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Diet-Induced Neuropeptide Expression: Feasibility of Quantifying Extended and Highly Charged Endogenous Peptide Sequences by Selected Reaction Monitoring

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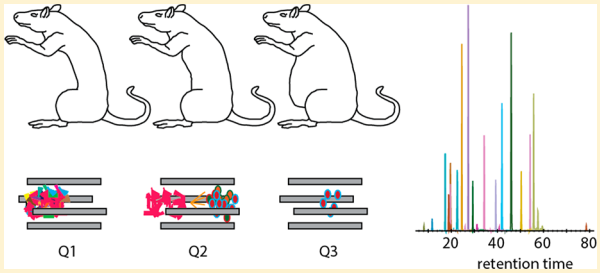
Supporting Information

ABSTRACT: Understanding regulation and action of endogenous peptides, especially neuropeptides, which serve as inter- and intracellular signal transmitters, is key in understanding a variety of functional processes, such as energy balance, memory, circadian rhythm, drug addiction, etc. Therefore, accurate and reproducible quantification of these bioactive endogenous compounds is highly relevant. The biosynthesis of endogenous peptides, involving multiple possible trimming and modification events, hinders the *de novo* prediction of the active peptide sequences, making MS-based measurements very valuable in determining the actual active compounds. Here, we report an extended selected reaction monitoring (SRM)-based strategy to reproducibly and quantitatively monitor the abundances of a set of 15 endogenously occurring peptides from *Rattus norvegicus* hypothalamus. We demonstrate that SRM can be extended toward reproducible detection and quantification of peptides, bearing characteristics very different from tryptic peptides. We show that long peptide sequences, producing precursors with up to five and MS2 fragment ions with up to three charges, can be targeted by SRM on a triple quadrupole instrument. Using this approach to quantify endogenous peptide levels in hypothalami of animals subjected to different diets revealed several significant changes, most notably the significant upregulation of VGF-derived signaling peptide AQEE-30 upon high caloric feeding.



The advent of mass spectrometry (MS)-based proteomics has caused a significant increase in the amount of quantitative data on thousands of proteins, including dynamic post-translational modifications, across a plethora of conditions. This has given the scientific community both a powerful new tool but also a new challenge.[1] While MS driven discovery experiments provide new views on the molecular mechanisms occurring in cells, the need for validation tools for many new targets rises.[2] Whereas the production of antibodies for all targets of interest is costly and time-consuming, the development of selected reaction monitoring (SRM) resulted in a fast, robust, and relatively inexpensive tool to quantify, in principal, almost any target of interest, and especially the use of heavy labeled peptides enables straightforward assay development.[3−5] Compared to shotgun proteomics this enables more accurate and reproducible quantification of numerous proteins across large populations. However, the technology is still limited in sample complexity it can handle and requires peptides amenable to robust MS analysis and hypothesis-based assay development.[2]

The workflow to establish SRM assays has been standardized in recent years. Especially the use of heavy labeled peptides made assay development and quantification of tryptic peptides more straightforward.[5] However, the use of alternative enzymes beyond trypsin is gaining popularity, especially in the field of global proteomics.[6−9] Moreover, experiments ranging from middle-down using different kind of proteases[10,11] to top-down experiments analyzing intact proteins[12−14] and the analysis of endogenous peptides[15−18] has been the focus of an increasing number of MS-based proteomics studies. One important group of endogenous peptides are neuropeptides, which serve as inter- and intracellular neuronal signal transmitters. They are involved in a variety of processes, such as energy balance, memory, circadian rhythm, and drug addiction.[19] Their biosynthesis involves multiple steps of proteolytic cleavage of a precursor protein and subsequent posttranslational modifications.[20] The variety of possible trimming and modification events impedes the *de novo* prediction of the active neuropeptides from the genome or even the precursor protein.[21] Therefore, mass-spectrometric measurement of such peptides can be very

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Table 1. List of Endogenous Neuropeptides (and Their Precursor Proteins) Targeted by Selected Reaction Monitoring

precursor protein	peptide name	sequence
Cbln1	[des-Ser1]-Cerebellin	GSAKVAFSAIRSTNH
Cbln1	Cerebellin	SGSAKVAFSAIRSTNH
Gal	Galanin	GWTLNSAGYLLGPHAIDNHRSFSDKHGLT(amide)
Gnrh1	Gonadoliberin-1	-*Pyr*-HWSYGLRPG(amide)[a]
Nts	Neurotensin	-*Pyr*-LYENKPRRPYIL
Pdyn	Dynorphin A(1−8)	YGGFLRRI
Pdyn	Dynorphin A(1−13)	YGGFLRRIRPKLK
Pdyn	α-neoendorphin	YGGFLRKYPK
Pdyn	β-neoendorphin	YGGFLRKYP
Pdyn	Dynorphin B	YGGFLRRQFKVVT
Pdyn	Dynorphin A(1−17)	YGGFLRRIRPKLKWDNQ
Pmch	Neuropeptide EI	EIGDEENSAKFPI(amide)
Pnoc	Nociceptin	FGGFTGARKSARKLANQ
Sst	Anthrin	APSDPRLRQF
Vgf	AQEE-30	AQEEADAEERRLQEQEELENYIEHVLLHRP

