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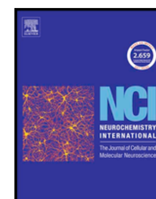

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Determination of morphine and norlaudanosoline in murine brain regions by dispersive liquid-liquid micro-extraction and liquid chromatograpy-electrochemical detection

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ABSTRACT

Morphine can be synthesized endogenously by mammals from dopamine via the intermediate norlaudanosoline. Previously, both compounds have been detected separately in whole brains of mice and brain regions of rats, and in urine of humans. Here, we report a novel method for the analysis of both compounds in single murine brain regions. Initially, a variant of dispersive liquid-liquid microextraction was established by using methanol as an extractant, cyclohexane as solvent, and tributylphosphate as disperser. The extraction method was applied to murine brain regions homogenized with perchloric acid while the subsequent detection was carried out by HPLC with electrochemical detection. In the thalamus of C57Bl/6J mice (n = 3, male, age 4–8 months), morphine and norlaudanosoline could be detected at levels of 19 ± 3.9 and 7.2 ± 2.3 pg/mg, respectively. Overall, we provide a novel method for the simultaneous extraction and detection of both morphine and norlaudanosoline in single murine brain regions.

Abbreviations

DA	Dopamine
DLLME	Dispersive Liquid-liquid Microextraction
DoE	Design of Experiment
ECD	Electrochemical detection
HG	Higenamine
HPLC	High Performance Liquid Chromatography
IS	Internal Standard
L-DOPAL	L-3,4-Dihydroxyphenylalanine
LOD	Limit of Detection
LOQ	Limit of Quantification
MA	Monoamine
MeOH	Methanol
MO	Morphine
NL	Norlaudanosoline
NP	Nalorphine
RSD	Relative Standard Deviation
TBP	Tributylphosphate
THP	Tetrahydropapaveroline

1. Introduction

Opioid peptides and molecules are found throughout the brain. They are known to contribute to pain, mood disorder, and addiction

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(Toubia and Khailfe, 2019). Further, a contribution of the opioid system to the manifestation of non-motor symptoms in Parkinson's disease is possible, however, remains to be elucidated (Thobois et al., 2018). In this context it is highly interesting that the synthesis of morphine (MO) seems to be dependent on dopamine (DA) produced by dopaminergic neurons, the neuronal population succumbing to cell death in the nigrostriatal pathway during the course of Parkinson's disease (Neri et al., 2008). In addition, the treatment of Parkinson's disease patients with L-DOPA leads to elevated levels of MO and norlaudanosoline (NL) (also called tetrahydropapaveroline (THP), a precursor to MO) in urine, indicating a direct interaction between the opioid system and the dopaminergic system (Matsubara et al., 1992). Indeed, L-DOPA is a precursor to DA, that likely feeds the formation of endogenous MO via the reaction of dopamine and L-DOPAL, an intermediate of dopamine degradation, to NL (Hoover et al., 1991). Several steps lead to the intermediate thebaine and to the synthesis of morphine by two possible pathways (Fig. 1).(Laux-Biehlmann et al., 2013) (Poeaknapo et al., 2004) Emerging evidence shows that the synthesis of MO and its pathway intermediates is affected by Parkinson's disease (Charron et al., 2011; Stefano et al., 2012). However, molecular mechanisms for this functional impact remain largely elusive. Moreover, how Parkinson's disease affects MO synthesis in individual brain regions has not been systematically addressed.

Research on Parkinson's disease is often conducted in mice as an important laboratory animal species, but to date, a method for the detection of MO and NL in specific disease associated murine brain regions has not been reported. MO and NL have only been measured in rat brain regions and whole mice brains, mice cerebellum (Cashaw et al., 1987; Cashaw 1993; Guarna et al., 2002; Muller et al., 2008), as well as in urine and blood of mice and rats (Groenendaal et al., 2005; Grobe et al., 2010). Accordingly, the aim was to develop a simple method for the analysis of MO and NL in individual brain regions of mice.

A HPLC with an electrochemical detector (ECD) was used to quantify MO and NL. Both substances have a hydroxy group neighboring an aromatic ring, making them prone to oxidation. They can thus be detected by ECD with high selectivity and sensitivity. The high sensitivity of ECD, which has been reported for a number of other biological compounds (Van Dam, Vermeiren et al., 2014; Bidel et al., 2015; Allen et al., 2017; Nagler et al., 2018), makes this detection method especially suitable for MO and NL.

Due to their sufficient concentrations in murine tissues, the analysis of monoamines (MA) by HPLC with ECD is usually based on a simple one step homogenization with perchloric acid and direct injection of the supernatant into HPLC. As the expected concentration of MO and NL is low, a more elaborate approach with an extraction and concentration step was required for these compounds. However, a simultaneous pre-concentration of MO and NL by solid phase extraction (SPE) did not seem viable due to different molecular properties of the compounds, possibly requiring separate cartridge materials. Thus, dispersive liquid-liquid microextraction (DLLME), a method that offers fast extraction across a wide range of analytes with low amount of solvents, was chosen. DLLME was first introduced by Rezaee in 2006 (Rezaee et al., 2006). An aqueous sample is filled with a mixture of an extractant that is not soluble in water, and a disperser that is soluble in both the extractant and water. The result is a fine dispersion of extractant droplets in the sample, which the analytes diffuse into. The advantages are a very fast equilibrium state and a low amount of necessary solvent.

