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Understanding neuropeptide transmission in the brain by optical uncaging and release

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Abstract

Neuropeptides are abundant and essential signaling molecules in the nervous system involved in modulating neural circuits and behavior. Neuropeptides are generally released extrasynaptically and signal via volume transmission through G-protein coupled receptors (GPCR). Although substantive functional roles of neuropeptides have been discovered, many questions on neuropeptide transmission remain poorly understood, including the local diffusion and transmission properties in the brain extracellular space. To address this challenge, intensive efforts are required to develop advanced tools for releasing and detecting neuropeptides with high spatiotemporal resolution. Owing to the rapid development of biosensors and materials science, emerging tools are beginning to provide a better understanding of neuropeptide transmission. In this perspective, we summarize the fundamental advances in understanding neuropeptide transmission over the past decade, highlight the tools for releasing neuropeptides with high spatiotemporal solution in the brain, and discuss open questions and future directions in the field.

Keywords

neuropeptide transmission; neuropeptide release; neuropeptide sensor; light; brain

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Author contributions

All authors contributed to writing and revising this Perspective.

Competing interests

The authors declare that they have no competing financial interests.

What we know and don't know about neuropeptide volume transmission

Neuropeptides are a diverse class of endogenous molecules that are synthesized, stored, and secreted by neurons in the central and peripheral nervous systems.[1] They have attracted great interest over the years owing to their unique structure and function in a wide range of physiological processes.[2] Neuropeptides are usually packaged into dense-core vesicles and can be released in non-specialized synaptic sites.[3] In contrast to small molecule transmitters such as glutamate or GABA, neuropeptides can diffuse over a long distance to act far from the release site. This widespread mode of intercellular communication is referred to as volume transmission.[1, 4] Although over 100 neuropeptides have been discovered and probably more would be identified from genomic data,[5] many fundamental questions remain. For example, what is the release pattern and how far does a particular neuropeptide act relative to its release site? What constraints limit the spread of neuropeptides?

In a pioneering work nearly ten years ago, Banghart and Sabatini measured the spatial profile of enkephalinergic volume transmission in acute brain slices.[6] They found that enkephalin (LE) could diffuse as far as 150 μm to activate the opioid receptor in rat locus coeruleus of acute slices. In other words, the enkephalin signal spread rapidly through approximately 70,000 μm^2 of tissue, which is approximately 200-fold larger than the area of release. Recently, Xiong et al. used new optical tools and cell-based sensors to determine the spatiotemporal scale of somatostatin-14 (SST) volume transmission in the mouse cortex in vivo.[7] They revealed reduced but synchronized SST transmission within 130 μm, and delayed, reduced transmission at longer distances. The maximal diffusion distance of SST to activate the receptor was approximately 220 μm. Note that similar diffusion distance constraints were observed for LE and SST; however, the onset (0.25-1 s for 0-150 μm) of the evoked currents for LE diffusion is much shorter than the time of peak response (5-20 s for 0-150 μm) of cell-based sensors for SST diffusion. This difference in kinetics could be due to the different detection methods (electrophysiology vs downstream Ca^{2+} imaging) and the different preparations (slices vs in vivo). These measurements provide new insights into neuropeptide volume transmission in the brain of living animals.

Unlike primary neurotransmitters that are actively recycled, neuropeptides are released and signal until they are degraded. What are the factors limit that the diffusion of neuropeptides in the brain extracellular space? First, it is well known that neuropeptides are subject to degradation by peptidases. However, the degradation rate of neuropeptides in the brain extracellular space is largely unknown. To date, based on radioimmunoassays, the half-lives of oxytocin and vasopressin in cerebrospinal fluid is around 20 min.[8] Banghart and Sabatini measured somatic currents evoked by uncaging LE in acute slices of rat locus coeruleus and found that peptidases limit the peptide signaling released in large volumes (>70 μm), while diffusion is dominant in limiting the spread in smaller release sites.[6] Xiong et al. compared the distance-dependent SST signaling measurements in mouse cortex with a theoretical point-source diffusion model and estimated the loss rate of SST due to peptide degradation and binding in the range of 0.023 s^{-1}–0.048 s^{-1}. Although some progress has been made, additional studies are needed to better elucidate the effect of peptidase degradation on neuropeptide transmission. Second, the extracellular space (ECS) and extracellular matrix (ECM) can hinder extrasynaptic molecular diffusion,[9, 10] and play

an important role in neuropeptide transmission. For example, brain tissue with a chemically-degraded extracellular matrix provides less hindrance to peptide transmission.[7] However, there are many factors including the charge and size of the peptide that can lead to very different transmission profiles.