[a]Pyr = N-terminal pyroglutamate.

valuable in determining the actual active compounds present.[16,22−26] Especially, the growing use of the electron driven fragmentation technique electron transfer dissociation (ETD)[27] allows for in depth investigation of the neuropeptidome by mass spectrometry, as ETD is less influenced by peptide sequence, length, and post-translational modifications than collision-based fragmentation techniques.

Several reports describe quantification of endogenous peptides, reviewed recently by Buchberger et al.[28] Because of the endogenous nature of the peptides of interest, resulting in arbitrary peptide sequences, and the maturing of label-free quantification methods, most studies utilize this approach,[16,29−31] although chemical labeling strategies have been employed[32,33] and recently also data-independent acquisition (DIA).[34] However, to date, targeted MS-based tools for robust quantification of endogenous peptides are mostly lacking, as they show very different mass spectrometric characteristics from tryptic peptides. Monitoring target peptides by SRM already dates back several decades and has occasionally been used to quantify a limited number of neuropeptides, mainly in whole rat brain samples. In 1999, Jäverfalk-Hoyes et al.[35] used SRM assays to quantify Met-Enkephalin and Substance P 1-7 in rat brains, and in 2009, Kosanam et al.[36] used SRM to quantify endogenous α- and β-endorphins. Both of these studies monitor comparably short, trypsin like peptides using a single transition per peptide only. So far the only attempt to measure longer neuropeptides was performed by Dass et al.[37] using SRM in combination with fast atom bombardment to quantify β-endorphin, a peptide containing 31 amino acid residues, however, using tryptic digestion prior to the measurement.

The use of SRM for the targeted quantification of peptides evolved substantially throughout the last years. In current typical SRM assays, multiple parameters are taken into account. Several transitions per peptide are acquired and their relative intensities as well as their LC elution time and profile result in a large gain in specificity.[38] These strict SRM criteria result in extraction of quantitative information from complex biological backgrounds with high selectivity and thus confidence.[5] Additional instrumental and software developments enable a high degree of multiplexing.[39] Collision energy optimization for individual transitions additionally allows a boost in sensitivity for each peptide of interest,[40] thus creating the most sensitive MS-based detection method available.[41]

Taken these developments together have made SRM, used in combination with stable isotope dilution, the quantitative "gold standard" MS-based quantification method for tryptic peptides.[42,43] Since antibody based analysis of neuropeptides is cumbersome, targeted MS analysis could be very valuable; however, selection of SRM friendly peptides is not possible. Here, we show that the general concept of SRM can be expanded to measure long and highly basic endogenous peptides with precursor and fragment charges much higher than usually targeted. While long and highly basic peptides occur in tryptic digests as well, they are mostly omitted as they often carry missed cleavages.[4] We show the capability of SRM on a triple quadrupole MS to reliably quantify peptides with up to 30 amino acid residues and multiple basic sites. The selected endogenous peptides require transitions containing precursor charges of up to 5+ and fragment charges of up to 3+. To the best of our knowledge, the study performed here is the first to show the capability of SRM to simultaneously quantify multiple endogenous peptides using high precursor charge and fragment ion charge states in hypothalami.

We selected a set of 15 peptides (Table 1) to be targeted by SRM. The peptides were chosen based on a good detectability and potential regulation patterns from our recent shotgun study,[16] comparing endogenous peptide expression changes in different brain areas of *Rattus norvegicus* in response to diverse feeding conditions. In the previous study, we detected over 1700 unique endogenous peptides by combining higher energy collisional dissociation (HCD) and ETD fragmentation. The variations between individuals of the same feeding schedule, however, were relatively high (CVs up to 124%), thus hampering the detection of significant diet-induced changes in neuropeptide abundance. To overcome this limitation and determine significant changes in peptide expression, a higher number of replicates is required, necessitating a high-throughput analysis method, like SRM. Therefore, we set up a targeted SRM method, expanding the number of biological replicates by preparing in total 24 new hypothalamic neuropeptide samples, to resolve confident quantification of selected neuropeptides.

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EXPERIMENTAL SECTION

Materials. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Steinham, Germany). Synthetic peptides were purchased from JPT (Berlin, Germany).