In the past, extractants with higher density than water such as chloroform, dichloromethane, and tetrachloroethylene have been used (Saraji and Boroujeni 2014). After centrifugation, the extractant is found at the bottom of the tube and is then collected with a syringe. Newer variants also use extractants with lower density than water that are found at the top of the solution, thus requiring special equipment for their collection. Common dispersers consist of methanol (MeOH), acetonitrile, and acetone (Saraji and Boroujeni 2014).Due to the properties of the extractants, DLLME has mostly been used for the extraction of non-polar compounds such as pesticides or steroids. However, DLLME is also used for the extraction of compounds out of biological samples and common for large water samples. (Mansour and Khairy 2017).

Here, a novel DLLME technique for the simultaneous extraction and enrichment of NL and MO from murine brain homogenates was devel-

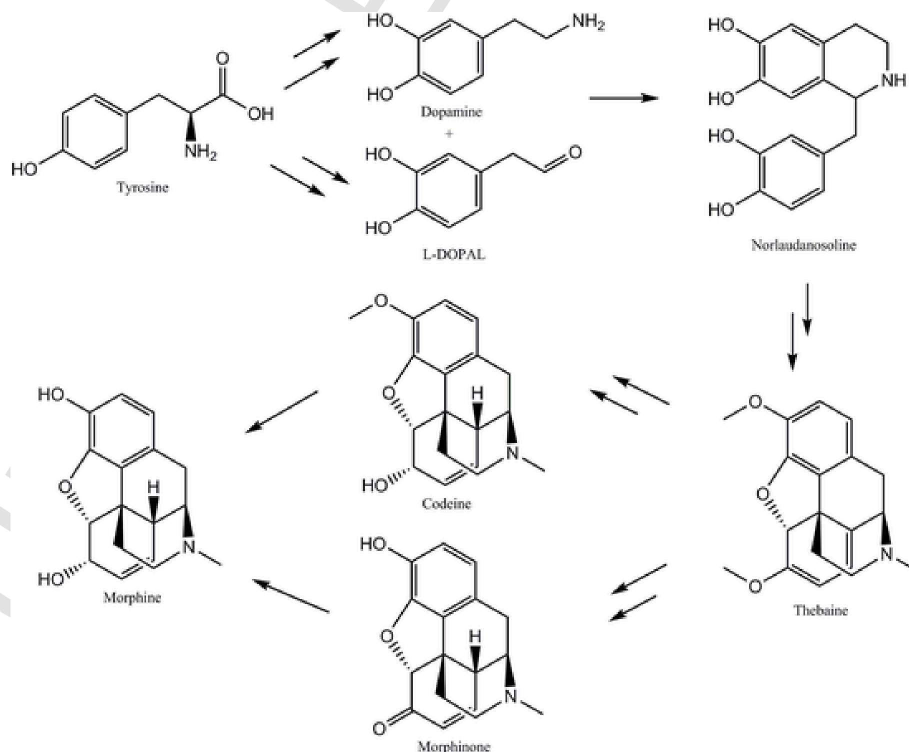

Fig. 1. Biological formation of morphine via norlaudanosoline.

oped. In a two-step protocol, where the second step is a DLLME, MeOH is used not as a disperser but as a second-step extractant that can be directly injected into the HPLC system. This falls into the category of so called reversed phase DLLME (Hashemi et al., 2010; Godoy-Caballero et al., 2013; Ziyaadini et al., 2016). However, so far methanol has not been used as an extractant in this variant. For the optimization of the variables of DLLME, a Design of Experiment (DoE) approach was used to identify optimal conditions for the extraction.

2. Experimental

2.1. Chemicals and reagents

Morphine, nalorphine, tributylphosphate, cyclohexane and perchloric acid ($HClO_4$, 70%) were purchased from Sigma Aldrich. Norlaudanosoline was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Higenamine, acetonitrile and cyclohexane were purchased from Promochem (Wesel, NRW, Germany). A commercially available Mobile Phase from the company RECIPE as part of the ClinRep® kit (Munich, BY, Germany) was used during the experiments. Merck supplied methanol and LC-MS water (Darmstadt, HE, Germany). All chemicals were acquired in the highest available purity and used without further purification.

