triangular. In our study, the most common shape was oval (73 % of the studied neurons). This is in accordance with the earlier studies, in which all these shapes (Hayashi et al. 1988, Reiner et al. 1988, Airaksinen et al. 1991), or only oval and round shapes (Ericson et al. 1987, Zimatkin et al. 2006), oval and multipolar neurons (Bajic et al. 2004) or mainly oval type of cells (Wouterlood et al. 1986) have been detected. The cell shape might be correlated to the number of dendrites. Most of the histaminergic neurons are probably bipolar, which means that one neurite begins from each side of the elongated cell body. In case of multipolar cells, the higher number of dendrites may indicate that histaminergic neurons receive input from the more widespread brain region.

6.2.2. Axon of the histaminergic neuron

In most histaminergic neurons, all neurites, which started from the cell soma, were MAP2-positive indicating that they were all dendrites. In some cases, a thin MAP2-negative process beginning from the cell soma was detected. Occasionally, the axon was detected to arise from dendrite, which is in accordance with the earlier studies. (table 1 in page 19) (Wouterlood et al. 1986, Reiner et al. 1988).

The unusual morphology of the dendritic tree and initiation of the axon from one of the dendrites actually challenges the classical picture of the neuronal activation, which starts in dendrites, summates in the cell body, and if the threshold is exceeded, the action potential initiates in the axon hillock, and propagates along the axon. In the substantia

nigra dopaminergic neurons, an action potential has been shown to initiate in the axon, which grows from one of the dendrites. From there, the action potential backpropagates to the dendrite connected to the axon, and continues to the cell soma and other dendrites (Häusser et al. 1995). In this study, the authors suggest that the dendrite bearing the axon represents a privileged dendrite, since its synaptic input may influence to the initiation of the action potential. Furthermore, since the action potential backpropagates from axon to the privileged dendrite and the cell soma, the privileged dendrite actually regulates the input to the soma and other dendrites (Häusser et al. 1995).

6.2.3. Distribution of the histamine-containing vesicles

According to our finding, histamine-containing vesicles can be detected all over the neuron including the axon, cell soma and dendrites, which is in accordance with the *in vivo* findings (Michelsen and Panula 2002). This kind of neurotransmitter distribution may indicate that histamine is released nonsynaptically from axon varicosities, dendrites, and soma. In other monoaminergic neurons, in which the transmitter release has been measured in the awake animals by using microdialysis and *in vitro* studies, somatodendritic release has been shown to occur in dopaminergic (Kalivas and Duffy 1991, Heeringa and Abercrombie 1995, see review Adell and Artigas 2004), serotonergic (see review DeMiguel and Trueta 2005), and noradrenergic neurons (Huang et al. 2007). Furthermore, subcellular location of VMAT2 in all subcellular compartments of the dopaminergic neurons in the substantia nigra and ventral tegmental area also suggests the possibility of the somatodendritic release mechanism (Nirenberg et al. 1996). In fact, replacement of certain amino acid residues in the C-terminal domain of VMAT2 disrupts sorting of VMAT2 to LDCV, and eliminates the regulated exocytosis of VMAT2 in dendrites, but only partly impairs exocytosis in axons (Li et al. 2005). Functionally, somatodendritic release of monoamines is suggested to be dependent on the stimulus frequency. For example, in accompanied amperometry and patch-clamp study from noradrenergic neurons in the locus coeruleus brain slices, high frequency bursts (20-50 Hz) caused significantly increased secretion of noradrenaline from the cell soma, when compared to low frequency (4 Hz) or spontaneous activity (Huang et al. 2007).

6.2.4. Existence of GABA and galanin in the histaminergic neurons

GABA and galanin have previously been shown to be located in the histaminergic neurons *in vivo* (Ericson et al. 1991, Airaksinen et al. 1992), but their intracellular location and functional importance in the histaminergic neurons has been less clear. Our results showed that both GABA and galanin are colocalized in the histaminergic neurons in a way that GABA was stored in the distinct vesicles, whereas galanin was partly colocalized with histamine in the same vesicles.