Animals. The experiments were approved by the animal ethics committee of the University Medical Center Utrecht, following Dutch legislation. Male Wistar rats (Charles-River, Erkrath, Germany) were used (N = 24), ranging in initial weight from 200 to 250 g. The animals were housed individually (378 mm × 217 mm × 180 mm) in a controlled environment under a 12:12 light/dark cycle with lights on at 07:00 h. Rats were allowed to adapt to their environment for 7 days, after which they were divided into three experimental groups that were matched for body weight. Control (CTRL) and high-fat high-sucrose (HFHS) animals had ad libitum access to water and chow (Special diet services, Essex, U.K.). In addition, HFHS animals had ad libitum access to saturated fat (Vandemoortele, Eeklo, Belgium) and a 30% sucrose solution (Suiker Unie, Hoogkerk, The Netherlands). Restricted feeding schedule (RFS) animals received chow from 13:00 to 15:00 daily and had ad libitum access to water. Detailed information about the food intake and the body weight of all 24 individuals can be found in Table S-8.

Sample Preparation. All animals were decapitated between 12:15 h and 12:45 h within 10 s after they had been taken from their home cage. Immediately after decapitation, the heads were heated using a 800 W microwave (Sharp Co., Bangkok, Thailand)[26,44] for 5 s, after which the brains were removed from the skull and the hypothalamic areas were dissected, snap-frozen in liquid nitrogen, and stored at −80 °C until further use.

Peptide Extraction. Neuropeptides were extracted from rat hypothalami by a double-extraction protocol as previously described.[16] In short, hypothalami were subjected to two subsequent extractions using acidified acetone and diluted acetic acid, respectively. For the first step, endogenous peptides were extracted with 300 μL of ice-cold acidified acetone (acetone/water/hydrochloric acid 40:6:1), lysed on ice by microtip sonication, incubated on ice for 1 h, and centrifuged for 25 min at 14 000g. Supernatants were neutralized with NaOH and dried down. For the second extraction step, endogenous peptides from the remaining pellets were extracted with 0.25% acetic acid, incubated on ice for 1 h, and centrifuged for 25 min at 14 000g. For each hypothalamus, the two extracts were pooled and passed through a 10 kDa cutoff filter (Amicon ultra YM-10, Millipore) for 45 min at 14 000g. C18 solid-phase extraction (Sep-Pak C18 cartridge 1 cc, Waters) was used for desalting prior to MS analysis.

Liquid Chromatography−Mass Spectrometry Settings. All SRM experiments were performed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher). Upfront chromatographic separation was performed using an EASY-spray system consisting of an Easy-nLC 1000 coupled to a 25 cm, 75 μm i.d. PepMap RSLC, C18, 100 Å, 2 μm particle size column (Thermo Scientific, Odense, DK). The LC was configured in one-column setup. Formic acid (0.1%, Merck, Darmstadt, Germany) in deionized water (Milli-Q, Millipore) was used as solvent A, 0.1% formic acid in acetonitrile (Biosolve, Valkenswaard, NL) as solvent B. All measurements were performed at 200 nL/min flow rate. Different gradients were used for assay development and samples. For detailed gradient information, see section S5 in the Supporting Information. Samples were loaded in 10% formic acid at a volume of 2 μL. Loading on the column occurred at 600 bar. The mass spectrometer was set to 0.7 Da peak width (fwhm) for Q1 and Q3.

HCD spectra were acquired on a Q Exactive (Thermo Fisher) coupled to an Easy-nLC 1000 (Thermo Scientific, Odense, DK) equipped with in-house packed columns. Peptides were trapped (Reprosil C18, Dr Maisch, GmbH, Ammersbuch, Germany, 3 μm, 2 cm × 100 μm) at 800 bar with 100% solvent A (0.1% formic acid in water) before separation on the analytical column (Agilent Poroshell 120 EC-C18, 2.7 μm, 40 cm × 50 μm) on a 31 min gradient from 7% to 30% solvent B (0.1% formic acid in ACN) at a flow rate of 150 nL/min.

Spectral Library Acquisition. Two different fragment spectral libraries were acquired, once on a triple quadrupole mass spectrometer and once on a Q Exactive, which has been shown to accurately reflect the fragmentation events happening in a triple quadrupole mass spectrometer.[45] The Q Exactive mass spectrometer was set up as described above and programmed in data-dependent acquisition mode. Full scans were acquired at a resolution of 35 000 at 400 m/z. Fragmentation was induced for the Top 10 peaks using a 10 s dynamic exclusion. Target peaks were isolated in a 1.5 Da isolation window and subjected to HCD fragmentation with a normalized collision energy of 25%. MS/MS spectra were acquired in the Orbitrap at a resolution of 17 500 at 400 m/z.