2.2. Analytical instrumentation and chromatographic conditions

Measurements were conducted on an Ultimate 3000 HPLC–ECD system from Thermo Fisher. The system comprised a gradient pump (ISO 3100 BM), a cooled autosampler (WPS 3000 RS) with an automatic injection valve with a 100 μL sample loop, and a coulometric, electrochemical detector (ECD 3000 RS with 6011 RS Cell) consisting of two channels with a membrane working electrode. The signal area of peaks was used for the analysis of the respective compounds. The injection volume for all measurements was 10 μL. Separation of the compounds was carried out at a flow rate of 1 mL/min on a reversed phase C18-column from Waters (Atlantis T3, 150 × 4.6 mm, 3 μm). A C18 security cartridge from Phenomenex (4 × 3.0 mm) prevented blocking of the column from residues. The column oven was set to 30 °C. The draw and injection speed of the autosampler were set to 0.05 μL/s. For measurements, an isocratic elution with a commercially available buffered aqeuous mobile phase from RECIPE (pH of 4.2) with 15% v/v MeOH and 5% v/v acetonitrile was used. The mobile phase was degassed under vacuum in an ultrasonic bath (Bandelin, Sonorex RK 156) prior to use. The potential of the detector was set to 0.5 V. The gain range was 10 μA and the data transfer rate to the computer 10 Hz. The Chromeleon software 7.2 SR3 from Thermo Fisher was applied to control the system and to process the chromatograms.

2.3. Tissue collection

All procedures involving animal handling were approved by the committee for Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany. Male C57Bl/6J mice (age 4–8 months) were sacrificed via cervical dislocation and brains were dissected into the specific regions, immediately snap frozen in liquid nitrogen and stored at −80 °C until further processing.

2.4. Extraction procedure

2.4.1. Homogenization

The brain samples (Thalamus: 6–17 mg; Hypothalamus: 10 mg; Hippocampus: 22–29 mg; Striatum: 8–10 mg; Olfactory Bulb: 13–16 mg; Prefrontal Cortex 25–47 mg) were thawed on ice and 105 μL of 0.3 M $HCLO_4$ and 5 μL of a mixture of the internal standards (IS) higenamine (HG) and nalorphine (NP) (2 ng/μL each) were added to the tubes. The mixtures were homogenized via ultrasonication on ice for 10 s and centrifuged for 5 min at 4000 × *g*. The supernatants were transferred into a 2 mL collection tube.

2.4.2. MO/NL extraction

300 μL tributylphosphate (TBP) was injected rapidly into the supernatant with a Hamilton syringe. The solution was shaken until a cloudy dispersion was formed and then centrifuged for 10s at 3500 × *g*. The lower water phase was taken and discarded. The remaining TBP phase was taken and transferred into a 10 mL conical tube.

2.4.3. MO/NL re-extraction

5 mL of pre-cooled (4 °C) cyclohexane was added to TBP in the 10 mL tube. Then, 60 μL of pre-cooled (4 °C) MeOH was added with a Hamilton syringe and the tube was briefly shaken. The resulting cloudy dispersion was centrifuged for 1 min at 3200 × *g*. The lower MeOH phase was carefully taken with a 10 μL Hamilton syringe and transferred into a storage vial. An aliquot was transferred into a sample vial and injected into the HPLC system.

2.5. Method validation

2.5.1. Linearity, LOD, and LOQ

The linearity of the HPLC method was tested in standard solution ranging from LOQ to 20 ng per injection.

The limit of detection (LOD) was determined by diluting a standard solution of both analytes until a signal to noise ratio of 3 was reached. The noise was calculated by a H_2O blank injection. The limit of quantification (LOQ) was determined during the same experiments as the LOD with a signal to noise ratio of 10.

2.5.2. Precision

Precision was tested during spiking experiments. For this, 4 thalamus samples were pooled and homogenized. Due to limited WT material availability, each day thalami of two Dusp8tm1a het, one Dusp8tm1a wt, one Ghrelin KO and one Ghrelin; GHSR dKO male C57Bl6J mice were pooled in an Eppendorf tube and homogenized together.

Their supernatants were diluted threefold, aliquots were spiked with four different concentrations (blank, 0.5 ng, 1 ng, and 5 ng), and extracted three times for the intraday precision. The tests were repeated three times over three days to get the inter-day precision.

2.5.3. Recovery

In order to assess the recovery in real samples, internal standards were added in known concentrations at the beginning of the clean-up. The chromeleon software adjusts the analyte values according to the recovery of the IS with the following formula:

$$Amount_{j,k} = f(Response_{j,k}) \times \left(\frac{Dilution\ Factor_j}{Weight_j}\right) \times Factor_k \times IS\ Factor_{j,k}$$

f is the inverted calibration function, $Response_{j,k}$ the amount detected for component k in injection j, *Dilution Factor* and *Weight* are factors defined in the injection list (set to 1), $Factor_k$ is a scaling factor (set to 1), and $IS\ Factor_{j,k}$ is the correction factor for the recovery.

$$IS\ Factor_{j,k} = \frac{Amount_{j(injection\ List)}}{Amount_{j,\ IS\ (k)}}$$

The amount of IS is calculated with the same formula as the other components but without the weight correction. Five standards of 10 ng MO/NL and 10 ng NP/HG in 110 μL HClO4 were subjected to the DLLME procedure to test the variability between the analyte and the according internal standard, to preclude errors in the calculation due to

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differences in the extraction. This was also tested in brain samples to assure that the biological matrix also does not affect analyte and IS differently.