Both GABA and glutamate have been shown to coexist in dopaminergic, serotonergic, and cholinergic neurons (see review Torrealba and Carrasco 2004). It is

poorly understood, in which circumstances monoamines are coreleased with glutamate or GABA, and what are the consequences of the corelease. However, it has been known already in the 1970's that direct application of dopamine inhibits firing of neurons in the striatum, while stimulation of the dopaminergic neurons in the substantia nigra generally produces excitation in the same region (Siggins et al. 1976). Recently, the functional relevance of corelease of dopamine and glutamate has been hypothesized to be connected to the formation of temporally precise information during learning process (see reviews Lapish et al. 2007, Schultz et al. 2007). It has previously been shown that dopaminergic neurons are important in prediction of the reward during learning, and if the reward is not predicted correctly, the phenomenon is called reward-prediction error (see review Schultz et al. 2007). Glutamate is suggested to be important in the precise timing of the dopaminergic action in the reward-prediction error signal (Lapish et al. 2007). The role of GABA in the histaminergic neurons is not known, but it would be interesting to speculate that the same kind of behavioural correlation might exist, when histamine is coreleased with GABA.

In general, monoaminergic neurons contain several neuropeptides, which are usually co-localized with the amine transmitter in the LDCV (see review Zupanc 1996). Galanin for example has been shown to be colocalized with monoamines including histamine (Staines et al. 1986, Köhler et al. 1986, Airaksinen et al. 1992), serotonin (Xu et al. 1998, see review Hökfelt et al. 1998), noradrenaline and dopamine (Melander et al. 1986a), and acetylcholine (Melander et al. 1986b). Release of neuropeptides seems to be highly dependent on frequency of the stimulus (Whim and Lloyd 1989, Karhunen et al. 2001), but in which circumstances neuropeptide and monoamine are released and what is the net effect of corelease, is not yet understood.

6.3. Mechanisms of KA-induced neuronal death

The mechanism of KA-induced neuronal death is difficult to define, since apoptosis (Pollard et al. 1994, Simonian et al. 1996, Tuunanen et al. 1999, Dong et al. 2002), necrosis (Sperk et al. 1983, Fujikawa et al. 2000), and neuronal death having features from both apoptosis and necrosis (Portera-Cailliau et al. 1997, Humphrey et al. 2002), have been reported. In general, the KA-induced neuronal death depends on how KA has been administered, what is the concentration of KA, how far away is the brain region from the site of KA injection, how long after KA injection the samples have been studied, and what is the species, strain, and age of the studied animals.

6.3.1. KA-induced excitotoxic cell death in the immature hippocampus

The cultured hippocampal slice taken from P7 rat and cultured for 7 days can be considered as immature. Different cell types and connections as well as functions related to the structural changes are still developing at that age and during the culture period (see reviews Frotscher 1995, Holopainen 2005, Gähwiler et al. 1997, Holopainen 2008).

In other studies, immature slices are considered the ones prepared from the animals not older than 10 days (see review Gähwiler et al. 1997). There is a simple technical reason for that. The slices taken from older animals are difficult to maintain in culture conditions. Also slices from older animals have been successfully cultured, and morphology as well as the functional characteristics resemble those seen in the more mature animals (Xiang et al. 2000). However, we should keep in mind that separated brain slice cultured in an artificial environment forms an individual unit with reformed intrinsic connections and slightly different functional properties.

The culture system was created to study the mechanisms of neuronal death in the developing hippocampus. However, even though hippocampal sclerosis has been detected in pediatric patients with epilepsy, it is usually not known whether the hippocampal sclerosis occurred before the onset of seizures, thus being cause of seizures, or if the hippocampal sclerosis follows after the recurrent seizures thus being the consequence of seizures. In general, the highest incidence of epilepsy is during the first year of life (see reviews Hauser 1994, Haut et al 2004), even though at that time the brain is less vulnerable than later in life (see review Lado et al. 2002). During maturation, the seizure-induced neuronal degeneration gradually increases (see review Haut et al 2004), which is in accordance with the impairment in cognition observed in pediatric patients, who suffer from epilepsy (see review Holmes 2004).

In our model, the KA-induced cell death was detected in the CA3 pyramidal neurons, whereas CA1 pyramidal neurons remained intact. This is in agreement with the previous studies in epileptic animals (Buckmaster and Dudek 1997, Zhang et al. 2002), and in the organotypic slice cultures (Bruce et al. 1995, Routbort et al. 1999, Liu et al. 2001). Moreover, the KA-induced damage of CA3 neurons was augmented during the 48 h culture time in normal medium, the finding which is partly in accordance with a study in immature cultured hippocampal slices (Bruce et al. 1995). In this study, the initial insult (KA 50 μ M, 3 h) followed by the 48 h resting phase in normal culture medium resulted in a progressive neuronal loss not only in CA3 but also in CA1. Although the mechanisms of increased damage after the withdrawal of KA are not known, one reason could be that pathological electrical activity continues within the intrahippocampal network after KA withdrawal, and finally leads to neuronal death as recently suggested for glutamate-induced toxicity in the cultured hippocampal slices (Lahtinen et al. 2001).