Tools for controlled release of neuropeptide in vivo

Despite its importance in brain function, very few measurements of peptide release are available due to the lack of tools. Rough estimates suggest that each dense core vesicle contains ~10^4 peptides, and hundreds of vesicles are released per neuron over seconds (10^3 vesicles per second in hypothalamic neurons).[5, 11] This suggests that a neuron releases ~10^6 peptide molecules per second upon stimulus, possibly higher in the hypothalamic neurons (10^7 molecules per second).

Tools to control the timing and spatial release of neuropeptides in vivo are needed to investigate the transmission and function of neuropeptides. The optogenetic approach is an elegant method to control the neurotransmitter release, such as acetylcholine,[12] serotonin,[13] and dopamine,[14, 15] with millisecond precision and cell type-specific resolution. Dao et al. successfully induced and inhibited the SST release from SST-positive neurons in acute slices using optogenetics as validated by an enzyme-linked immunosorbent assay.[16] Interestingly and importantly, Al-Hasani et al. measured the endogenous opioid peptide release (absolute concentration in dialysate: 0.13-8.69 pM) in freely moving rodents with a customized optogenetic-microdialysis probe.[17] They controlled cell-type selective opioid release in different brain regions and detected several opioids such as dynorphin A_{1-8} and LE (leu- and met-). The new approach moves the field forward; however, one complication to optogenetic-driven neuropeptide release is the co-release of other small molecule transmitters (such as dopamine, GABA and glutamate),[18, 19] which requires more specific detection of neuropeptides in real-time to measure the diffusion.

Controlled release or uncaging of exogenously supplied neuropeptide has the advantage of releasing a certain amount of a specific neuropeptide. A widely used method of using light to release specific neurotransmitters is to ‘cage’ the neurotransmitter with a photo-cleavable group. Caged compounds, such as caged glutamate or GABA,[20, 21] have been used to study cell signaling or physiology under one-photon or two-photon stimulation. Banghart and Sabatini measured the LE transmission using the caged-LE (CYLE) modified with carboxynitrobenzyl (CNB) chromophore, which responds to UV light illumination (Figure 1A).[6] The binding affinity of CYLE to the delta and mu receptors was decreased by 100- to 500-fold with respect to LE, while CYLE enabled rapid (onset of response: ~350 ms) and robust delivery of LE under photolysis. Taking advantage of the high spatial resolution (2 μm light spot), the photoactivatable opioid peptide provides a useful tool to investigate the spatiotemporal dynamics of peptidergic signaling. Later, the same group synthesized a new caged analog of LE (N-MNVOC-LE) to reduce the residual activity and demonstrated the feasibility in brain slices of rat locus coeruleus.[22] These can be extended to other caged analogs, including dynorphin,[6] gastrin-releasing peptide, oxytocin,[23] cholecystokinin, and substance P. However, caged peptides have some limitations. First, each caged compound requires a separate optimization process to ensure

biological inertness, solubility in physiological pH, resistance to aqueous hydrolysis, fast uncaging speed, and high uncaging efficiency.[21] For example, it is difficult to control the position and number of protecting groups since peptides may contain multiple reactive groups.[24] Second, caged compounds offer good spatial and temporal control but may be limited by one-photon uncaging of *ortho*-nitrobenzyl photolabile protecting groups with UV or blue light.[25] Furthermore, caged peptides are also subject to peptidase degradation which can limit their in vivo application.[6] Thus, other approaches to uncage or photo-release neuropeptides are needed to better understand their transient and localized effects in the nervous system.