2.6. Design of Experiment

For the DoE, the program R with the Rcmdr package version 2.4–4 was used to design the optimization experiments and model the results. A separate design was used for each extraction step according to the parameters. For the analysis, the standard response surface model was used.

3. Results and discussion

3.1. Method development

In order to develop the DLLME method for the extraction of MO and NL, first, a number of extractants were tested for their ability to extract NL out of a standard solution. As NL has lipophilic properties with a logP of 1.9, accordingly, the lipophilic extractants dichloromethane, chloroform, tetrachloroethylene, undecanol, and TBP were chosen. Only TBP was able to extract NL, while the others achieved little extraction. Likely, NL forms strong hydrogen bonds with water due to its four hydroxy groups, which hinders extraction by lipophilic extractants. TBP, which is well known for its use in uranium purification, has been used in DLLME for the extraction of phenols (Hu et al., 2010). It is a Lewis base and might form a complex with the hydroxy groups of NL to extract it out of water. The addition of perchloric acid in the first step was used to lower the pH value, thus facilitating extraction of NL due to full protonation of its hydroxyl groups. As disperser, MeOH, acetonitrile, and acetone were tested. In preliminary trials MeOH achieved the best extraction of NL. Acetone was at 50% of MeOH, while acetonitrile was at 40%.

The second compound of interest, MO, could also be extracted with TBP, but with a lower efficiency due to its lower logP of 0.89. Therefore, to achieve full extraction of MO, an increased amount of TBP had to be used, compared to extraction of more lipophilic compounds alone.

TBP is a solvent lower in density than water and the extractant needs to be collected from the top of the tube. The water phase was removed with a Hamilton syringe until only TBP was left. However, TBP cannot be directly injected into the HPLC/ECD system as it lowers the sensitivity drastically, possibly due to dispersion in the mobile phase and reduced access of analytes to the ECD. Further, TBP cannot be evaporated due to its low vapor pressure. Thus, a second extraction was performed to transfer analytes into a suitable solvent. For this re-extraction of MO and NL, a DLLME procedure with interchanged roles of the phases was devised. Cyclohexane was added to TBP in a higher volume to act as the solvent. MeOH, which is normally used as a disperser, is not soluble in cyclohexane and can therefore act as an extractant in this second step. TBP is soluble in both solvents and acts as the disperser. Both analytes are not soluble in cyclohexane and diffuse into the MeOH phase, which can then be collected from the bottom of the tube and directly injected into the HPLC system.

3.2. Optimization of the extraction of NL and MO by DoE

The procedure was optimized by adjusting the volumes of TBP as extractant and MeOH as disperser. The optimization of the extraction was done out of standard solution, where 5 μL of standard solution of MO/NL (2 ng/μL each) and 5 μL of NP/HG (2 ng/μL each) were added to 100 μL $HClO_4$. As TBP could not be injected directly, recovery of the first step was assessed by analyzing the analyte content of the remaining H_2O phase after the extraction procedure and calculating the amount of extracted analytes. The compounds NP and HG were chosen as internal standards due their similarity to the target compounds while also not being present endogenously.

The results for the optimization of the extraction of MO and NL with DLLME are presented in the form of surface models. The factors optimized for the first extraction step were TBP and MeOH in the range of 160–440 μL and 3–116 μL, respectively. The standard response surface model of the Rcmdr package was used with the first order, quadratic, and interaction terms of the variables.

Fig. 1 in the supplemental shows the result of the response surface model for the extraction of MO. The model shows that MeOH affects the extraction negatively. TBP has a positive relation, however, there is a drop off after 350 μL. To test this model, extractions with TBP around 350 μL without MeOH were performed. Here, the optimal amount was found at 300 μL TBP with an extraction of 90%. The model for the extraction of NL (Supplemental Fig. 2) shows that MeOH has a negative effect here as well. However, it was much less pronounced than for MO, as the extraction was above 90% for the whole factor range. TBP also has a drop off above 350 μL, which was not there in test measurements with higher amounts of TBP without MeOH. With 300 μL TBP complete extraction was achieved.

TBP has a much higher affinity for NL than for MO. Therefore, for more lipophilic compounds a lower amount of TBP could be used. As the goal was the simultaneous extraction of NL and MO, 300 μL TBP, without addition of MeOH as a disperser, was chosen as the optimal amount. Even without any MeOH, there was a fine dispersion of TBP in the water phase after using a Hamilton syringe due to the dual properties of TBP with its aliphatic chains and the phosphate group. Technically, this is not DLLME anymore, but a conventional liquid-liquid extraction.