6.3.2. Apoptosis vs. necrosis

KA-induced excitotoxic neuronal death in the developing animal is usually first necrotic showing later also apoptotic features (Humprey et al. 2002, Dong et al. 2003, Portera-Cailliau et al. 1997). However, the type of death (apoptotic or necrotic) has been shown to depend on KA concentration and the time of the KA treatment (Humprey et al. 2002). Therefore, the concentration and time of the treatment we used is probably more suitable

for studying necrotic cell death, but at the lower KA concentration or by using a shorter treatment, it would be possible to detect also features of apoptosis.

In spite of the massive neuronal death comprising the entire CA3 region, the expression of the active caspase-3 and uncleaved and cleaved PARP proteins were not significantly changed after the KA treatment at any time point studied (4 up to 24 h) (Fig. 14). Moreover, active caspase-3 immunoreactivity was negative in both acute and resting groups, and no ultrastructural signs of apoptosis were detected after the 24 h KA treatment.

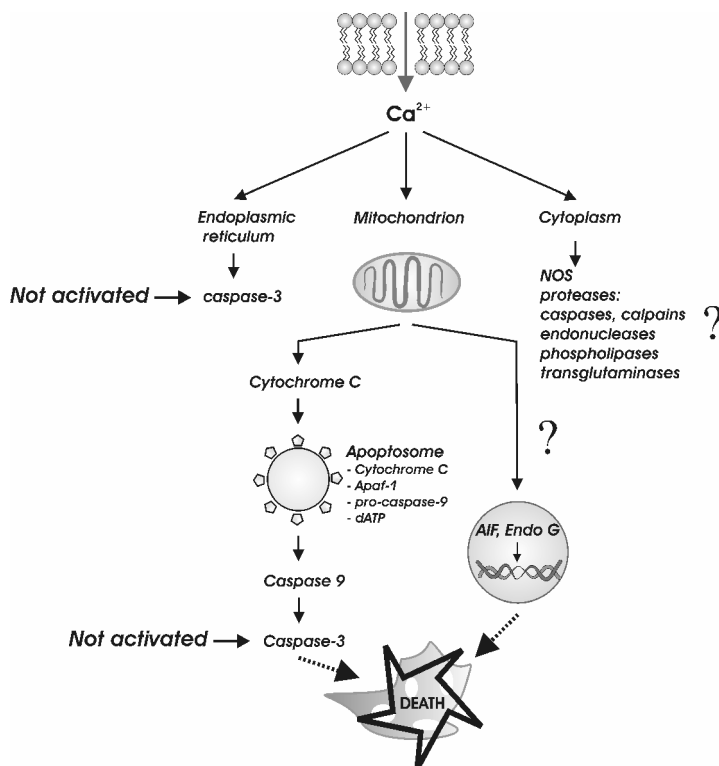


Figure 14. Pathway of the cell death mechanism in our model. The caspase-3 activation was not changed after the KA treatment indicating that the mitochondrial pathway through the cytochrome c release and caspase-3 activation is not involved. However, the caspase-independent pathway through AIF and EndoG activation may be involved in KA-induced neuronal death, as well as pathways related to the NOS and different proteases, which are activated by increased intracellular Ca^{2+} levels. Abbreviations: AIF=apoptosis inducing factor, Apaf-1=apoptotic-protease-activating factor 1 (Apaf-1), dATP=deoxyadenosine triphosphate, Endo G=endonuclease G, NOS=nitric oxide synthetase. (Based on Orrenius et al. 2003, Syntichaki and Tavernarakis 2003).

Activation of the mitochondrial apoptotic pathway has been earlier detected in the KA-treated hippocampal slices, which were taken from 6-8 day-old rats and cultured for a longer period (20–25 DIV) (Liu et al. 2001). In this study, pyramidal cell death, induction of Bax expression, and increased cytochrome c and caspase-3 levels were detected within

24 h after KA (50 μ M) application. Here apoptotic mechanisms are activated within hours after the initial insult (Liu et al. 2001), thus excluding the possibility that we might have missed the appropriate time for detection. Also in the *in vivo* study, a more delayed apoptotic cell death, i.e. 75 days after KA injection, has been reported in the developing animals (P7) (Montgomery et al. 1999, Humphrey et al. 2002). The fact, that we did not detect any changes in the caspase-3 activation, may indicate that neuronal death was either necrotic or caspase-independent apoptosis. However, electron microscopic studies showed mainly necrotic changes in the dying neurons indicating that cell death was mainly necrotic.