An alternative approach to chemical caging is to encapsulate neuropeptides in the photosensitive nanovesicles (Au-nV, Figure 1B). These are 100~200 nanometer-sized structures that consist of a natural phospholipid membrane that surrounds an aqueous core. To control the release of neuropeptides, nanovesicles are coated with small gold particles (3–5 nm),[26] which enables near-infrared light-triggered photo-release. Ultrashort laser pulses (picosecond or femtosecond) can activate gold nanoparticles to generate nanoscale cavitation bubbles that are effective to burst the vesicle, releasing the encapsulated neuropeptides.[26, 27] Physiological concentrations of SST (~100 nM, 1.2×10^8) could be photoreleased from nanovesicles at a depth of 200 μm in the mouse cortex,[7] which is close to the level of endogenous released peptides from ~10^4 dense core vesicles or 10 neurons per second (~10^4 peptides per vesicle, ~10^3 vesicles per neuron).[5] The photosensitive nanovesicles (Au-nV) have several features that are complementary to current methodologies for neuropeptide release. First, nanovesicles allow in vivo measurement by protecting peptides from rapid enzymatic degradation. Second, the in vivo optical stimulation provides a high spatial (μm, or fL volume) and temporal (sub-second) resolution to control neuropeptide release by the laser power and duration using a two-photon microscope. Furthermore, our recent work on photoswtichable nanovesicles demonstrated that it is possible to switch on and off the photorelease so providing the opportunity to consecutively release the molecules over several cycles.[28] Third, photo-stimulation of nanovesicles releases a bolus of a specific neuropeptide instead of a mixture with co-released transmitters. Furthermore, near-infrared light is more accessible for in vivo studies due to the deep tissue penetration and reduced photo-damage[29] and the photosensitive liposomes are suitable to package a wide range of neuropeptides.[30] However, since the Au-nV are much larger than the narrow width of the brain extracellular space (40-60 nm),[31] large Au-nV have limited penetration or diffusion in the brain. The development of small and brain-penetrating nanovesicles could allow investigation of neuropeptide transmission across a large brain region with a single minimally invasive injection.[32]

Integrating neuropeptide release with neuropeptide sensing

Integrating neuropeptide release with neuropeptide monitoring would provide a powerful set of tools to study neuropeptide transmission. Monitoring the neurotransmitters or neuropeptides by optical approaches is appealing to neuroscientists due to the high spatiotemporal resolution compared to analytic chemical methods such as fast scanning cyclic voltammetry and microdialysis.[33, 34] Cell-based fluorescent sensors can detect nM concentrations of neuropeptides in vivo by the co-expression of specific GPCR and Ca^{2+}

indicators (Figure 2A).[7, 35–38] With a new SST2 CNiFERs (cell-based neurotransmitter fluorescent engineered reporter) to detect SST in vivo in real-time,[7] Xiong et al. integrated the SST-encapsulated plasmonic nanovesicles and CNiFEERs (PACE) and probed the neuropeptide transmission in vivo by the synchronization of NIR stimulation and two-photon imaging. Since SST2 CNiFERs provide a proxy for G protein activation via increases in intracellular Ca^{2+}, PACE provides new insights into neuropeptide extrasynaptic volume transmission, as it includes neuropeptide release, extracellular diffusion, GPCR binding, and intracellular downstream signaling. PACE is an excellent example of integrating neuropeptide release with neuropeptide monitoring to map neuropeptide transmission. The development of genetically encoded GPCR-based fluorescent sensors for neuropeptides provides another new tool for probing neuropeptide signaling and diffusion. Recently, several groups have reported new genetically encoded sensors to monitor the release and dynamics of dynorphin,[39] orexin,[40] and oxytocin[41, 42] in living animals, respectively. Li's group reported a toolkit of G protein-coupled receptor activation–based (GRAB) sensors for several neuropeptides, including SST, cholecystokinin, corticotropin-releasing factor, neuropeptide Y, neurotensin, and vasoactive intestinal peptide.[43] CNiFERs with FRET-based Ca^{2+} indicators have a high signal-to-noise ratio in vivo, but require multiple implantations at different distances for the diffusion measurement. Genetically encoded GPCR-based fluorescent sensors are simpler to implement and may have a higher spatial resolution for measuring neuropeptide diffusion. Since genetically encoded sensors are more widely used, GPCR-based sensors for neuropeptides are under rapid development and will likely play a more important role in future diffusion measurements. With the photorelease technique and brighter and more sensitive genetically encoded GPCR-based sensors for neuropeptides (Figure 2B), the integrated approach will allow for a better understanding of the neuropeptide volume transmission in the brain at a cellular resolution.

