MEDS 370 – INTRODUCTORY NEUROSCIENCE

Neurotransmitters
September 14, 2001 9:00 am EG052
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Reading: all three texts do a fine job of covering this material
Kandel, Schwartz and Jessell, Principles of Neural Science, 4th Ed (2000) Chapter 15, Neurotransmitters, pp.280-297
Zigmond et al., Fundamental Neuroscience (1999) Chapter 8, Neurotransmitters, pp.193-234
Purves et al., Neuroscience, 2nd Ed (2001) Chapter 6, Neurotransmitters, pp.117-140

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What Makes a Molecule a Neurotransmitter? Are over a hundred

localization - present at presynaptic site; usually made in that neuron
release - usually expect dependence on **extracellular Ca^{++}**, increase with **frequency**
mimicry – given exogenously, get same effect; **quantitation** important here
inactivation - by enzymatic degradation (ACh, peptides), reuptake (NE, DA, 5-HT, GABA, Gly, Glu) or diffusion (all)

enough has to be released by the synaptic ending to do the job postsynaptically

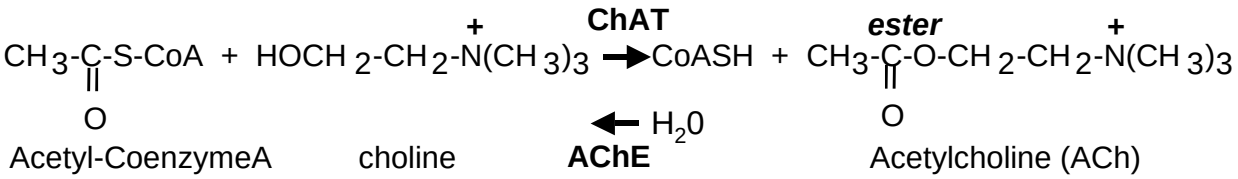
What Molecules Are Used as Neurotransmitters?
Small molecules: acetylcholine, biogenic amines (dopamine, norepinephrine, epinephrine, serotonin, histamine), amino acids (glycine, glutamate, GABA), ATP and adenosine

Peptides: fall into families of molecules with similar sequences – far out number the small molecule transmitters.

Small, clear synaptic vesicles store small molecule transmitters. Peptides are in large dense core vesicles; small molecule transmitters can also be stored here. ATP is present in both type of vesicle.

ACETYLCHOLINE

ACh - is the neurotransmitter at **nicotinic AChR's** (ligand-gated ion channels), notably the skeletal neuromuscular junction; and at **muscarinic** (7 transmembrane domain, second-messenger-mediated) receptors throughout the autonomic nervous system and the brain. ACh is probably a key player in nicotine addiction/dependence, may improve performance on some learning tasks, and is central to many symptoms of Alzheimer's disease. AChE inhibitors are the key ingredients in many pesticides and nerve gases; AChR blockade is a common result of many snake venoms. AChE inhibitors are being tested clinically for treating glaucoma, myasthenia gravis, and Alzheimer's disease.

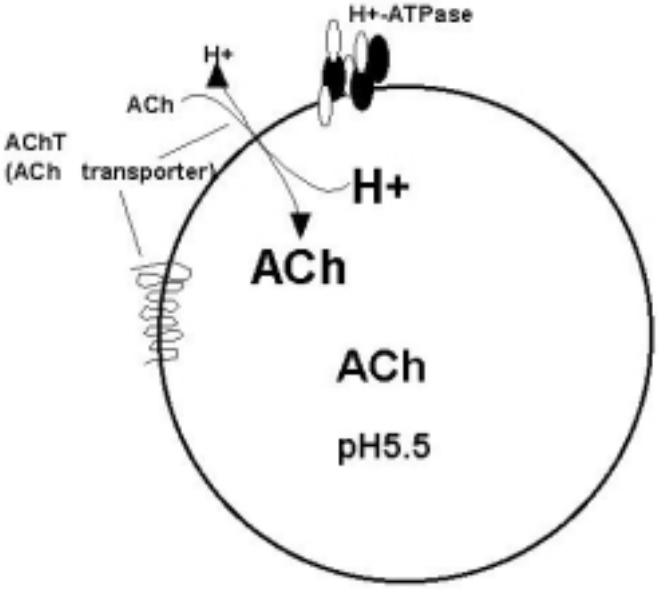


Choline Acetyltransferase (ChAT)

a soluble, cytoplasmic enzyme; the only enzyme unique to ACh synthesis
fast (not rate limiting); turnover ~250 moles ACh/mole enzyme/sec
choline supply is limiting; K_D of ChAT for choline is ~ 1 mM; K_D for AcCoA ~ 10 µM
neurons DO NOT make choline - choline must come from diet
choline uptake at plasma membrane is the rate limiting step in ACh synthesis
hemicholinium-3 blocks high affinity choline uptake system
K_m for uptake is below the blood [choline] ~10 µM

Vesicular ACh Transporter (AChT) – moves ACh from cytosol into synaptic vesicle
A 12 transmembrane domain protein - architecture related to bacterial drug resistance proteins and sugar transporters

Transport is ACh^+ exchange for H^+
1 or 2 H^+ leave granule as 1 ACh^+ enters
vesamicol blocks this process
K_m for uptake ~0.25 mM ACh
(remember cytosolic [ACh] ~ 4 mM)
= always full speed ahead!



