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# Broad characterization of endogenous peptides in the tree shrew visual system

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ABSTRACT

Endogenous neuropeptides, acting as neurotransmitters or hormones in the brain, carry out important functions including neural plasticity, metabolism and angiogenesis. Previous neuropeptide studies have focused on peptide-rich brain regions such as the striatum or hypothalamus. Here we present an investigation of peptides in the visual system, composed of brain regions that are generally less rich in peptides, with the aim of providing the first broad overview of peptides involved in mammalian visual functions. We target three important parts of the visual system: the primary visual cortex (V1), lateral geniculate nucleus (LGN) and superior colliculus (SC). Our study is performed in the tree shrew, a close relative of primates. Using a combination of data dependent acquisition and targeted LC-MS/MS based neuropeptidomics; we identified a total of 52 peptides from the tree shrew visual system. A total of 26 peptides, for example GAV and neuropeptide K were identified in the visual system for the first time. Out of the total 52 peptides, 27 peptides with high signal-to-noise-ratio (>10) in extracted ion chromatograms (EIC) were subjected to label-free quantitation. We observed generally lower abundance of peptides in the LGN compared to V1 and SC. Consistently, a number of individual peptides showed high abundance in V1 (such as neuropeptide Y or somatostatin 28) and in SC (such as somatostatin 28 AA1-12). This study provides the first in-depth characterization of peptides in the mammalian visual system. These findings now permit the investigation of neuropeptide-regulated mechanisms of visual perception.



## 1. Introduction

Neuropeptides are evolutionarily old neurotransmitters present in all species possessing a nervous system [1]. They are involved in regulating many physiological pathways [2–6]. Their functions and expression levels can differ considerably across particular brain areas [7]. In the visual system, peptides were found to carry out important functions related to development [8], neural plasticity, regulation of blood flow and energy metabolism [7,9]. Neuropeptides also play a key role in the development and function of the inhibitory circuits that shape the visual system in response to experience [10] and their expression patterns are different in relation to morphologically and physiologically distinct interneuron classes [11].

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To advance our understanding of peptide functions in the visual system, we sought to provide an in-depth simultaneous characterization of a large set of peptides present in different substructures of the visual system. Compared to peptides previously identified in peptide-rich areas such as hypothalamus [12] or striatum [13], peptides in the visual system have so far received relatively little attention using mass spectrometry approaches.

Neuropeptides, from an analytical chemistry point of view, have been characterized predominantly by employing immunoassay or radioimmunoassay [14,15]. Classical immunochemical approaches are usually used to characterize only a few peptides or peptide families within a single study, but it is usually difficult to distinguish between similar peptides from one peptide family. Contrastingly to mass spectrometric approaches, immunohistochemical or radiography also cannot provide a complete image of peptides in the studied brain region. Mass spectrometric approaches offer several advantages including higher sensitivity and throughput compared to traditional methods [16–21]. For example, recent neuropeptidomics studies have demonstrated high sensitivity characterization of peptides in diverse tissues such as human brain [22–25], and rat brain [26–30]. Moreover, several protocols for peptide characterization using mass spectrometry as analytical tools have been developed and are used routinely for biomedical applications [16,17,31,32].

Here, we thus sought to characterize the peptide population and its distribution across three important parts of the visual system using tree shrews (*Tupaia belangeri*) as model animals. Tree shrews are phylogenetically close relatives of primates, and similar to primates, they have a well-developed visual system and therefore are frequently used as animal models for the study of visual structure and function [33]. The three relevant visual centers targeted for neuropeptide characterization in this study are the primary visual cortex (V1), lateral geniculate nucleus (LGN) and superior colliculus (SC); the regions are schematically shown in the Fig. 1A. We have identified 52 peptides from 21 precursor families in the tree shrew visual system in the three targeted brain regions. This peptide population includes 21 classical neuropeptides that have been previously identified, as well as 26 peptides previously not reported in visual system. We have also used a label free quantification approach to evaluate the relative abundances of a subset of 27 peptides in the studied brain regions. We discuss our findings in relation the possible peptide functions in the visual system in light of previous literature.

## 2. Methods

### 2.1. Chemicals

LC-MS grade acetonitrile and formic acid were purchased from Fisher Scientific (New Jersey, USA) and Fluka (Wisconsin, USA), respectively. Pure water was prepared by GenPure system (TKA, Niederelbert, Germany) Siliconized micro centrifuge tubes (2 mL) were purchased from Eppendorf (Hamburg, Germany). Microcon centrifugal filter devices (Vivacon 500) were purchased from Sartorius AG (Goettingen, Germany Germany).

### 2.2. Animals

Tree shrews (*Tupaia belangeri*) were used as model animals. They were housed at constant temperature and humidity with free access to food and water. All procedures were in compliance with applicable European Union (EUVD 86/609/EEC) and Swiss regulations. Three male tree shrews, six years old, were sacrificed by a decapitation after having been anaesthetized with ketamine (Streuli Pharma AG, Uznach, Switzerland).

### 2.3. Sample preparation

During sample preparation, temperature control was used to minimize the degeneration of the endogenous neuropeptides and also to reduce the interference of peptides produced by degradation of proteins. Tree shrew heads were immediately heated up to 80 °C for 16 s using microwave irradiation [34]. Their brains were removed and stored at −80 °C, and visual system parts were dissected from the denatured brain, right and left half brains separately. For each extraction, 10.0 mg of brain tissue was used. Neuropeptides were extracted from 6 samples from both brain hemispheres (V1, LGN and SC). We used a four step extraction procedure that had been developed previously[35]. Briefly, it is based on a distinct gradient of organic phase (methanol) solutions, which were used as follows: firstly an aqueous solution of 0.2% acetic acid was used two times, then a methanol–water-actic acid solution (20:79.8:0.2, v/v/v) was selected and finally the last extraction part was performed using a methanol–water-acetic acid solution (50:49.8:0:2, v/v/v). Each of the four steps used 15 micro liters of solvent per one mg of brain sample. Before an extraction, samples had been homogenized twice (each time for 20 s) within 1 min by a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). After homogenization, they were centrifuged at 22, 000 g for 60 min at 4 °C. Before analysis, obtained supernatants from the first two steps (aqueous phase) as well as the supernatants from the last two steps (organic phase) had been mixed together and filtered using a 10 kDa cut-off filter (Vivacon 500, Sartorius AG, Goettingen, Germany) by centrifuging for 90 min with 14.000 g at 4 °C. Finally, aqueous and organic peptide extracts were pooled together on a trap column (100 μm ID, 2 cm long), which had been previously packed with a C18 AQ particles (5 μm, 100 Å;) and afterwards they were injected on an analytical column based on C18 AQ (3 μm, 100 Å) stationery phase which was packed in a Picofrit capillary with an emitter tip of 10 μm (NewObjective) and analyzed.

### 2.4. LC-FT-MS/MS data acquisition

For all mass spectrometric experiments, LTQ-Orbitrap Discovery (Thermo Fisher Scientific, Bremen, Germany) hyphenated with a 2D NanoLC (Eksigent Technologies, USA) was used. Firstly, samples were injected 5 times on a trap column in a peek column holder (Upchurch). Each injection was 5 μl and the interval between injections was 3 min. Trapped analytes were eluted using 2% acetonitrile and 98% water containing 0.2% formic acid. The mobile phase A and B in