3.3. DoE for re-extraction of MO and NL

For the re-extraction of the compounds into a suitable solvent, cyclohexane and MeOH were used. For each sample, the first extraction step was performed as described above. Therefore, the factors optimized for the second extraction step were cyclohexane and MeOH with ranges of 2–6 mL and 0–80 μL respectively. The standard response surface model of the Rcmdr package was used with the first order, quadratic, and interaction terms of the variables.

Fig. 2 shows the response surface model of the re-extraction of MO. It shows that cyclohexane has only a minor, non-significant effect, with more being better. It seems that the optimum for cyclohexane is outside of the tested range. As an optimum was found for NL (see below) within the range, this range was not further expanded. MeOH, however, has a clear optimum at 60 μL. At zero μL MeOH, there is still some recovery, as a little H_2O is solved in TBP during the first extraction step and is separated after addition of cyclohexane.

Fig. 3 shows the response surface model of the re-extraction of NL. Here, the addition of cyclohexane appears to improve NL recovery with an optimum at 5 mL. MeOH shows optimum NL recovery at 60 μL. With 85 ± 2%, recovery of NL over 5 measurements was comparable to that of MO. The two internal standards NP and HG were also tested during the experiments and resulted in the same response surface model and recoveries as their counterparts (Supplemental Figs. 5 and 6).

After identification of the optimum cyclohexane to MeOH ratio, it was tested next whether the pre-concentration of the method could be further increased by evaporation into smaller volumes of MeOH. However, this was not successful, as small amounts of TBP and water remained in the extracted MeOH. Once the MeOH was evaporated, the TBP/water mixture likely caused dispersion upon injection and lowered the sensitivity at the detector. However, as the amount of recovered MeOH was 35–40 μL and the injection volume was 10 μL, it was decided to continue directly injecting the MeOH eluate of the initial extraction. This also allowed multiple injections per sample. Notably, the presence of TBP and water in our MeOH eluate was also the reason for

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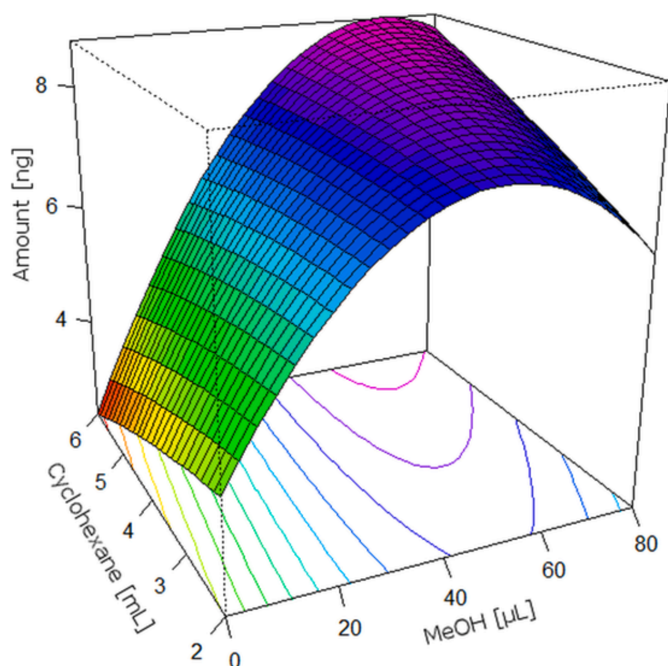

Fig. 2. Response surface model of MO re-extraction; Factor MeOH ($p = 0.001$) and second order interaction of MeOH ($p = 0.028$) were statistically significant; Adjusted $R^2 = 0.97$.

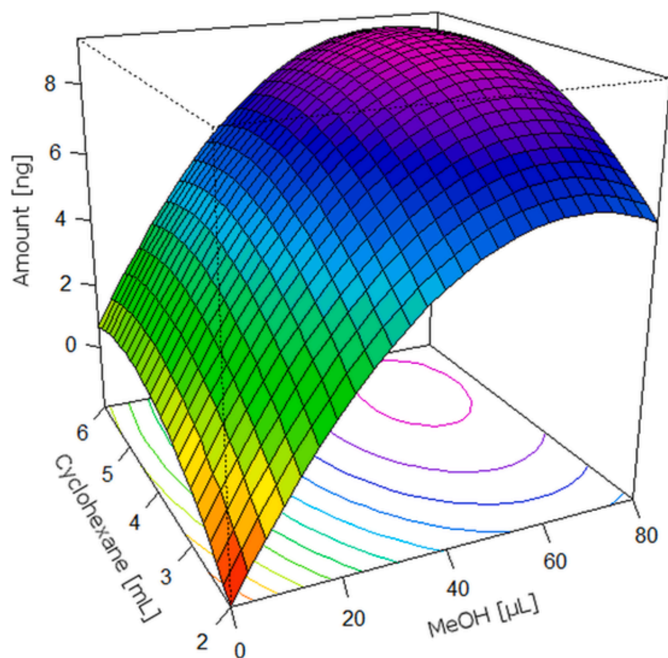

Fig. 3. Response surface model of NL re-extraction; Factors cyclohexane ($p = 0.011$), MeOH ($p = 0.0002$), and the second order interaction of MeOH ($p = 0.0175$) were statistically significant; Adjusted $R^2 = 0.98$.

the lowered draw and injection speed of the autosampler, as a fast injection of the extractant also lowered the sensitivity. This presence of TBP and water was also the reason for adding acetonitrile as an additional modifier to the mobile phase, to help prevent peak splitting due to different eluting strengths of sample and mobile phase.