Store about 2000 ACh molecules per vesicle in CNS. One quantum is <10,000 molecules of ACh or about the same as content of one vesicle.

ATP is stored in ACh vesicles and released with ACh; acts on targets as ATP (brain) or after hydrolysis to adenosine (periphery, especially muscle).

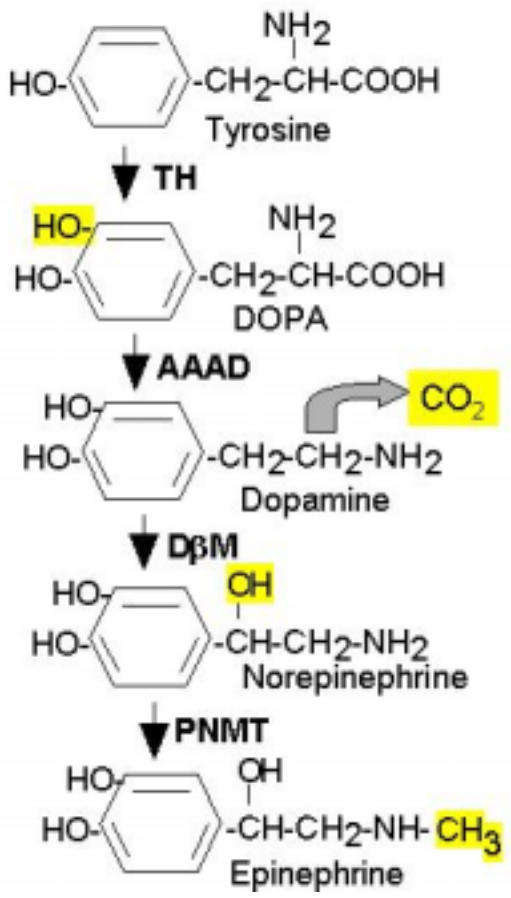
Acetylcholinesterase (AChE) – enzymatic inactivation is critical for ACh
AChE is an extremely fast enzyme – one molecule can degrade 5000 molecules of ACh/sec
Sources of AChE: neurons, target cells, glia
Idea of an ACh cloud in CNS gaining some support (like DA in some areas of the brain)
Choline generated by AChE is retrieved by plasma membrane choline transporter

BIOGENIC AMINES Catecholamines (dopamine, norepinephrine, epinephrine): <1% of neurons in brain make catecholamines, but all neurons in CNS are within 30 µm of a catecholamine ending; many CA endings are 5-30 µm from target; some are 50 nm.

Catecholamines are made from tyrosine by a total of 4 key enzymes:

Rate-limiting step is first one – catalyzed by
Tyrosine Hydroxylase (TH)

TH requires Fe^{+++}, molecular O_2, and tetrahydrobiopterin (BH_4) – regenerated by Pteridine Reductase (uses NADH)
TH is a cytosolic protein
TH activity is regulated by phosphorylation and by rate of synthesis



Next is a non-specific fast step catalyzed by
DOPA decarboxylase

also called **aromatic amino acid decarboxylase (AAAD)**
requires pyridoxal phosphate (PyrP)

DA neurons stop here!

To make norepinephrine, must have
Dopamine β-monooxygenase (**DβM**) =
Dopamine β-hydroxylase (DβH)

Present in neurons in locus ceruleus, sympathetic neurons, adrenal chromaffin cells
requires Cu^{+}, molecular O_2 & ascorbate

odd thing from cell biology point of view – it that DBM is inside granules
occurs in membrane associated and soluble forms

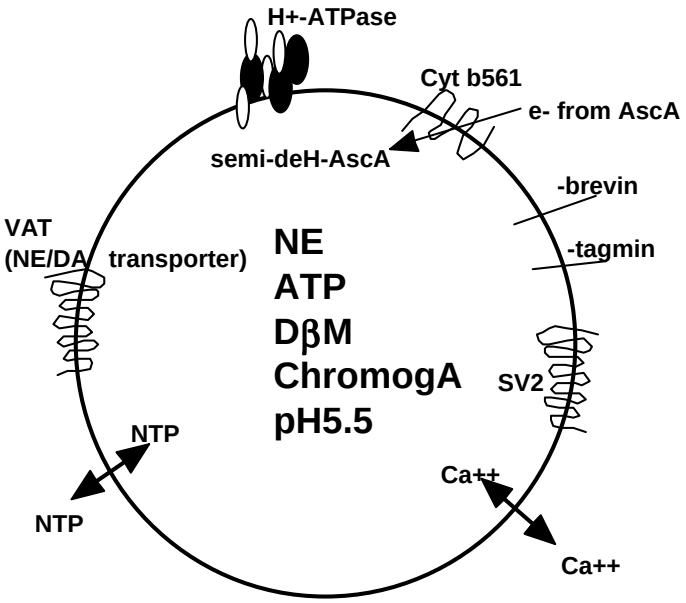
Sympathetic neurons mostly stop here!