Triple quadrupole fragment ion spectral libraries were acquired on a Thermo TSQ Vantage. The mass spectrometer was therefore programmed in QED mode. This contains SRM type of measurements for potential transitions determined from HCD libraries or theoretically. Upon surpassing a threshold of 1000 counts per second, a fragmentation event is triggered for the corresponding precursor. For each triggered fragmentation event, an identical repeat is programmed 15 s later before a 50 s exclusion duration. MS/MS spectra were acquired in the range of 200−1250 m/z at a scan time of 1.1 s.

MS/MS spectra for both libraries were partly analyzed using MASCOT[46] with a customized database containing only peptides of interest to restrict the search space and partly by manual fragment annotation.

Assay Development for Selected Reaction Monitoring. The most intense fragment ions found in the spectral libraries for each peptide were directly used as transitions, multiplexing up to 10 transitions per precursor if present. Those initial SRM assays were applied to the synthetic peptide library, enabling subsequent optimization of multiple parameters such as collision energy and LC gradient. For collision energy optimization, instrument specific parameters were used as a starting point (CE = 0.03m/z + 2.905 for doubly charged precursors and CE = 0.038m/z + 2.281 for precursor charges of three and higher) to scan through different normalized collision energy values using a step size of one.[40] The chromatographic gradient was adjusted to achieve an equal distribution of the peptides across 75 min of measurement time.

Peptide Detection in Hypothalamic Samples. In total, 10% of all peptide extracts were analyzed in randomized order using a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher) with the same setup as described above. Retention times were determined from the peptide library and each transition was run in a scheduled 8 min window. Potential carryover has been monitored by blank runs in between each sample subjected to identical measurement settings. In total, 10

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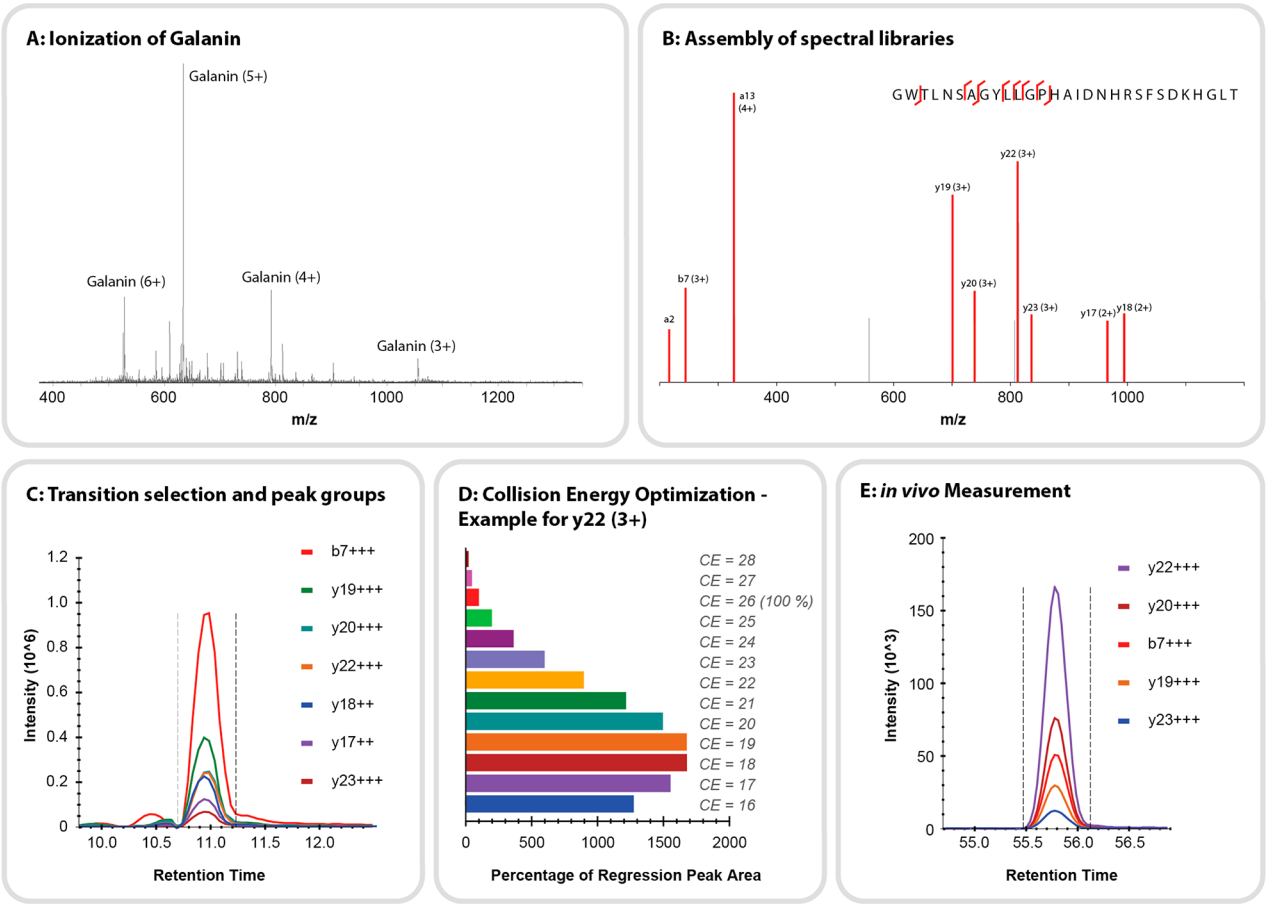