Open questions about neuropeptide transmission and future directions

There are several important unanswered questions about neuropeptide transmission. First, how do the diverse physicochemical properties of neuropeptides impact their transmission in the brain? Factors such as molecule size or weight,[31, 44, 45] the strength of transient binding interactions,[46] and charge[47] can also affect diffusion in the brain extracellular space. As such, it is likely that neuropeptides' physicochemical properties could affect how they diffuse through brain extracellular spaces. There are nearly 300 unique human neuropeptides recorded in the NeuroPep database that exhibit a broad range of physicochemical properties (Figure 3A).[48] Coupled with differences in peptide structure and folding (Figure 3B), such differences in neuropeptide physicochemical properties could potentially contribute to differential diffusion and volume transmission in the brain. Currently, there are very limited data on the diffusion and transmission properties and thus requires future work in this direction. We will likely need high throughput methods to investigate these important questions.

Second, how different is the transmission across the brain regions? Both electron microscopy of chemically fixed tissue and the super-resolution imaging of living brain slices demonstrate that ECS is diverse and heterogeneous.[55–57] The measurements from cation tetramethylammonium (TMA^+) diffusion directly revealed that the tortuosity of

ECS is heterogenous in different brains.[9] For example, the cerebellum exhibits significant heterogeneity between the molecular layer and granule cell layer, and both the tortuosity and volume fraction are different in the two layers.[58] There is also increasing evidence that diffusion is anisotropic in several regions. For instance, TMA^+ diffuses more readily along an axon bundle than across it, as observed in the myelinated corpus callosum.[59] The anisotropic diffusion in the brain has also been confirmed by magnetic resonance imaging (MRI).[60, 61] Furthermore, the neuropeptide GPCR expression levels are anticipated to differ significantly across brain regions.[62, 63] Therefore, neuropeptide transmission across different brain regions is expected to be heterogeneous but has yet to be experimentally confirmed. Future work could explore this aspect of neuropeptide transmission and determine how it impacts the function of different brain regions.

Lastly, how is neuropeptide transmission under different brain states? It has been reported that sleep induces an increase of ECS volume fraction and drives metabolite clearance in mice and humans.[64–66] MRI of healthy adult brains also provides evidence for the reduction in ECS volume of large parts of human white matter after wakefulness.[67] Therefore, it is reasonable to hypothesize that the sleep/wakefulness state modulates the extracellular diffusion of neuropeptides and their actions in brain circuits. The diffusion properties in pathological brain states are also of high interest since they can serve as an indicator of pathological processes and perhaps offer insights into their underlying mechanisms. Several studies have shown that the diffusion coefficients of TMA^+ vary in brain diseases such as ischemia, spreading depression, and Alzheimer's disease.[9] Changes in the ECM and ECS in these diseased states might affect neuropeptide transmission.[68–70]

To address these questions, we need to build a database for the parameters of neuropeptide transmission in the brain. First and foremost, the toolkit to control the timing and spatial release of neuropeptides and in vivo sensors to monitor neuropeptides in real-time needs to be expanded. With the rapid development of optogenetics, the photo-stimulation of the endogenous genetically encoded neurons is more widely used by neuroscientists to investigate the release and dynamics of neuropeptides. However, co-transmission has been inescapable until now. the caged compound or photosensitive nanovesicles focuses on the transient release of neuropeptides (sub-second), while endogenous neuropeptide release in the physiological conditions could last longer. The photoswitchable release from liposomes with azobenzene-containing phosphatidylcholine shows promise for controlling the neuropeptide release in seconds.[28] We anticipate that integration of these new opto-chemical tools with newly developed genetically encoded neuropeptide sensors (e.g., Light, GRAB) will significantly advance our understanding of neuropeptide signaling in the brain.