Adrenal chromaffin cells and a few CNS neurons make epinephrine – must have
Phenylethanolamine N-methyltransferase (PNMT) – cytosolic enzyme
uses S-adenosyl-methionine (SAM) as methyl donor

Localization of enzymes and substrates is important:
TH, AAAD, PNMT are cytosolic while **DβM is INSIDE the granule**

Breakdown of catecholamines is not at all neuron-specific, nor does it occur only at the synapse. Instead, CAs are broken down inside many cells by monoamine oxidase

(MAO) and by catechol-O-methyltransferase (COMT). MAO inhibitors areused as anti-anxiety drugs).



Catecholamine vesicles – small or large, always dense
- 500 mM CA = 10^6 molecules in granule
- 125 mM ATP
- 24 molecules DβM
- chromogranins – complex with CAs
- 50 opiate peptide molecules
- 10-20 mM ascorbate
- pH 5.5
- Vesicle membranes – must take up catecholamines; use H+ pump to do this

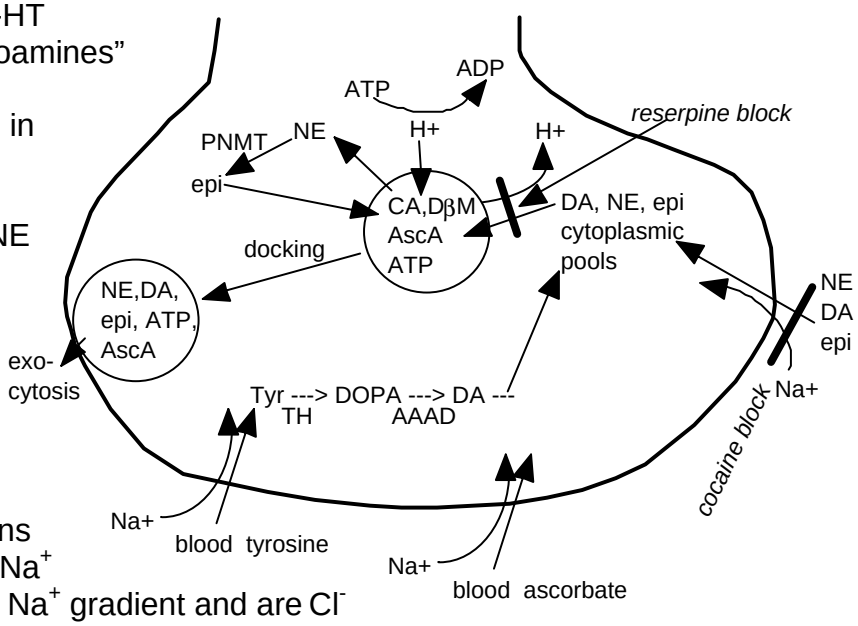
Vesicular uptake

VMATs (VAT) - vesicular monoamine transporters
- reserpine (1st anti-psychotic) blocks VMAT

Are 12 TMD proteins; chromaffin granules have VMAT1; CNS has VMAT2

Transport monoamines: NE, DA, epi, 5-HT
- are somewhat nonspecific for "monoamines"
- VMATs are 40% identical to VAChT

VMAT2 knockout -/- mice **-** born normal in normal Mendelian ratio
- die ~a day after birth
- +/- mice have ½ the expected NE content in vesicles!



Plasma membrane uptake – key for termination of action of many neurotransmitters – also allows for re-use of transmitter

For catecholamines, are 12 TMD proteins
- Are driven by inward gradient of Na^+
- Transporters for NE, DA require Na^+ gradient and are Cl^- dependent
- Plasma membrane transporters are targets for psychotropic drugs
- DA transporter is inhibited by cocaine
- Chronic cocaine up-regulates dopamine transporter (surface transporter), but *NOT* VMAT1 or 2
- Tricyclic anti-depressants block reuptake of NE

Inject [^{3}H]NE into blood; 1/2 broken down by MAO and COMT --> metabolites and 1/2 found in sympathetic endings, brain

despite having 12 TMD and similar ligands, there is no similarity between plasma membrane catecholamine transporters and VMATs