Figure 1. SRM assay development for highly charged neuropeptides using the example Galanin. (A) The preferred charge state of the precursor ion was determined based on full scans acquired on a TSQ Vantage. (B) Thereof HCD fragmentation spectra were acquired on a Q Exactive. (C) The most intense fragment ions were used as transitions for an initial SRM assay. (D) Further assay refinement by optimizing collision energy for each transition. Response equations for transitions combining highly charged precursor and fragment ions are unknown, hence signal improvements of more than 1700% are possible as shown for the transition y22 (3+). (E) Optimized transitions were used to measure endogenous peptides in rat hypothalamus samples. SRM transitions showing interference were removed.

fmol of fully digested bovine serum albumin has been spiked into each sample to control reproducibility of the retention time.

Quantification and Significance Analysis. Data analysis has been performed using Skyline.[47] The chromatographic quality has been assessed by visual inspection of the peak groups. Transitions showing clear interference have been deleted. Peptide quantification was performed label-free, where the summarized area under the curve of all transitions is used as a quantitative readout. Significance analysis has been performed using SRMstats.[48] This includes transforming intensities into $\log_2$-scale and subsequent intensity normalization based on equalizing medians. A linear mixed-effects model is successively used to test for abundance changes between different feeding conditions. A FDR cutoff of $\leq$0.05 in combination with a fold change of $\geq$2 were considered significant.

RESULTS AND DISCUSSION

Ionization, Fragmentation, and Initial SRM Assay Development. For a working set of SRM assays it is crucial to know the most abundant precursor charge state and the most abundant fragment ions. For tryptic digests, this information is often directly extracted from shotgun experiments measured on mass spectrometers capable of producing fragment spectra highly similar to the ones acquired in a triple quadrupole instrument (i.e., utilizing beam-type collision-induced dissociation (CID)[45]). An alternative is the use of SRM-triggered MS/MS[42] on either complex samples or peptide libraries. As the discovery based study used here was performed using, in part, ETD,[16,15] a direct transfer into SRM assays was not possible. Therefore, for assay development we constructed a peptide library containing the synthetic counterparts of the neuropeptides of interest. Measuring full scan spectra enabled us to determine predominant precursor charge states (Figure 1A). As expected overall observed charge states are much higher than observed for peptides originating from tryptic digests (Supplementary Figure 1 and Supplementary Table S-6). Fragmentation spectra were subsequently obtained for each peptide on a Q Exactive or triple-quadrupole TSQ Vantage, using beam-type CID. As expected for long peptides with high charge states, CID is not the optimal fragmentation method providing less informative fragment spectra than, e.g., ETD. The fragmentation spectra for most peptides contain only a few peaks, which however could be annotated clearly to specific fragment ions. Much unlike tryptic peptides, those fragment ions are often multiply charged (Figure 1B). Combining the most intense precursor and fragment m/z gave rise to an initial set of transitions used as a base for optimization (Figure 1C)