The fact, that levels of the cleaved (85 kDa) and uncleaved PARP (116 kDa) did not show any KA-induced changes, is confusing, since according to the recent theories PARP should be activated both in apoptosis and necrosis. However, several studies show that PARP is activated in excitotoxic cell death, but mainly by the NMDA receptor agonist (Mandir et al. 2000, Wang et al. 2005, Andrabi et al. 2006, Duan et al. 2007). The reason, why KA-induced neuronal death does not activate PARP, is not known.

In addition to the importance of apoptosis in the pathological cell death, it also plays a central role in controlling cell numbers during the brain development (see review Kuan et al. 2000). We detected in our Western blots signals of active caspase-3, and the uncleaved and cleaved PARP in both control hippocampal slices, and in samples prepared from P14 rat hippocampi. These results suggest that the normal, developmentally regulated programmed cell death is still to some extent operational at this age in the rat hippocampus.

6.4. Mechanisms of histamine-mediated protection on excitotoxic cell death in the developing hippocampus

6.4.1. Endogenous vs. exogenous histamine in neuroprotection

Our results showed that neuronal histamine is important in induction of neuroprotective effect in the KA-induced excitotoxicity in the *in vitro* model, since neuronal death was significantly decreased in the hippocampal slice when it was cultured with the posterior hypothalamic slice containing histaminergic neurons. Furthermore, inhibition of histamine synthesis by α -FMH eliminated the protective effect. However, commercial histamine applied to the culture medium was neuroprotective only at low nanomolar concentration. The concentration-dependent effect in neuroprotection suggests that neuronal histamine release and consequent increase in histamine concentration in the extracellular space is strictly regulated by H3 autoreceptors (van der Vliet et al. 1990, Morisset et al. 2000). High, not-neuroprotective concentrations of histamine probably inhibit histamine/corelease from the histaminergic neurons. The direct evidence of this is that application of histamine reduces firing of the histaminergic neurons (Brown et al. 2001).

Histamine has been shown to have both neuroprotective and neurotoxic effects. In the earlier studies, pre- or post-treatment with histidine or histamine has attenuated neuronal

death, whereas depletion of neuronal histamine by α -FMH or pre-treatment with H3 receptor agonist has aggravated neuronal degeneration in the animal models of ischemia (Sugimoto et al. 1994, Fujitani et al. 1996, Adachi et al. 2004), and in NMDA-induced death of cultured cortical neurons (Dai et al. 2006). As a neurotoxic compound, additional histamine causes death of cultured cerebellar neurons (Gepdiremen et al. 2003), and the dopaminergic neurons of the substantia nigra (Vizuete et al. 2000). Furthermore, histamine can cause NMDA-dependent neuronal swelling in the neostriatum (Colwell and Levine 1997).

Toxicity of histamine seems to depend on the histamine concentration, and on the brain region studied. In the cultured cerebellar neurons, additional histamine induces dose- dependent neuronal death, and for example, 10 μ M histamine kills more than 50 % of cells (Gepdiremen et al. 2003). Furthermore, in the study of Colwell and Levine (1997), 100 μ M of histamine together with 10 and 100 μ M NMDA caused neuronal swelling. In addition, different brain regions show different vulnerability, since 100 nM histamine induces selective damage of dopaminergic neurons in the substantia nigra without having any effect on dopaminergic neurons in the striatum, on cholinergic neurons in the medial septum, or serotonergic neurons in the medial lemniscus (Vizuete et al. 2000).

According to our results, the proposed effect of histamine on KA-induced neuronal death is depicted in Fig. 15.