Concentration of transmitter in terminal generally 10,000-fold higher than concentration in synaptic cleft – transporter operating in reverse can release transmitter in a non-vesicular process

Catecholamines and disease

TH may contribute to oxidative stress as a monooxygenase -> idiopathic Parkinsonism
Uncouple hydroxylation of Tyrosine from link to biopterin --> make H_2O_2

Likewise argue CA themselves are part of the problem in schizophrenia,
contributing to neuromelanin, and possibly to myocardial atrophy also

In CA neurons and chromaffin cells, [TH] is ~50 μM or >1% of protein in cytosol!
That gives production rate of 2 mM DOPA/min. If only 95% couples to biopterin, then production of H_2O_2 can be 0.1 mM/min, plenty to cause damage!

TH and DBM knockout mice

TH knockout mice die in gestation; completely saved by L-DOPA given to mother

- require continued L-DOPA after birth, or become inactive, stop eating, die

DBM knockouts also die in utero, saved by dihydroxyphenylserine to mother

- severely cold intolerant, poor peripheral vasoconstriction [to conserve heat]
- cannot induce thermogenesis in brown fat through uncoupling protein
- increased food intake, but do not get fat, due to elevated basal metabolism

AMINO ACIDS AS NEUROTRANSMITTERS

Glutamate: excitatory transmitter – asymmetrical contacts on dendritic shafts/spines
Made from glutamine or as part of TCA cycle; 20 mM in synaptic vesicles
Nearly all excitatory neurons in the CNS are glutamatergic
Over half of the synapses in the brain release Glu
After release, removed from cleft by several high affinity Glu transporters on glial cells and nerve terminals – differ in structure from transporters for CAs

Glycine and GABA: inhibitory transmitters
As many as one third of synapses in brain use GABA – in local circuit interneurons
Glutamate is converted to GABA by Glutamic Acid Decarboxylase (GAD) – are two GAD genes
About half of the inhibitory synapses in spinal cord use Gly
GABA and Gly are removed from cleft by specific transporters

A key feature for neurotransmitters that also play roles in intermediary metabolism is keeping metabolic supply and transmitter supply separate. Vesicular transporters play a key role in this process. Plasma membrane transporters are also critical.

Peptides

A. Many neuropeptides were originally identified as pituitary hormones, hormones of the gastrointestinal tract or bioactive frog skin peptides.

First was vasopressin from the posterior lobe of the pituitary
Gut peptides like cholecystokinin
Hypothalamic releasing factors – e.g. corticotropin releasing hormone (CRH)
Substance P - the first purified as "neuropeptide"

B. Neuropeptides far outnumber the classical neurotransmitters. Since there are so many, must focus on generalities and how to think about peptidergic systems. Knock-out experiments eliminating a single prohormone or a single receptor sub-type are identifying specific roles.

Selected bioactive peptides

Hypothalamic releasing factors
CRH: corticotropin releasing hormone
GHRH: growth hormone releasing hormone
GnRH: gonadotropin releasing hormone
Somatostatin
TRH: thyrotropin releasing hormone

Opiate peptides
β-endorphin
dynorphin
leu-enkephalin
met-enkephalin

Neurohypophyseal peptides
oxytocin
vasopressin

Pituitary hormones
ACTH: adrenocorticotropic hormone
αMSH: α-melanocyte stimulating hormone
β-endorphin
GH: growth hormone
PRL: prolactin
FSH: follicle stimulating hormone
LH: luteinizing hormone
TSH: thyrotropin [thyroid stimulating hormone]

GI and brain peptides
CCK: cholecystokinin
gastrin
GRP: gastrin releasing peptide
motilin
neurotensin
substance K; substance P
(tachykinins)

GI and pancreas
glucagon
PP: pancreatic polypeptide

Neuronal and endocrine
ANF: atrial natriuretic peptide
CGRP: calcitonin gene-related peptide
VIP: vasoactive intestinal peptide

Neurons only?
galanin
neuromedin K
NPY: neuropeptide Y
PYY: peptide YY

Endocrine only?
calcitonin
insulin
secretin
parathyroid hormone

Circulating
angiotensin
bradykinin

Frog skin
bombesin
caerulein
ranatensin

Can group into structurally related families:

OT/VP - duplication early in evolution; genes close but in opposite orientation
Opioids - core sequence of Tyr-Gly-Gly-(Phe/Leu)-Met
3 precursors - proenkephalin, prodynorphin, POMC
Gastrin/CCK - sulfated CCK-8, CCK-4 in brain; Trp-Met-Asp-Phe-NH_2 at COOH
Glugacon-related - VIP, GIP, glucagon, secretin, GHRH
homology scattered over entire acid peptide
Tachykinins - SP; core sequence - Phe-X-Gly-Leu-Met-NH_2 at COOH-terminus.