The enrichment factor of the method was calculated with $EF = \frac{c_f}{c_s}$ with c_s being the initial concentration in the sample and c_f the final concentration in the extract. It was 2.5 for analytes and IS. The enrichment factor is lower than expected, however, is still useful for the low amount of target analytes. Fig. 4 shows the final work flow for the extraction method.

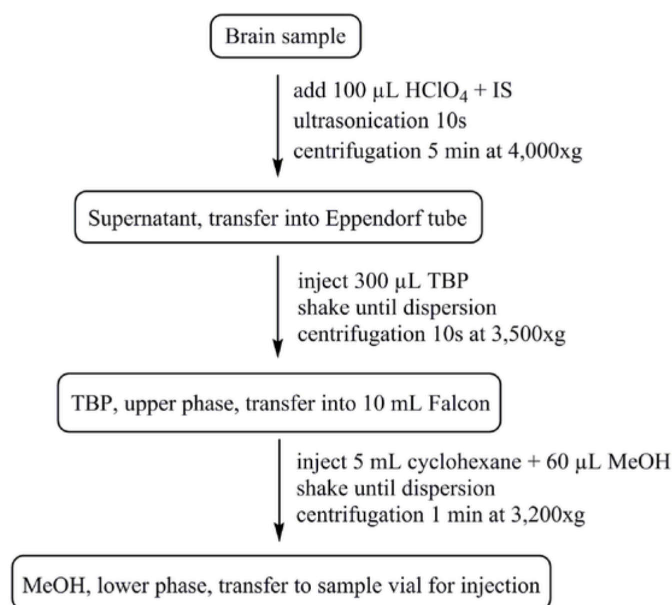

Fig. 4. Extraction flow path of MO and NL from brain samples.

3.4. Method validation

3.4.1. LOD/LOQ, linearity, and precision

In order to validate the method and the instrument, the LOD/LOQ, linearity, precision, and accuracy were tested. The LOD of the instrument for MO and NL was 3.6 and 1.3 pg/µL, while the LOQ was 10 and 3.6 pg/µL, respectively. The detector was linear in the tested range from LOQ to 20 ng with an $R^2 > 0.99$ for both MO and NL (Supplemental Figs. 3 and 4). The validation showed that the method is suitable for the extraction of MO and NL. The low sensitivity allows for detection of small expected amounts of analyte present in the brain samples. The linearity allows reliable detection of the analytes well above the expected concentration range.

The variability between the signal area of the analyte to the respective IS was between 95 and 105% (Table 1), showing that the recoveries of analyte and IS are identical, meaning they behave comparably during the DLLME procedure.

Validation of the method was done in a pool of spiked thalamus samples of genetically altered mice. Table 2 shows the precision, accuracy, and recovery for MO and NL conducted over the course of three days in thalamus samples spiked with fixed amounts of the analytes and IS.

The low RSD value show that the method is very precise and little deviation occurs between analyses. The accuracy is shown as the deviation between the calculated amount of analyte after IS adjustment and the expected value. Both the standard deviation as well as the accuracy deviation were below 10% and fulfilled the requirements of the US Food and Drug Administration for bioanalytical method validation (FDA, 2018). Recovery is shown as percentage of IS to the expected spiked amount. This shows the loss of IS during the extraction procedure and is used to adjust for loss of its according analyte. The previous

Table 1
Percentage of analyte to the respective IS over 5 extractions.

Analyte/IS	1	2	3	4	5	Mean
MO/NP	98.1	100.7	98.4	100.6	103.4	100.2 ± 1.9
NL/HG	103.7	101.7	96.8	100.1	101.8	100.8 ± 2.3

Table 2
Combined calculated intraday and inter-day mean, relative standard deviation (RSD), accuracy, and recovery of IS in spiked thalamus samples. Measurements were done in triplicates on three days.

