Identification of neuropeptides in mouse spinal cord using mass spectrometry

Karl Sköld[3], Jie Su[1,2], Katalin Sandor[1], Camilla Svensson[1], Kim Kultima[4]

Department of Physiology and Pharmacology[1] and Neuroscience[2] Karolinska Institutet, Stockholm, Sweden [3]Denator AB, Uppsala, Sweden [4]Department of Medical Sciences, Cancer Pharmacology and Computational Medicine, Uppsala University, Uppsala, Sweden

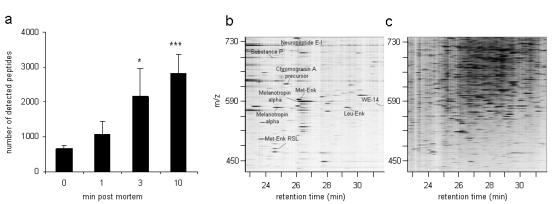
Background

Neuropeptides and hormones are often difficult to detect and measure, often due to low levels, small size, and their rapid degradation. Previously combinations of heat stabilization, extraction and filtering of homogenate and detection using LCMS have been successfully used for the detection of 50-100 peptides / run. Despite the possibility to detect and measure a large number of peptides some peptides are not detected. In this study we have focused on method development, and have experimented on the extraction method to be able to detect more peptides and especially targeted galanin.

Heat Stabilization

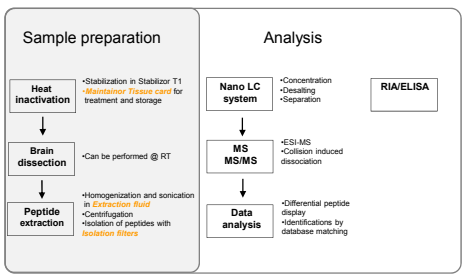
Conductive heat stabilization has been used to rapidly cause loss of protein structure to render the degradative enzymes inactive. This method may be used on both fresh and frozen tissue samples without the use of hazardous chemical inhibition cocktails. Generally speaking, the samples are subjected to as little handling as possible.

Reduction of sample complexity

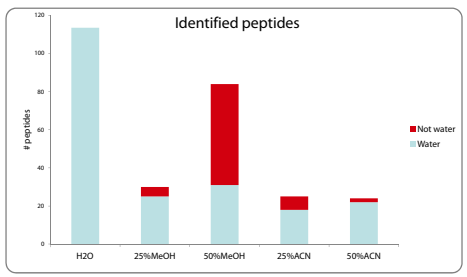


A. *Post mortem* time delay effect on the number of detected peptides in the hypothalamus of mouse (*P<0.05, ***P<0.001, ANOVA, t-test). B. LCMS 2D plot of heat stabilized hypothalamus of rat. C. LCMS 2D plot of snap frozen hypothalamus of rat.

Workflow



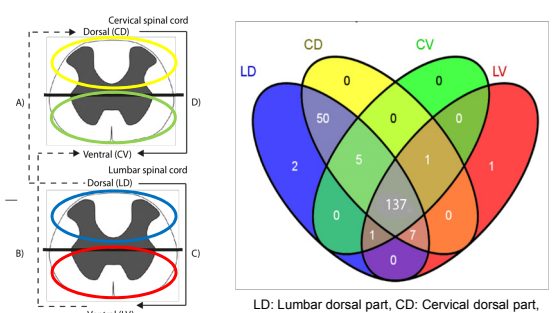
Extraction of peptides from heat stabilized spinal cord



Family	Precursor	Peptide name	Peptide sequence	Peptide modification
Bombesin/neuromedin-B/ranatensin family	GRP	Neuromedin-C	GSHWAVGHLM	Amid(C-term)
Bombesin/neuromedin-B/ranatensin family	NMB	Neuromedin-B	GNLWATGHFM	Amid(C-term)
Cerebellins	CBLN1	Cerebellin	SGSAKVAFSAIRSTNH	
Cerebellins	CBLN1	[des-Ser1]-cerebellin	GSAKVAFSAIRSTNH	
FARP (FMRFamide related peptide) family	NPFF	Neuropeptide SF	SPAFLFQPQRF	Amid(C-term)
Granins	7B2	C-terminal peptide	SVPHFSEEEKEAE	Phos(S6)
Granins	CMGA	WE-14	WSRMDQLAKELTAE	
Kinin and tensin gene family	TKN1	Substance P	RPKPQQFFGLM	Amid(C-term)
Kinin and tensin gene family	TKN1	Neurokinin A	HKTDSFVGLM	Amid(C-term)
Kinin and tensin gene family	TKN1	C-terminal-flanking peptide	ALNSVAYERSAMQNYE	
Neurotensin family	NEUT	Neuromedin N	KIPYIL	
Neurotensin family	NEUT	Neurotensin	QLYENKPRRPYIL	pGlu(Q1)
Neurotensin family	NEUT	Tail peptide	GSYYY	
NmU family	NMU	Propeptide	STSFI	
No-family neuropeptides	MCH	Neuropeptide-glutamic acid-isoleucine	EIGDEENSAKFPI	Amid(C-term)
No-family neuropeptides	PCSK1	Big SAAS	ARPVKEPRSLSAASAPLVETSTPLRL	
No-family neuropeptides	PCSK1	KEP	ARPVKEP	
No-family neuropeptides	PCSK1	Little SAAS	SLSAASAPLVETSTPLRL	
No-family neuropeptides	PCSK1	Big LEN	LENPSPQAPARRLLPP	
Opioid gene family	PDYN	Alpha-neoendorphin	YGGFLRKYPK	
Opioid gene family	PDYN	Beta-neoendorphin	YGGFLRKYP	
Opioid gene family	PDYN	Rimorphin	YGGFLRRQFKVVT	
Opioid gene family	PDYN/PENK	Leu-enkephalin	YGGFL	
Opioid gene family	PENK	Met-enkephalin	YGGFM	
Opioid gene family	PENK	Met-enkephalin-Arg-Ser-Leu	YGGFMRSL	
Opioid gene family	PENK	Propeptide	SPQLEDEAKELQ	
Opioid gene family	PENK	Propeptide	VGRPEWWMDYQ	
Opioid gene family	PENK	Met-enkephalin-Arg-Phe	YGGFMRF	
Opioid gene family	PNOC	Nociceptin	FGGFTGARKSARKLANQ	
Opioid gene family	PNOC	Propeptide	TLHQNGNV	
Somastostatin gene family	SMS	Somatostatin-14	AGCKNFFWKTFTSC	
Thymosin beta family	TYB10	Thymosin beta-10	ADKPDMGEIASFDKAKLKKTETQEKNTLPTKETIEQEKRSEIS	Acet(K3)