related peptides in frog skin
Calcitonin/CGRP/amylin/adrenomedullin
NPY - PP, NPY, PYY

C. The use of peptides as messengers is evolutionarily very old - yeast mating factors
Cnidarians (e.g. sea anemones, corals, jellyfishes, Hydra)
lowest animal group with nervous system – nerve net
Hydra have peptides but no acetylcholine, catecholamines or serotonin
nerve net is strongly peptidergic
Precursors have multiple copies of same peptide - biosynthetic machinery for bioactive peptides well conserved from yeast to man

D. A variety of techniques have been used to identify neuropeptides:
1. Bioassay/radioreceptor assay - oldest way; purify peptide, then sequence
2. Purified from relatively homogeneous population of cells - melanotropes, chromaffin granules; mass spec from single cells
3. Find elsewhere and later identify in CNS - e.g. frog skin as source
4. Find based on structure - amide; NPY, PHI; purify
5. Molecular biology approaches:
Screen for ligands to orphan receptors:
bioassay – purify active factor from brain extracts – orexins/hypocretins; nociceptin/orphanin FQ
Subtractive hybridization/differential display - way to induce system; peptide precursors have characteristic features - signal, paired basics
CART - cocaine and amphetamine regulated transcript
Single identified cell library - RT-PCR with RNA from single cell; peptide such a major product, precursor mRNA easily found
Not clear from human genome project how many there are

E. The neuropeptides exhibit a few key differences from the classical neurotransmitters.
1. Present at much lower levels than ACh, catecholamines or amino acids.
BUT peptides are active at correspondingly lower concentrations
2. Probably key difference – biosynthesis – occurs only in cell body.
3. Released in response to different stimuli.
4. No concentrative re-uptake – peptides enzymatically inactivated or diffuse away

F. Neuropeptides are found in many neurons, and often in the same synapses with classical neurotransmitters.
Neuropeptide expression is extremely plastic.
Concept of plurichemical transmission. LDCVs and SVs also have ATP.
Chemical coding: look at paravertebral ganglion neurons and can tell function by mixture of transmitters present:
norepinephrine and NPY = vasoconstriction
acetylcholine and VIP = vasodilation in sweat gland

G. The **biosynthesis** of neuropeptides is fundamentally different from the biosynthesis of classical neurotransmitters.

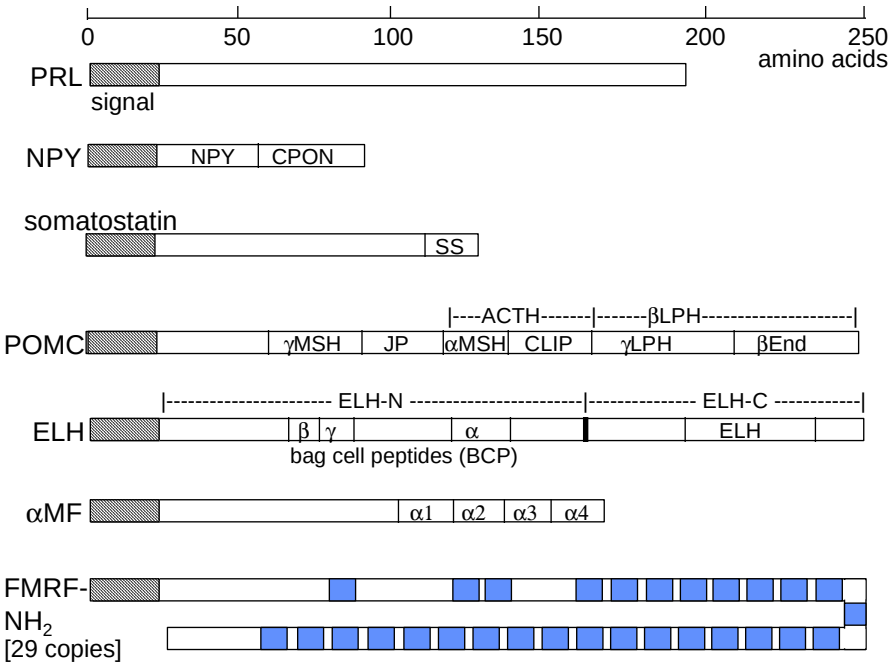
Neuropeptides derived from **inactive precursors - PREPRONEUROPEPTIDES**

Simplest - one product; only remove signal sequence - e.g. prolactin

Single product - requires cleavage; preproNPY and preprosomatostatin

Multiple peptides - coordinate activities - POMC (pro-opiomelanocortin) and ELH (egg laying hormone)