Analyte	Spiked amount [ng]	Mean calculated amount [ng]	RSD [%]	Accuracy [%]	Recovery of IS [%]
MO	0.5	0.5	3.6 ± 1.6	99.6 ± 10.4	81.9 ± 3.8
	1	1	3.4 ± 2.1	100.4 ± 4.5	81.1 ± 1.9
	5	4.8	3.1 ± 0.7	96.6 ± 5.6	80.4 ± 7.9
NL	0.5	0.5	3.4 ± 1.1	108 ± 2.5	82.1 ± 1.8
	1	1	2.2 ± 0.3	107.9 ± 4.5	77.6 ± 6.8
	5	5.	2.3 ± 1.1	103.9 ± 5.6	77.8 ± 5.1

measurements of the variability between IS and analyte in standard solution during DLLME as well as the high calculated accuracy in the spiked samples show that analyte and IS indeed behave the same during extraction and that the IS can be used to adjust for loss of analyte. The recovery was well above the generally accepted value of 50%. Each day, a blank extraction without addition of analyte and IS was performed to ensure that no underlying peaks disturbed the analysis. The measurements were done with pools of brain samples, as not enough animals to use one sample per measurement point were available. However, the homogenate could not be aliquoted equally due to differential amount of liquid and solid particles, so spiking of the analytes before the homogenization, as normally done, was not possible. The validation still shows no matrix effects are present in the supernatant and accurate estimation of the analytes was achieved. The pool of various genotypes has no influence on the analysis of MO and NL and it is unlikely that a disturbing effect exists only in WT mice.

3.4.2. Application in biological samples

After validation, the method was applied to single murine brain regions. Table 3 shows the results of MO and NL in thalamus, hypothalamus, hippocampus, striatum, olfactory bulb, and prefrontal cortex of C57Bl/6J mice (n = 3, male, age 4–8 months). The recovery was calculated with the IS and is also shown in cases were no MO and NL could be detected. In cases where no RSD is presented, the compound could only be detected in one sample. Only in the thalamus, MO and NL were detected consistently. Results are based on wet tissue weights.

For each brain region, one sample without the addition of IS was analyzed, to ensure that no underlying peaks are present. In general, the recoveries are lower than during the validation, which may be due to analyte residues remaining in the precipitate after centrifugation of the homogenate. Nonetheless, washing the precipitate in test samples with 0.3 M $HClO_4$ or MeOH did not show any analytes in the wash solutions. Possibly, some of the analytes are bound irreversibly to proteins or other compounds during ultrasonication and remain unavailable for analysis. A thalamus sample was spiked with analytes and IS before the homogenization, to ensure that analyte and IS also behave the same during this step, which was the case.

Fig. 5 shows a chromatogram of a thalamus sample. The signal of MO peak 11 and NL peak 15 are very close to the LOD, but still clearly distinguishable from the baseline. Some of the peaks 1 to 10 preceding the MO peak 11 might be monoamines. They were not

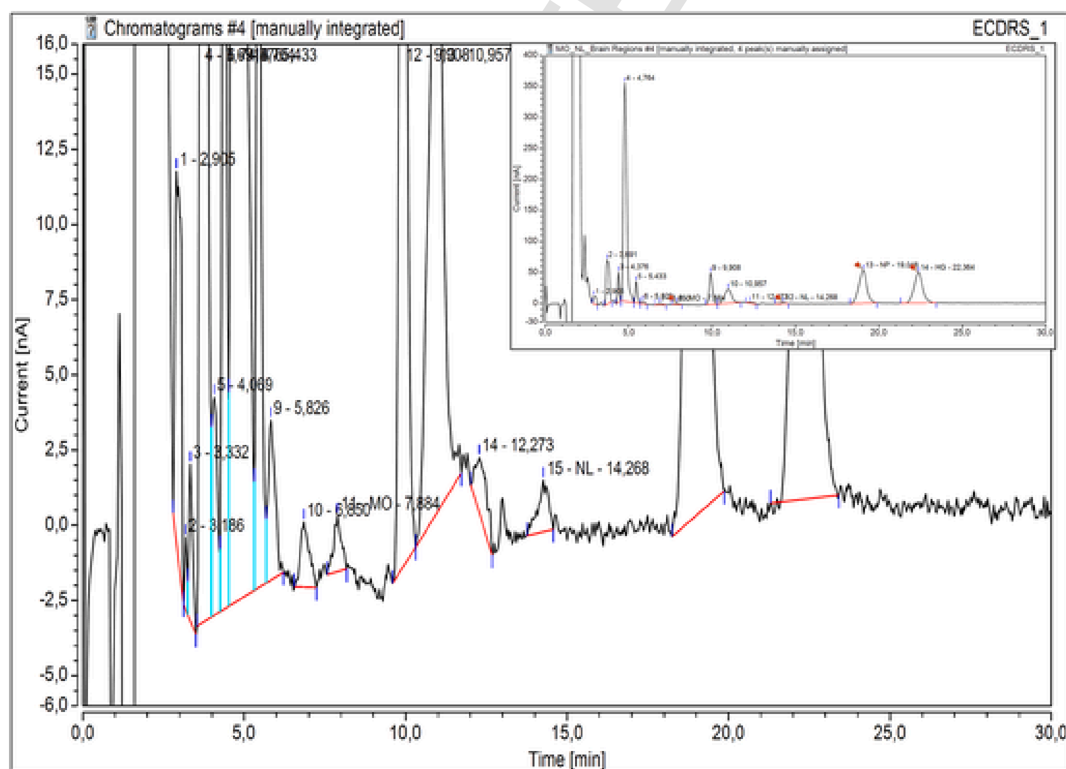

Fig. 5. Chromatogram of one thalamus sample; MO: Morphine; NL: Norlaudanosoline; NP: Nalorphine; HG: Higenamine.