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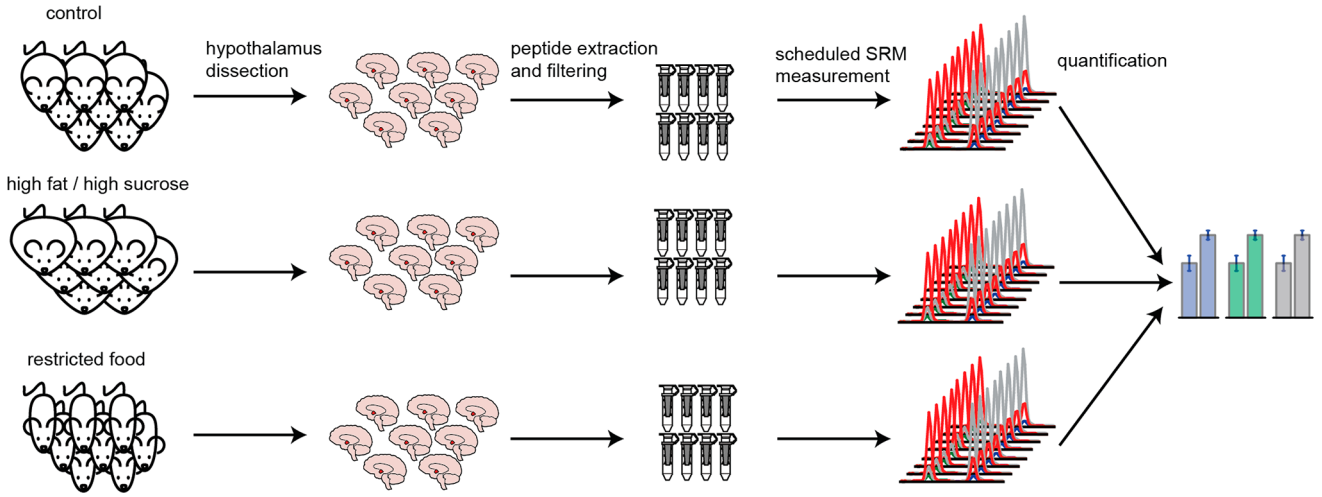

Figure 2. Workflow for measuring neuropeptide abundances in rat brain *ex vivo*. In total, 24 rats were divided into three groups of 8 animals each and subjected to different feeding schedules including standard diet (control), standard diet plus *ad libitum* access to lard and sugar (high fat/high sucrose), and time restricted food access (restricted food). After 8 days of dietary exposure, hypothalami were dissected and subjected to peptide extraction. Neuropeptides of interest were measured in SRM mode on a triple quadrupole mass spectrometer using the area under the curve for quantification.

Optimizing Collision Energy. Equations to predict the optimal collision energies for SRM assays exist for most instrument platforms capable of measuring SRM type experiments. However, they are mostly limited to predictive equations for precursor charges of 2+ and 3+ only. For transitions combining both highly charged precursor and fragment ions, those equations are of little use. By stepwise screening through different normalized collision energy percentages, we were able to optimize the signal for most of our transitions substantially. Signals increasing by up to 1700% for individual transitions were observed throughout our dataset (Figure 1D). These results clearly show that parameters for optimization of collision energy for larger multiple charged peptides cannot be extracted from parameters used for tryptic digests. Implementing the optimized settings into the final SRM assay considerably increased our sensitivity when measuring the endogenous neuropeptides. This however can drastically change the relative intensity patterns from the ones observed in the spectral library, as they were acquired using fixed collision energies depending only on the precursor m/z. Therefore, comparing relative intensities of our optimized SRM transitions and relative fragment ion intensities in the spectral library, which is often used as a quality control step to validate the SRM traces, is no longer possible. Instead, we verified relative intensities of the transitions measured in hypothalamus samples (Figure 1E) against SRM experiments with pure synthetic peptides.

In Vivo Neuropeptide Detection. Once the SRM assays were optimized, we applied them to measure endogenous neuropeptides from rat brain samples. Previously we investigated diet-induced changes in abundance of endogenous peptides in hypothalamus and striatum from rats from four differentially treated dietary groups including a high-fat/high-sucrose diet, mimicking diet-induced obesity. In that study the high variation observed put a strain on the quantification of the data, which we believe to be caused by the low number of animals per group (n = 3). The development of targeted SRM assays for several selected peptides from this initial study now allows us to increase the number of animals used, keeping the size of the experiment controlled while, as we believe, improving the reliability and significance of the quantification. The feeding conditions used here were regular diet (CTRL), high-fat/high-sucrose diet (HFHS), and restricted chow (RFS),[16,49,50] with the three dietary groups each consisting of eight animals. A schematic workflow is depicted in Figure 2.

The individual retention times of all peptides were determined using their synthetic standard counterpart, enabling us to measure the endogenous peptides in a scheduled manner and thus increasing the dwell time for each transition. All peptides were detectable *in vivo* except for Dynorphin A (1-13), which did not show any peak group across all 24 samples. The absence of this peptide can be explained as its endogenous processing and thus presence is unclear and often synthetic versions are described.[51,52] All other peptides were clearly detectable, showing distinct peak groups in each sample with mostly little to no visible interference (section S2 in the Supporting Information).