Tissue specificity of products

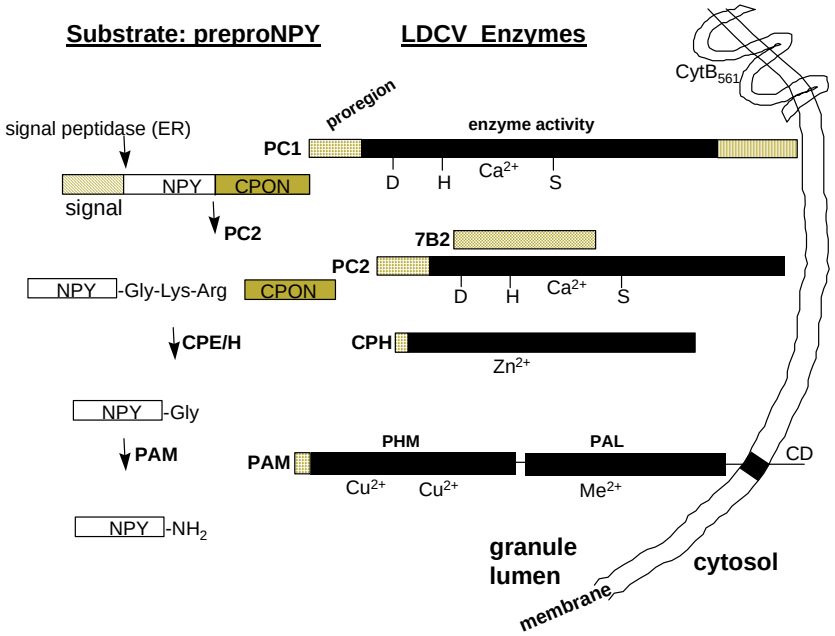


Multiple copies of same peptide - FMRF-amide - common in primitive nervous systems

α-mating factor - peptides in yeast

H. Most of the **enzymes involved in peptide biogenesis** have been identified:

COMMON STEPS in the POST-TRANSLATIONAL PROCESSING of PRE-PRONEUROPEPTIDES



The most common steps and enzymes involved are shown; PC1 and PC2, prohormone convertases 1 and 2; CPE/H, carboxypeptidase E (also CPH); PAM, peptidylglycine α-amidating monooxygenase; $CytB_{561}$ cytochrome b_{561},, transports reducing equivalents across membrane.

Many steps not unique to neuropeptides: signal peptide cleavage, folding, disulfide bond formation, N- and O-glycosylation, phosphorylation

Post-translational processing occurs as prohormones travel from ER to Golgi to storage site in large dense core vesicles (LDCV)
Later steps are more neuroendocrine-specific

Key enzymes in neuropeptide biosynthesis:

Endoproteases - Finding Kex2p, enzyme that cleaves yeast peptide pheromone, α-mating factor, was key; then identified furin and prohormone convertases (PCs)
All are subtilisin-like endoproteases - PC1/3, PC2, PC4, PC5/6, PC7/8 , PACE4
Specificity not that great; one enzyme cleaves many prohormones; means site of enzyme expression is critical
Regulated at many levels - folding; autoactivation; inhibitors; pH. Production of active PC2 requires synthesis of 7B2, a small protein that seems to serve as a chaperone/inhibitor
Could be more enzymes; proteases from other families also important
Can be rate-limiting - do see intermediates

CPE/H - Purified through use of a specific inhibitor; then cloned
Generally not rate limiting
Several homologs recently identified - inactivating mutation causes obesity and diabetes mellitus
Likely need an aminopeptidase also

PAM - Purified and cloned
Half of bioactive peptides have an essential $-NH_2$ at COOH-terminus
Resembles dopamine β-monooxygenase; requires ascorbate, Cu, molecular O_2

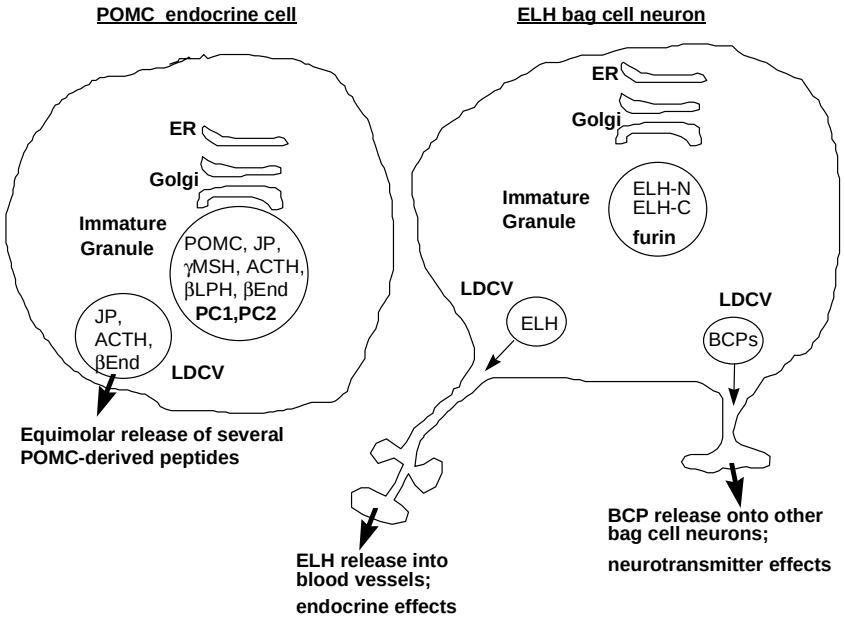
Less common - Glutaminyl cyclase, peptide α-N-acetyltransferase and tyrosylprotein sulfotransferase