Family	Precursor	Peptide name	Peptide sequence	Modification	Mr (calc)
Calcitonin gene family	CALCA	Calcitonin gene-related peptide 1	SCNTATCVTHRLAGLLSRSGGVVKDNFVPTNVGSEAF	Amid(C-term)	3805.898
Galanin gene family	GALA	Galanin	GWTLNSAGYLLGPHAIDNHRSFSDKHGLT	Amid(C-term)	3162.574
NPY family	NPY	C-flanking peptide of NPY	SSPETLISDLLMKESTENAAPETRLEDPSMW		3432.653
Opioid gene family	PENK	PENK[114-133]	MDELYPMEPEEEANGGEILL		2235.954
No-family neuropeptides?	PCSK1	PEN-20	SVDQDLGPEVPPENVLGALLL		2061.062
No-family neuropeptides?	PCSK1	PEN	SVDQDLGPEVPPENVLGALLRV		2316.232
Kinin and tensin gene family	TKNK	Neurokinin-B	DMHDFFVGLM	Amid(C-term)	1209.531

Vend diagram of identified peptides



LD: Lumbar dorsal part, CD: Cervical dorsal part, CV: Cervical ventral part, LV: Lumbar ventral part

Methods

Mice were deeply anesthetized with isoflurane and spinal cords collected by extrohydrusion. The spinal cord samples were immediately heat stabilized using the Stabilizor system (Denator AB, Gothenburg, Sweden) set at 95°C for 30-40 sec (Skold et al. 2007). After stabilization the dorsal and ventral parts of the cervical (C3-C8) and lumbar (L1-L6) enlargements were dissected under a dissecting microscope. Peptide extraction was performed using Peptide extraction Kit (Denator AB, Gothenburg, Sweden) according to the manufacturer's description. Spinal cords were transferred to low-retention Eppendorf tubes and suspended in pre-chilled extraction solution (7.5 µl 0.25% acetic acid/mg tissue) and homogenized by sonication (Vibra cell 750, Sonics & Materials Inc., Newtown, CT, USA) for 30 seconds (Svensson *et al.* 2003).

In order to investigate which peptides that are not extracted using mainly water, the extraction solution was replaced using 7.5 µl 0.25% acetic acid/mg tissue and 25% or 50% methanol or acetonitrile, respectively, utilizing ventral and lumbar dorsal horns. Five-microliter sample injections were made with an HTC-PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) connected to an Agilent 1100 binary pump (Agilent Technologies, Palo Alto, CA, USA). The peptides were trapped on a precolumn (45 x 0.100 mm i.d.) and separated on a reversed phase column, 200 x 0.050 mm. Both columns are packed in-house with ReproSil-Pur C18-AQ 3 µm particles (Dr. Maisch GmbH, Ammerbuch, Germany). The nanoflow LC-MS/MS were performed on a hybrid LTQ FT-ICR mass spectrometer equipped with a 7T ICR magnet (LTQ FT, Thermo Electron, Bremen, Germany). The obtained MS/MS files were searched against a compilation of mouse precursors containing known neuropeptides and peptide hormones by using X! Tandem (Craig & Beavis 2004) and MASCOT Daemon (version 2.3, Matrix Science, London, UK).

Results

In the extraction utilizing mainly water and 0.25% acetic acid a total of 204 protein precursor derived peptides were identified in mouse spinal cord. Out of these 36 were determined to be previously identified full-length neuropeptides. By extraction with organic solvents in mouse dorsal horns using acetonitrile or methanol in 25% or 50% concentrations, an additional seven previously identified full-length neuropeptides including galanin and an additional 16 protein precursor derived peptides were identified. Galanin was only detected using 50% methanol as extraction buffer. To conclude, we are able to measure galanin and up to 50 neuropeptides in spinal cord in one MS run. The method will be used to semi quantitatively compare neuropeptides between diseased / treated and control animals.

Conclusion

204 unique (neuro)peptides derived from prercursor-proteins (pro-hormones), 50 are only expressed in dorsal side, 36 full-length, previously known, neuropeptides,118 "novel" peptides with typical neuropeptide cleavage sites