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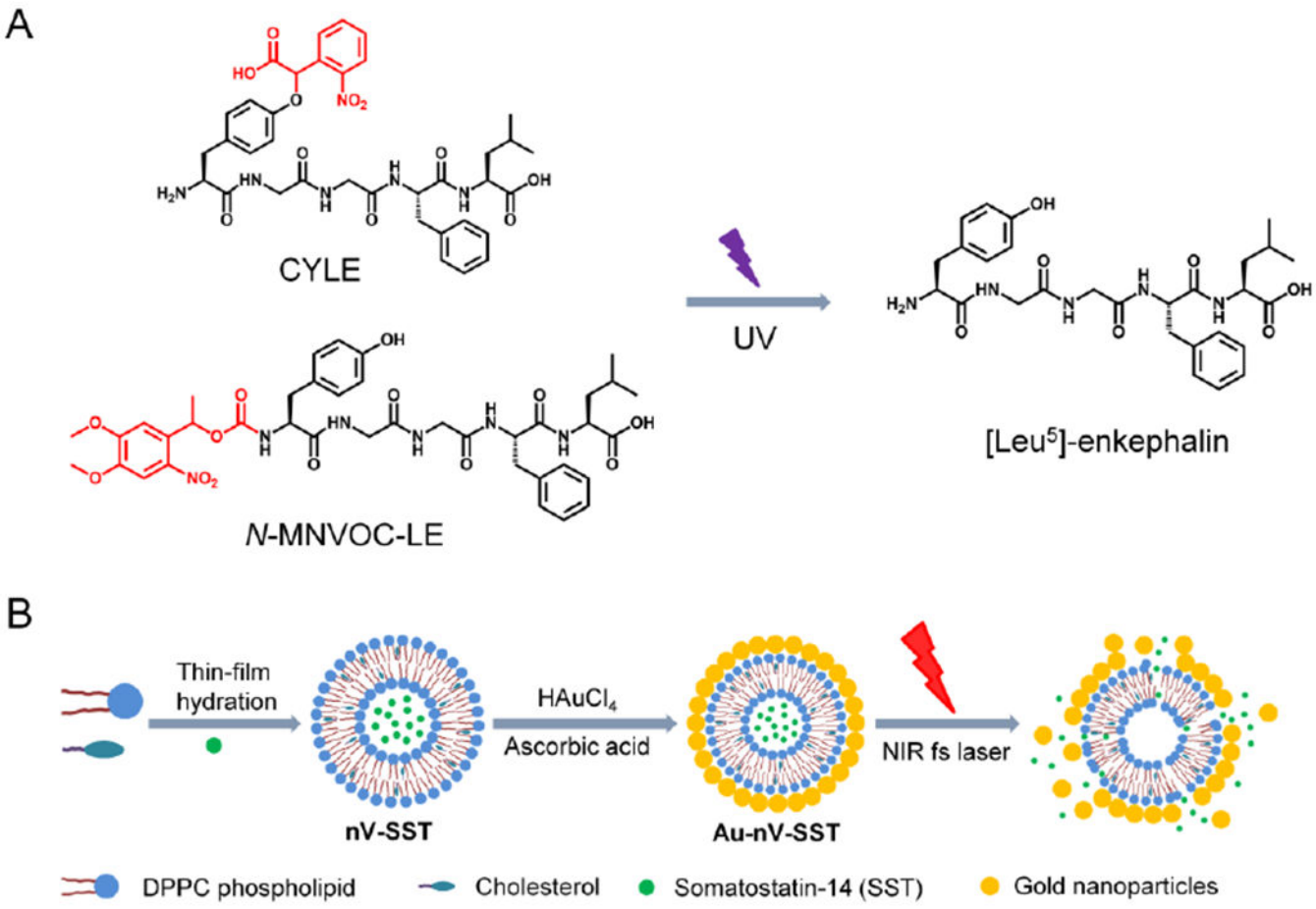

Figure 1.

(A) Chemical structures change of caged-peptides after UV light irradiation. CYLE: carboxynitrobenzyl modified [Leu^5]-enkephalin; *N*-MNVOC-LE N-(α-methyl-6-nitroveratryloxycarbonyl) modified [Leu^5]-enkephalin. The caging groups are indicated in red. (B) Schematic of preparation of somatostatin-encapsulated photosensitive nanovesicles (Au-nV-SST) and photorelease by the near-infrared laser pulses. The illustration of Au-nV-SST was adapted with permission from Ref [7].