I. Neuropeptides are packaged into large dense core vesicles (**LDCVs**)
In many cases, equimolar production of various product peptides; are secreted together
Need not always be this way - ELH and TRH

PEPTIDE PRODUCTS ARE STORED IN LDCVs

POMC products are released in equimolar amounts.

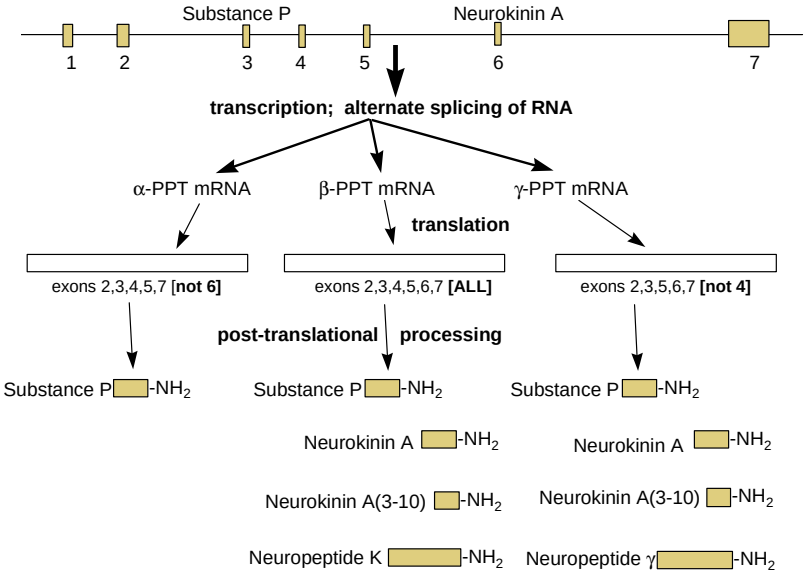
ELH NH_2- and COOH-terminal



fragments are sorted into different secretory granules released from different sites in the cell! What might happen if an ELH neuron lacked enzymes that could cleave in the immature granules and only had enzymes that could cleave later?

J. Diversity is generated by alternative splicing, proteolytic processing and post-translational modification.

1. Multiple family members with multiple copies of bioactive peptide:
   Opioid peptides - endorphin, enkephalin, dynorphin; 3 opioid receptor subtypes.
   FMRF-NH_2 family – in *C.elegans*, have 18 genes encoding as many as 53 family members – delete one gene and worms become uncoordinated, hyperactive. Genes are not functionally redundant.
2. Alternative splicing - neuropeptide genes often subject to tissue-specific alternative splicing --first seen for calcitonin/CGRP
3. RNA editing - looks like amphibian bombesin-like peptides use this mechanism



DIVERSITY IN NEUROPEPTIDES. Alternative Splicing of Preprotachykinin I (SP/NKA) Gene.

Rat gene is shown with boxes representing exons 1 to 7. Alternate splicing yields 3 mRNAs and the corresponding peptide products are indicated.

[simplified and adapted from Helke et al 1990 FASEB J 4:1606]

C. Neuropeptide receptors are not confined to synaptic regions. Peptidergic neurotransmission usually slow, non-synaptic. Mismatch of neuropeptide and receptor is common.

Neuropeptides can act at synapse, on self (autocrine), next door (juxtacrine), nearby (paracrine); in extreme, hypothalamic releasing factors act through circulation (endocrine).

> Example: Although some SP terminals contact membranes with SP receptor, no more than 15% of the SP receptor-laden membrane is apposed to synaptic terminals. SP may diffuse a considerable distance from its release site and still find a receptor with which it can interact.

Opioid receptors of mu [μ] and kappa [κ] subtype usually associated with plasma membrane of dendrites and cell bodies (post-synaptic); interpeduncular nucleus - enkephalin in one region and delta opioid receptor in neighboring subnucleus - may interact over hundreds of microns.

III. NEUROPEPTIDE FUNCTIONS

A. The study of peptidergic neurons requires a number of special tools. Can look for neuropeptides, for the enzymes specific to their biosynthesis and for their receptors.