Many of the 14 neuropeptides detected here exhibit features, so far not routinely implemented in SRM assays. For instance the peptides Neurotensin and Gonadoliberin-1 contain an N-terminal glutamate to pyroglutamate conversion (Table 1) while the transitions for the peptides Galanin (Figure 1E), AQEE-30, and Dynorphin A (1-17) are based on precursor charges of 5 in combination with fragment charges of up to 3. To the best of our knowledge, this is the first study showing the feasibility of a conventional triple quadrupole mass spectrometer to analyze peptides with very high charge states in SRM mode.

For each sample, all peptides were quantified individually using the area under the curve. At this point of the study, the peptide β-neoendorphine was excluded due to chromatographic peak broadening. The remaining 13 neuropeptides performed well under the optimized SRM conditions and could be confidently quantified in all dietary groups. Using the software package SRMstats,[48] the samples were subjected to statistical significance analysis, testing for significant changes in peptide abundance across the three different feeding conditions. The analysis pipeline includes global signal normalization, quality control based on variations of the relative intensities of all transitions, and the use of a linear mixed-effect model to test for

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condition specific changes in peptide abundance. Figure 3A depicts average fold changes between feeding conditions with

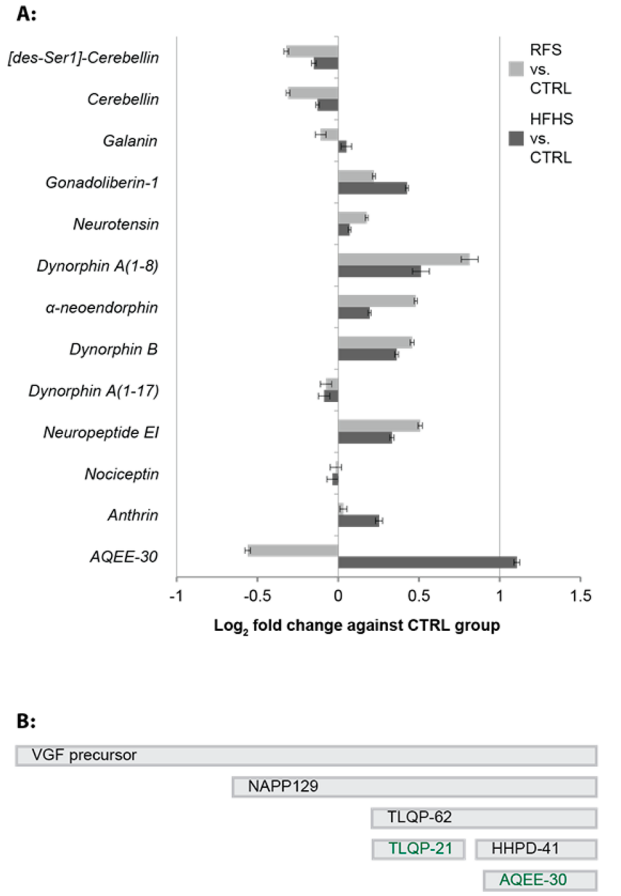

Figure 3. (A) Changes of neuropeptide abundance in brains of rats subjected to different diets. Bars indicate $\log_2$-fold change of each peptide upon restricted food access (light gray $n = 8$) and upon high caloric feeding (dark gray $n = 8$) against a control group ($n = 8$). By applying a significance cutoff of $p \leq 0.05$ and fold change cutoff of ≥ 2, the peptide AQEE-30 shows an elevated level upon high caloric feeding. (B) AQEE-30 is a product of multiple trimming steps guided by prohormone convertases. Its precursor polypeptide is VGF, a gene product often linked to food intake control and energy balance.

error bars representing standard errors. No overlap between the error bars indicates statistical significance ($p \leq 0.05$). When comparing group specific changes in abundance of the peptides detected in our study to the changes reported by Frese et al., we see similar trends for most of the investigated peptides. For instance, the increased abundances of Gonadoliberin-1, Neurotensin, Dynorphin A(1-8), α-neoendorphine, Dynorphin B, Neuropeptide EI, anthirin and AQEE-30 in high caloric feeding compared to the control group has been observed in both studies. The HFHS diet results in obesity,[49] whereas RFS rats loose weight.[53] These positive and negative energy balance states are reflected by Galanin and by AQEE-30. In contrast most peptides shown in Figure 3 show an effect in the same direction during positive and negative energy balance. The latter results are in line with previous results in which upon exposure to the HFHS diet for 1 week, POMC mRNA levels decreased whereas NPY mRNA levels increased in the arcuate nucleus. This change in neuropeptide mRNA expression was in the same direction as in rats that were food restricted.[54] Exposure to the HFHS diet results in leptin resistance,[55] in which high levels of plasma leptin fail to limit food intake and to suppress NPY and to increase POMC expression.[49] Taken together, although some neuropeptides reflect energy balance status (such as Galanin and AQEE-30), others (like Dynorphin) reflect leptin resistance in a state of positive energy balance.