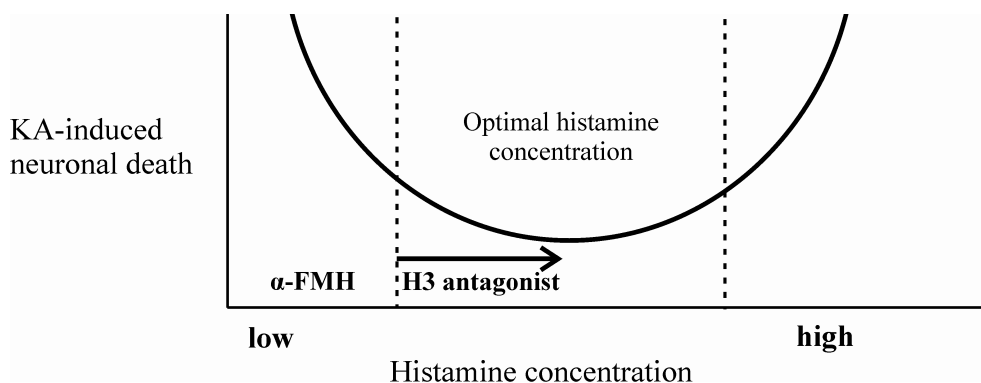


Figure 15. A hypothesized connection of histamine concentration to KA-induced neuronal death in the slice culture model. At the optimal histamine concentration the KA-induced neuronal death is minimal. H3 antagonist (or inverse agonist) blocks the H3 autoreceptor function in the histaminergic neurons inducing an increase in histamine release (an arrow from the left line to the central point of the area of the optimal histamine concentration), which further ameliorates the neuroprotective effect of the histaminergic neurons. Alpha-FMH in turn inhibits histamine synthesis and release, and increases KA-induced neuronal death in the coculture system. The histamine concentrations above the optimal range increase dose-dependently the KA-induced neuronal death. Abbreviations: α -FMH= α -fluoromethylhistidine.

The reason why high amount of histamine is not neuroprotective in our study may mimic the situation, in which high amounts of histamine are released from mast cells.

Increased histamine levels have been detected in the animal model of Wernicke's encephalopathy, the disease, in which inhibition of histamine synthesis by α -FMH is neuroprotective (Langlais et al. 1994, McRee et al. 2000). Furthermore, in Wernicke's encephalopathy, increased histamine release has been detected both in the hippocampus and thalamus, but granulocytes (including mast cells) were detected only in the damaged thalamic region (McRee et al. 2000). Moreover, when hippocampal neurons are co-cultured with mast cells, excitotoxic injury is enhanced by 60 %, and it is abolished in the presence of diamine oxidase, which catalyzes inactivation of histamine (Skaper et al. 2001). Finally, degranulation of histamine from mast cells and consequent cell death may be preceded by an inflammatory reaction such as activation of astrocytes, as it has been shown to occur in the substantia nigra after an injection of 100 nM histamine (Vizuete et al. 2001). However, in our study, the high concentration of histamine (10-100 μ M) was not toxic itself, although it eliminated the neuroprotective effect of histamine seen in the coculture system after the KA treatment.

Histamine decreases neuronal death at least partly by inhibiting the KA-induced epileptiform activity in the CA3 region. However, other mechanisms may also be involved. For example, histamine may modulate cell death cascades primarily as in case of dopamine 2 (D2) receptors, which activate anti-apoptotic pathway by replacing N-ethylmaleimide-sensitive factor from the AMPA receptor (see review Bozzi and Borrelli 2006).

Finally, GABA and galanin, which are both inhibitory neurotransmitters, may induce an inhibitory and neuroprotective net effect if coreleased with histamine during the seizures. Galanin inhibits epileptic seizures through galanin 2 receptors, whereas galanin 1 receptor shows proconvulsant activity (see review Mazarati 2004, Mazarati et al. 2005, McColl 2006). Interestingly, when galanin is coreleased with serotonin, both pro- and anticonvulsive activity of galanin can be detected (Mazarati et al. 2005). However, when serotonin is depleted, also the response to galanin is eliminated.

6.4.2. Histamine receptors in neuroprotection

H1 and H2 receptors are involved in neuroprotection, since blocking of these receptors enhances neuronal death both in the animal model of ischemia and in cultured cerebellar neurons (Adachi et al. 1993, Fujitani et al. 1996, Diaz-Trelles et al. 1999, Diaz-Trelles et al. 2000, Adachi et al. 2001, Otsuka et al. 2003), whereas H2 agonists have been shown to be neuroprotective (Hamami et al. 2004, Dai et al. 2006). Our results showed that H1 receptor is important in neuroprotection, but the exact mechanism is not known.