Table 3
Values of MO and NL for single brain regions; n = 3; Injection volume = 20 μL; mean [pg/mg]; recovery of IS [%], n.d. = non detectable.

Analyte	Thalamus		Hypothalamus		Hippocampus		Striatum		Olfactory Bulb		Prefrontal Cortex	
	Mean	Recovery [IS]	Mean	Recovery [IS]	Mean	Recovery [IS]	Mean	Recovery [IS]	Mean	Recovery [IS]	Mean	Recovery [IS]
MO	18.8 ± 3.9	69 ± 5	n.d.	75 ± 2	3.9	53 ± 7	25.6 ± 4.9	61 ± 2	n.d.	77 ± 4	n.d.	66 ± 10
NL	7.2 ± 2.3	61 ± 6	n.d.	63 ± 2	n.d.	47 ± 9	n.d.	56 ± 1	n.d.	56 ± 5	17.4	58 ± 6

identified by spiking in the biological samples, yet, out of standard solutions, 3-methoxy-4-hydroxyphenylglycol (MHPG), 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine (NE), epinephrine (E), 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxybenzylamine (DHBA) as IS, homovanillic acid (HVA), dopamine, 3-methoxytyramine (3-MT) and serotonin (5-HT) could be extracted and detected with the method in the same area of the chromatogram (see chromatogram and retention times of standard solution Supplemental Fig. 7 and Table 1). Most of these MA are similarly enriched to MO and NL (see Supplemental Table 2), but MHPG, DOPAC, 5-HIAA, HVA, and 3-MT show decreased enrichment factors compared to MO, possibly due to a higher number of polar groups. As these MAs were only identified after the validation, they were not included in the analysis. However future analysis could readily include these MAs, especially DA as the proposed precursor to MO and NL.

Dopamine is thought of as an essential precursor for the synthesis of MO. (Neri et al., 2008; Laux-Biehlmann et al., 2013). Interestingly, it also has been shown that MO and MO like compounds are not found in dopaminergic but in GABAergic neuronal processes and astrocytes, that do not contain tyrosine hydroxylase for the production of DA (Laux et al., 2011). Moreover, in our measurements, the regions with a high number of dopaminergic neurons, i.e. the hypothalamus and olfactory bulb, showed no MO and NL. The striatum, supplied by the substantia nigra with a high amount of DA, contained MO in every sample, but no NL. In one hippocampus sample, MO was present. Likewise, in one prefrontal cortex sample, NL was found. Muller et al. reported 0.4 pg/mg MO in the cortex, 0.6 in the olfactory bulb and 2.1 pg/mg MO in the hippocampus of mice with ELISA (Muller et al., 2008). In comparison, the one hippocampus sample with MO lies in that range, but the values for the cortex are below our LOD. The only brain region that showed MO and NL consistently was the thalamus. In the thalamus, dopaminergic neurons are relatively rare compared to GABAergic or glutamatergic neurons. Overall, highest MO and NL concentrations in the thalamus are in line with research showing the presence of opioid receptors within this brain structure (Brunton and Charpak 1998). Therefore, our results support the hypothesis provided by Neri *et* al. that MO synthesis is not completed in dopaminergic cells. Rather, MO precursors or the finished product may be transported by various cell types, including astrocytes, and distributed throughout the brain. This distinct distribution of MO and NL in different brain regions and its interdependency with the dopaminergic system needs to be addressed in further research to elucidate its functions and its possible linkage to Parkinson's disease. .

This paper presents a method for the simultaneous detection of MO and its precursor NL in single murine brain regions. With the novel variant of DLLME based upon MeOH as extractant, an extraction procedure that is faster and more cost efficient compared to traditional SPE procedures is provided. The elution time on the HPLC with 25 min is slightly longer than most methods, but provides the additional option of MA measurements. This variant of DLLME combined with HPLC/ECD may further enable the detection of additional precursor of MO or other biogenic compounds across a wider lipophilic range. Finally, considering the use of MeOH as an extractant in DLLME, the method might serve as a starting point for the development of other applications outside of biological organisms such as environmental analytics. In summary, based on our method, researchers will be able to examine the complex regulation of endogenous opioid production and utilization in mammalian brain structures and their contribution to psychiatric and neurodegenerative diseases.

Author contribution

JN developed the method, analyzed the samples, and wrote the manuscript. SCS and PTP took care of the animal handling, dissected the mice, and provided the samples. PTP and DVW revised the manuscript. AR, WW, and KWS co-conceptualized the project. Additionally, KWS supervised the work. All authors have approved the final version of the manuscript.

Declaration of competing interest

There are no conflicting interests and external payments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2021.105174.

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