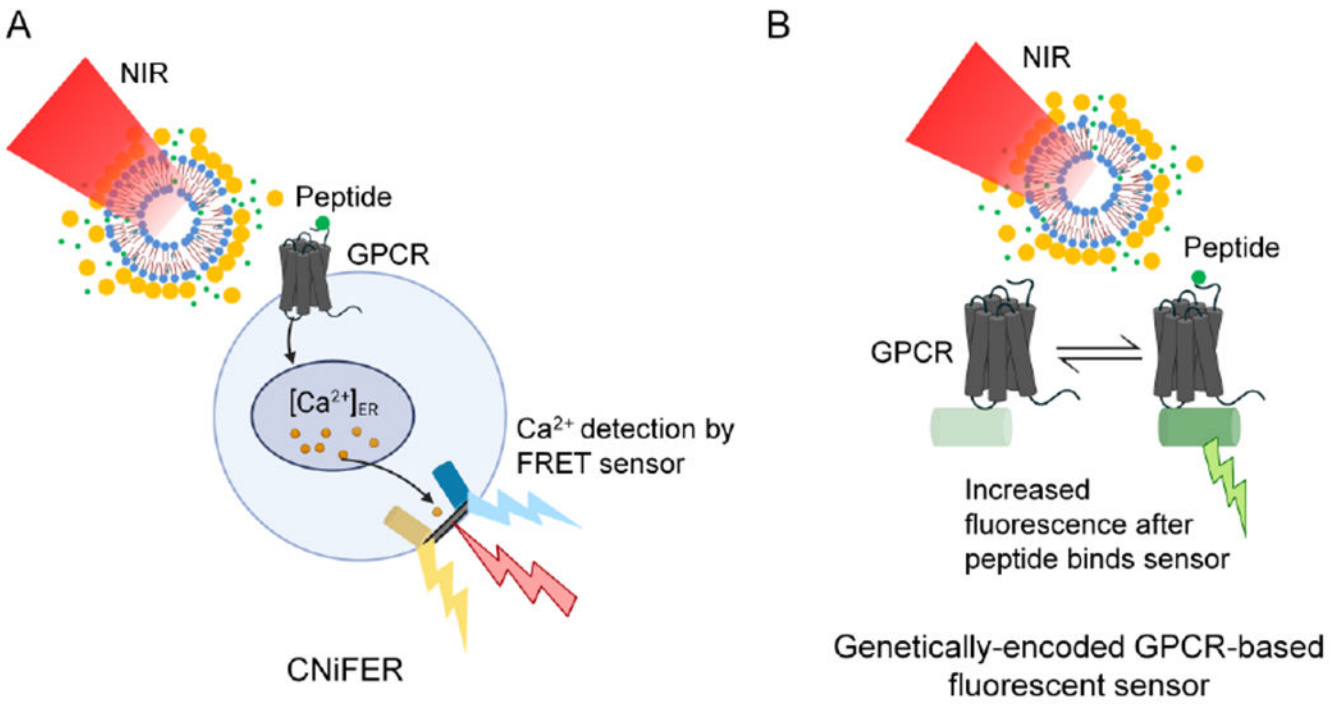

Figure 2.

Integrating neuropeptide release and sensing to probe neuropeptide transmission. (A) Schematic of cell-based neurotransmitter fluorescent engineered reporter (CNiFERs) for neuropeptide detection. (B) Schematic of genetically encoded GPCR-based sensors for neuropeptide detection.