Different effects of thioperamide and clobenprobit are difficult to explain, since they both are inverse agonists of H3 receptor, and they both increase histamine synthesis and release in the TM neurons (Moreno-Delgado et al. 2006). In our studies, thioperamide further decreased neuronal death in the coculture system, whereas clobenprobit did not have any effect. This suggests that H3 receptors might have different mechanisms, by which they respond on excitotoxicity. One explanation is the differences in their autoreceptor-mediated

effects on histamine synthesis and release. In addition, they may have also differences in the heteroreceptor function, for example in synthesis and release of GABA. The possible H1 and H3 receptor-mediated mechanisms of histamine-mediated protection against KA-induced death of CA3 neurons have been compiled in Fig. 16.

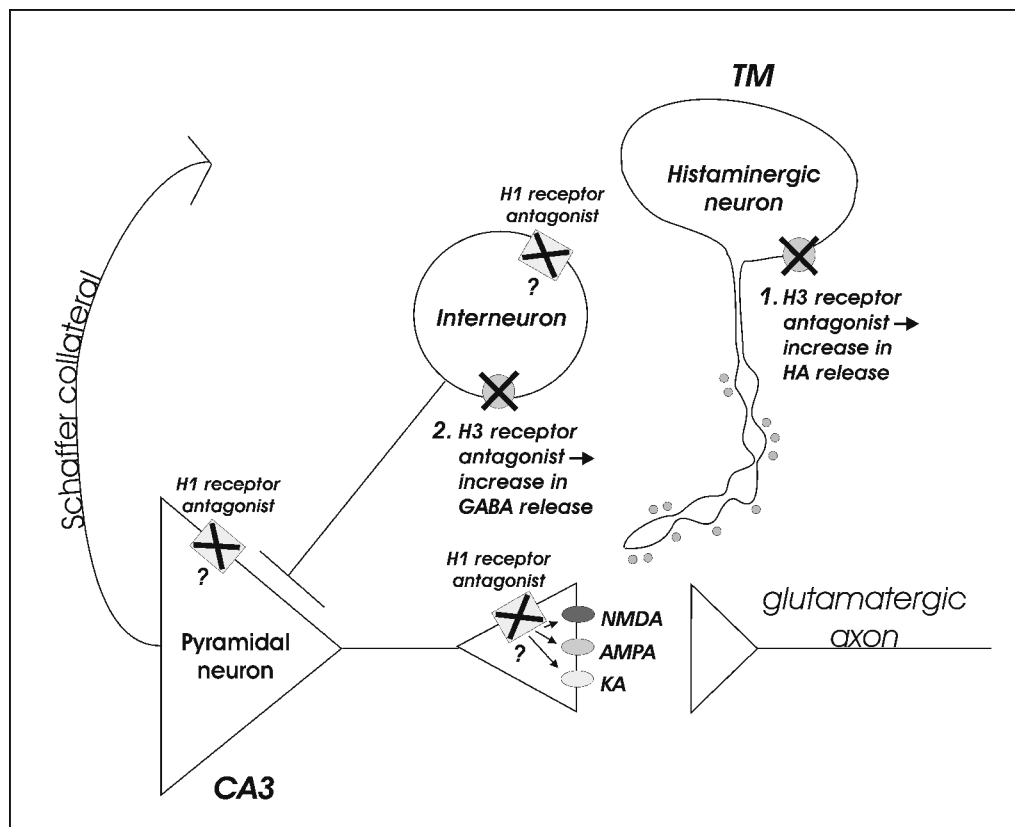


Figure 16. The possible H1 and H3 receptor-mediated neuroprotective mechanisms in the CA3 region of the hippocampus. Histamine is released nonsynaptically from the axon varicosities (volume transmission). In our model, H3 antagonist further decreased neuronal death, which may occur through two different mechanisms. First, the H3 antagonist can eliminate the autoreceptor-mediated inhibition of the histamine release resulting in enhanced histamine release. Second, the H3 receptor as a heteroreceptor can also inhibit GABA release. The H3 antagonist (or inverse agonist) in turn increases GABA release from interneurons, which might be neuroprotective. In our model, inhibition of the H1 receptor eliminated the neuroprotective effect of histamine, which indicates that H1 receptor at least partly regulates the neuroprotection. Whether the H1 receptor-mediated neuroprotective cascades are activated in the interneurons or pyramidal neurons, is not known. Abbreviations: AMPA=AMPA receptor, CA3=CA3 region of the hippocampus, GABA= γ -aminobutyric acid, HA=histamine, KA=KA receptor, NMDA=NMDA receptor, TM=tuberomamillary nucleus.