Peptides:
1. **antibody-based** detection - limitations relate to antibody sensitivity and cross-reactivity
- immunocytochemistry - qualitative, but good spatial resolution
- RIA - quantitative measure of release or content
- passive-immunization
2. **RNA-based** detection - Northern blot or *in situ* hybridization
3. **Direct** - metabolically label or chemically isolate peptides; measure levels, release
4. **Peptide agonists and antagonists** becoming available - an essential tool
5. **Knockout** of gene encoding single preprohormone – e.g flp-1 FMRFamide precursor in *C.elegans*

Receptors: same tools as for classical neurotransmitter receptors
receptor protein, *in situ* hybridization, ligand binding assay, ligand binding *in situ*

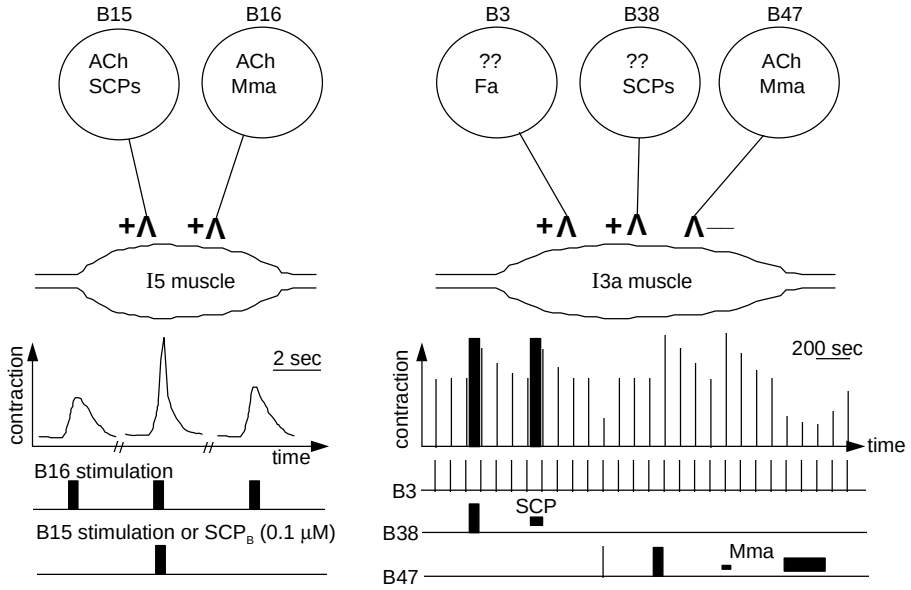
A key to understanding what peptides do is the development of highly selective agonists and antagonists with specificities that exceed those of the natural ligands

No single method will tell you everything.

B. Neuropeptides make a unique contribution to signaling. Peptides are generally released with classical neurotransmitters, but each transmitter has its own unique effects on target tissues.

Release of peptides from LDCVs generally requires a more intense stimulus than release of classical transmitters from SVs. As a result, the contribution of peptides to signaling can vary with the pattern of stimulation. Importantly, the stimulation patterns that evoke peptide release are within the range of patterns observed *in vivo*.

An example from Aplysia illustrates the unique role of peptides.



Aplysia motorneurons B3, B15, B16, B38 and B47 devote ~10% of their total protein synthesis to their respective peptide neurotransmitters:

B3 Fa = FMRF-NH_2 [4 amino acids]

B15, B38 SCPs = small cardioactive peptides [9, 11 amino acids]

B16, B47 Mma = myomodulin A [7 amino acids]

Adapted from Church et al 1993 **J Neurosci 13**:2790; Whim et al 1994 **Mol Neuro 7**:335

In addition to peptides, B15, B16 and B47 synthesize and secrete acetylcholine (**ACh**). The conventional transmitter from B3 and B38 is not known.

Only the SCPs elevate I5 muscle cAMP levels and enhance excitation/contraction coupling; ACh does not have this effect. Application of SCPs increases the amplitude and relaxation rate of I5 muscle contractions evoked by stimulating B15 or B16 (see left side of Figure). Thus release of peptide cotransmitters modifies the dynamics of the neuromuscular system.

Buccal muscle I3a (right side of Figure) has two excitatory motor neurons (non-cholinergic) and an inhibitory motor neuron that is cholinergic. All 3 peptides (SCPs, Mma and Fa) increase the amplitude of B3-evoked EJPs and I3a muscle contractions (high concentrations of Mma inhibit muscle contraction - far right side of figure). B47 is an inhibitory motor neuron that releases an excitatory modulatory peptide. When B3 and B47 are stimulated simultaneously, contraction amplitude is reduced. When B47 is stimulated at high frequency during the B3 interburst interval, subsequent B3-evoked contractions are enhanced in amplitude. The stimulation paradigm determines whether this motor neuron enhances or inhibits the effects of a second motor neuron at the same neuromuscular junction.

Stimulation patterns that cause release of peptide from one neuron will not necessarily release the same peptide from a different neuron.