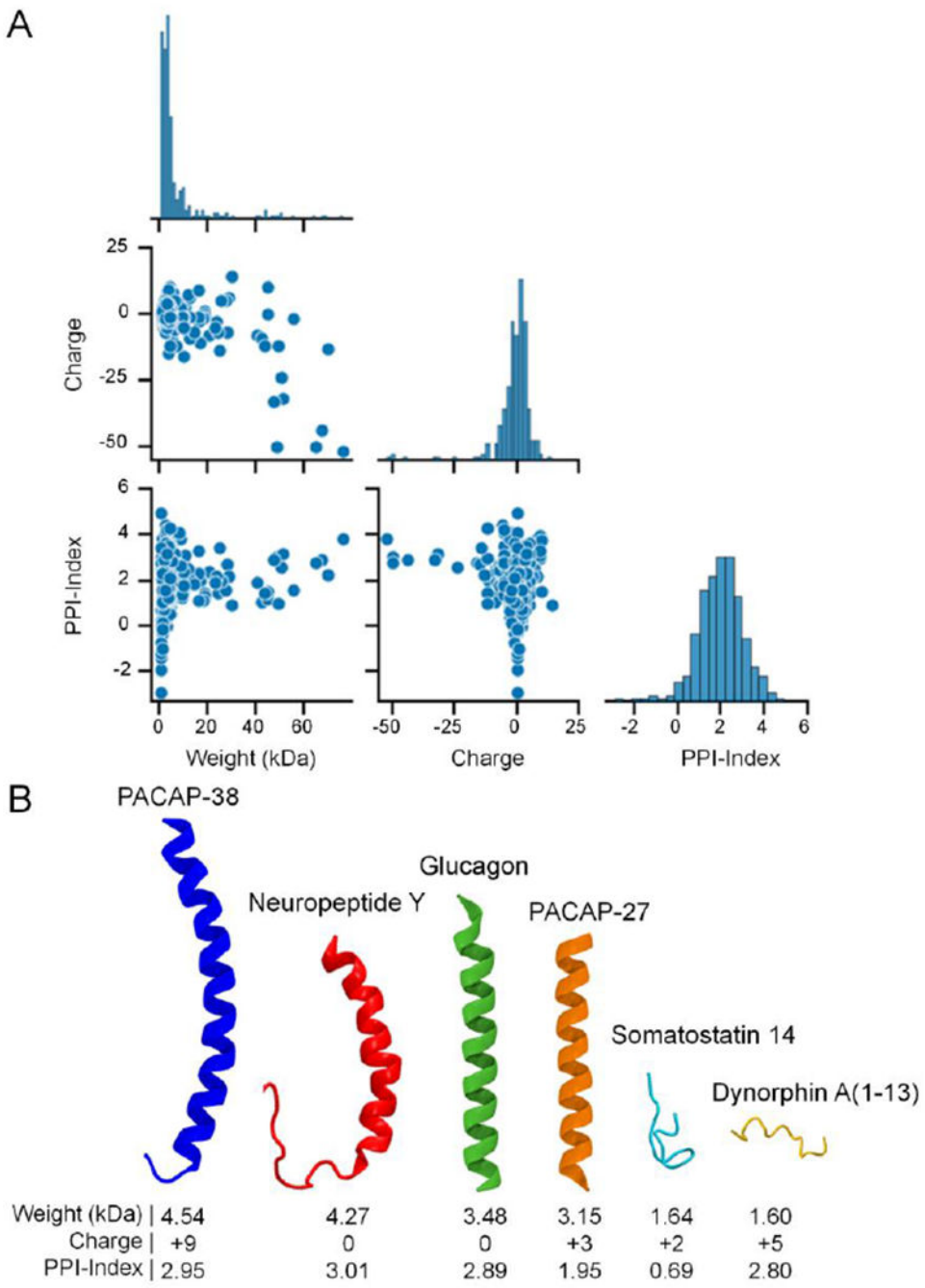

Figure 3.

The diverse physicochemical properties of neuropeptides. (A) Pair plot comparing the molecular weight (kDa), theoretical net charge at a physiologic pH of 7.4, and the Potential Protein Interaction Index (PPI-Index), a predictor of a polypeptide's propensity to bind other proteins/receptors,[49] for all 283 human neuropeptides in the NeuroPep database.[48] The properties were estimated from the peptide sequences using the peptides.py package (https://github.com/althonos/peptides.py).[50] Note that the diagonal edge of the pair plot shows the distributions of each property. (B) A selection of human neuropeptide

structures collected from the RCSB Protein Data Bank[51] and AlphaFold Protein Structure Database[52, 53] highlighting the diversity of neuropeptide structure and physicochemical properties, including the 38 amino acid variant (blue) of pituitary adenylate cyclase-activating peptide (PACAP), Neuropeptide Y (red), glucagon (green), the 27 amino acid variant of PACAP (orange), Somatostatin 14 (light blue), and Dynorphin A (1-13) (yellow). The neuropeptide structures were rendered using the Visual Molecular Dynamics (VMD) software.[54]