7. CONCLUSIONS

The central histaminergic neuron system is a powerful regulator of different homeostatic states of our body. However, the basic mechanisms of histamine storage, release and colocalization of other compounds in these neurons are poorly understood. Furthermore, in epilepsy, the histaminergic system has been shown to control the threshold, duration and strength of seizures, but the importance of the histaminergic neurons in the KA-induced seizures and consequent neuronal death have not been studied. Therefore, our purpose was to develop different *in vitro* culture models for the histaminergic neurons, to compare the morphology of these neurons to the previous findings *in vivo*, to understand the mechanisms of the KA-induced neuronal death in the immature organotypic hippocampal slice cultures, and finally, to study, whether or not the histaminergic neurons are neuroprotective in the KA-induced neuronal death.

We showed that the histaminergic neurons were maintained successfully in the *in vitro* conditions, and their morphological features were comparable to those detected previously *in vivo*. Furthermore, histamine containing storage vesicles were distributed throughout the neuron consistent with the idea of volume transmission and suggesting a possibility for the somatodendritic release mechanism. VMAT2 was partly colocalized with histamine in the same subcellular vesicles suggesting that VMAT2 transports histamine to the vesicles. Finally, in the histaminergic neurons GABA was localized in distinct vesicles whereas galanin was partly colocalized in the same subcellular vesicles as VMAT2 suggesting different corelease mechanisms for GABA and galanin with histamine. This is the first evidence about costorage of GABA and galanin with histamine at the level of a single vesicle. These results are important for future studies, in which the mechanisms of histamine release/corelease and its regulation are considered.

KA-induced neuronal death in organotypic hippocampal slice cultures was region-specific, restricted mainly to the CA3 region consistent with the previous results. Furthermore, the KA-induced death process was irreversible, since recovery period in normal culture medium did not save the cells but instead increased the damage. In addition, KA-induced neuronal death seemed to be necrotic, since the levels of the apoptotic markers did not change after the KA treatment, and ultrastructural changes were characteristic for necrosis.

The coculture system of the hippocampus and the posterior hypothalamus (HI+HY, POST) was created to mimic the physiologically relevant histaminergic regulation of hippocampal functions. In culture conditions, the histaminergic neurons innervated the hippocampal slice in a diffuse manner.

The histaminergic neurons significantly attenuated the KA-induced neuronal death of the CA3 pyramidal neurons in HI+HY, POST. The neuroprotective effect was eliminated when histamine synthesis was inhibited by the specific inhibitor α -FMH or when the hippocampus was cultured with the anterior hypothalamic slice devoid

of the histaminergic neurons. Furthermore, the KA-induced epileptiform activity recorded in the CA1 region significantly decreased in HI+HY, POST, when compared to the hippocampus cultured alone. Histamine when added to the hippocampal slices cultured alone was neuroprotective only at low nanomolar concentrations. This favours the idea that possible histamine-based antiepileptic and neuroprotective drugs should be designed to regulate the activity of the histaminergic neurons and not to increase histamine concentration in the brain.

The neuroprotective effect of the posterior hypothalamus was at least partly mediated through H1 and H3 receptors. The H3 antagonist thioperamide further increased this neuroprotective effect probably through autoreceptor-mediated increase in histamine synthesis and release. H1 receptor antagonists decreased dose-dependently the neuroprotective effect of the posterior hypothalamus. This suggests that H1 receptor has an important role in neuroprotection.

Epilepsy is not a homogenous disease, which can be treated by one certain type of drug, but different solutions for the drug development are needed. Therefore the central histaminergic system, which can regulate activity of the entire system effecting simultaneously on several ion channels and downstream signalling pathways might be a good target for designing new drugs against some epileptic disorders. We showed for the first time that the central histaminergic neurons can at the same time inhibit epileptiform activity and decrease neuronal death in the *in vitro* model. This kind of doubleprotective effect is an important strategy in the development of new antiepileptic drugs, since the currently used drugs mainly decrease epileptiform activity but seem not to have significant neuroprotective effect.

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Turku, May 2008

A handwritten signature in black ink, appearing to read 'Tiina-Kaisa Kukko-Lukjanov'. The signature is written in a cursive, flowing style with a large initial 'T' and 'K'.

Tiina-Kaisa Kukko-Lukjanov

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