The higher expression of the peptides Neurotensin, Dynorphin A(1-8), α-neoendorphine, Dynorphin B, Neuropeptide EI, and Anthirin under restricted food conditions compared to the control group could be confirmed with the targeted approach. In many of those cases these expression changes however showed no statistical significance in the shotgun approach. By using the approach of higher sample numbers in combination with SRM measurements, these trends could now be confirmed significantly for all of the above-mentioned peptides. Only in a minority of cases the quantification strategies used in the previous shotgun study and this study showed different outcomes. For instance, the increased abundance of Galanin and Dynorphin A(1-17) under high caloric diet and the increased abundance of Gonadoliberin-1 under food restriction as observed by us before could not be confirmed here, however the later was not significant in the shotgun study. Likewise the trends for the peptides Cerebellin and [des-Ser1]-Cerebellin vary between the two studies. Moreover, the observed diet-induced changes in peptide abundance are consistently less pronounced in the targeted SRM approach than in the shotgun experiments. We believe that this effect is likely due to the higher number of samples in combination with the more accurate quantification strategy, putting less weight on potential outliers. Especially in the above-mentioned cases of Galanin and Dynorphin A(1-17), the fold changes observed here were close to zero. The increased number of replicas used in this study is likely the cause of the different regulation patterns observed for the two forms of Cerebellin compared to Frese et al. It is however interesting to note that [des-Ser1]-Cerebellin and Cerebellin originating from the same precursor protein show very similar diet related abundance changes. Likewise for the four peptides originating from Pdyn, three show very similar abundance patterns with only Dynorphin A(1-17) deviating from this trend. This could suggest a higher diet induced effect on precursor synthesis than actual peptide processing.

As label-free SRM assays are inherently less accurate than experiments using internal standards, most studies using label-free SRM experiments make use of a fold-change cutoff of two. Here a cutoff of 2-fold was surpassed in one occasion. The peptide AQEE-30 showed a higher than 2-fold, significant increase in rats subjected to a high fat/high sucrose diet compared to the control group (Figure 3A). AQEE-30 is one of several neuro-endocrine peptides resulting from post-translational processing of the VGF protein[56] (Figure 3B). VGF is exclusively expressed in neuronal and neuroendocrine cells.[57] It has been linked to diverse biological processes throughout the last decades, including food intake and energy balance. Its mRNA levels have been shown to be affected by feeding conditions.[58,59] Likewise, *vgf*-knockout mice are thin, small, and hyperactive and lose the ability to become obese.[58]

So far most studies investigating the effect of VGF derived neuropeptides focus on another peptide product, namely, TLQP-21.[60] This is so far the only VGF derived peptide for which potential cell surface receptors have been identified.[61,62] While VGF itself has been related to the onset of obesity,

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interestingly TLQP-21 has the opposite effect.[63,64] A chronic intracerebroventricular (ICV) infusion of TLQP-21 in mice fed with a normal laboratory diet did not induce obesity and resulted only in a small increase in resting expenditure.[65] Similar effects were observed in rats[66] and Siberian hamsters.[57,67] These contradictory results regarding the role of VGF derived peptides in energy balance lead Lewis et al.[60] to suggest a biphasic role of VGF in energy regulation, requiring a further investigation into the role of other VGF-derived peptides.

CONCLUSION

In the work presented here, we demonstrate the potential of SRM to specifically monitor endogenous neuropeptides of interest. Despite current common practice, we could show that SRM is not limited to tryptic peptides but can be expanded to measure long and highly basic (endogenous) peptides. Precursor charges of up to 5 and fragment charges of up to 3 prove not to limit the quality of the SRM peak groups and can be easily used for accurate quantification, although they require a dedicated individual optimization of analysis parameters, in particular the used collision energies for peptide fragmentation.

Finding a significant upregulation of the VGF derived peptide AQEE-30 upon high caloric diet in rats strengthens the reliability of our approach, as VGF has often been shown to have an involvement in energy balance and obesity. However, the direct role of AQEE-30 on a peptide level has so far only sparsely been investigated. In view of contradictory results regarding the role of other VGF-derived peptides in energy balance, AQEE-30 and its potential involvement in obesity might become an interesting target for further investigation.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b03334.

Spectral libraries for peptides of interest; SRM traces of peptides libraries and hypothalamus samples; SRMStats parameters; SRMStats QC Plot; detailed mass spec and gradient information; SRM parameters for peptides of interest (PDF)

Quantitative SRM data of all peptide transitions (XLSX)

Data on rat body weight and feeding schedule (XLSX)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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