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Investigating the effects of a FAAH inhibitor in the laterodorsal tegmental nucleus using a new *ex vivo* mouse preparation

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ABSTRACT

As part of the brainstem reticular activating system, the laterodorsal tegmentum (LDT) is a central player in regulating sleep, arousal, and motivated behaviors including addiction. The neuronal lipids within the LDT that could regulate neuronal activity and LDT signaling are not fully characterized due, in part, to the complication of the presence of fatty acid amide hydrolases (FAAH). To determine the lipids in the LDT, mouse brain slices at different stages of brain development (16, 26 and 66 days) were exposed *ex vivo* to the FAAH inhibitor oleyl trifluoromethyl ketone (OTMK). Metabolomics and proteomic analyses were conducted on matched samples. Metabolomics analysis revealed differences between OTMK treated and untreated LDT. Furthermore, a distinct phenotype (proteomic profile) as a function of OTMK treatment was observed in LDT from adolescent (66 days) mice indicating an effect of treatment with OTMK at later stages of brain development. Our data indicate that this *ex vivo* preparation could facilitate screening of different FAAH inhibitors in mammalian tissues, and more importantly, this preparation should allow a deeper characterization of global mass spectrometry-based omics profiles within the LDT.

1. Introduction

The laterodorsal tegmental nucleus (LDT) is a pontine nucleus comprised of GABAergic, glutamatergic and cholinergic neurons [1]. The LDT is recognized as mediating states of arousal and is critical in wakefulness, REM sleep, locomotion, and motivated states including drug dependence, all of which have been extensively reviewed [2–6]. While the role played by each LDT neuronal phenotype in behaviors controlled by this nucleus has not been fully elucidated, the LDT controls arousal and motivated behavior through a diffuse cholinergic projection system which includes inputs directed to the thalamus, the ventral tegmental area and the nucleus accumbens [5,7–10]. Within the LDT, in addition to the three principle transmitters, several neuroactive peptides and mediators have been identified, which control activity of the LDT and subsequently, output to targets of the LDT responsible for mediation of behavior [11–13]. While lipids are known for their role in maintaining brain structure due to their chains of fatty acids which confer a hydrophobic nature, they also function as hormones or paracrine markers [14]. Thus, lipids are key mediators of signaling within many neuronal groups and could play a role in LDT functioning; however, very little is known about the presence of, or the signaling role played by, lipids in the LDT.

Arguably, the most widely studied neuronal lipids are the endocannabinoids (eCBs), which are generally synthesized and released at need [15]. eCBs act at two known types of eCB receptors, type 1 or type 2 (CB1/2 receptors) [16,17]. In an earlier study, we provided evidence of functional CB1 receptors in the LDT, which could be activated by endogenously applied synthetic agonist [18,19]. Further, we have previously detected two known endogenous CB1/2 receptor agonists,

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anandamide and 2-arachidonoyl glycerol (2-AG), within this nucleus [20]. As receptors and agonist are endogenously present, our data suggested that these two players of the eCB system could play a functional role in LDT neuronal signaling. In addition to eCBs, *N*-acyl acids, saturated palmitoylethanolamide (PEA) and unsaturated oleoylethanolamide (OEA) have been noted to act at CB1 receptors as neuromodulators [21]; however, their presence has not been reported in the LDT. Furthermore, age-dependent changes in the levels of eCBs and their degrading enzymes have previously been reported in other nuclei, but this information is lacking in the LDT [22]. Apart from eCBs, there are other physiologically important small fatty acids, such as omega-3, omega-6, eicosapentaenoic acid, and docosahexaenoic acid that are present in the brain and implicated in a number of diseases including cancer, and neurodegenerative and autoimmune diseases [23]. Whether any of these important fatty acids are present in the LDT is unknown. To gain a full understanding of the control of activity of LDT neurons, we need to understand the signaling molecules present in the LDT nucleus and whether they alter with age.

Rapid breakdown of lipids by fatty acid amide hydrolase or FAAH [24] poses a significant challenge in detection and quantification of lipids in the brain. To determine the molecular lipid signatures within the LDT as a function of age, we required a suitable sample preparation strategy. While exposure of mice *in vivo* to a potent FAAH inhibitor followed by harvesting of the LDT represents one approach, administration of drugs to mice is complicated by necessity of acquiring animal permits for chronic drug treatments, as well as handling and conducting injections in animals, which activates physiological stress systems. Therefore, in hopes of developing a suitable and relatively easy to use *ex vivo* model, we exposed LDT brain slices from different stages of brain development i.e from the early postnatal stage to adolescence (16, 26 and 66 days) [25] to oleyl trifluoromethyl ketone (OTMK), a potent FAAH inhibitor (Ki = 82 nM) [24]. Following a 1-h incubation in the FAAH inhibitor in physiological and oxygenated buffer, we conducted proteomics and metabolomics analyses to determine whether it was possible to detect changes in protein and metabolite profiles within the LDT using this *ex vivo* preparation across the three different stages of brain development.

2. Materials and methods

2.1. Brain sample preparation

All animal studies complied with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and with Danish laws regulating experiments on animals. Animal use studies were permitted by the Animal Welfare Committee, which was appointed by the Danish Ministry of Justice. The LDT was extracted from brain slices that were taken from mixed sex 16, 26 and 66 days old NMRI mice (Harlan Mice laboratories, Denmark). All mice were housed in a temperature-controlled room on a 12:12 light and dark schedule with lights on at 7 a.m. Tap water and laboratory chow were available *ad libitum*. All samples were collected in the morning, which is during the rest phase of the nocturnal mouse. All analysis was performed in triplicate.

2.2. Ex vivo model for oleyl trifluoromethyl ketone (OTMK) treatment in LDT

Slices containing the LDT were obtained according to previously published procedures that produced viable, living brain slices with ongoing, endogenous synaptic activity involving CB1R activation [18–20]. Tissues were collected from 16, 26 and 66 days old NMRI mice (n = 3, each age group), which were decapitated following loss of reflex to a pinch of the paw following induction of anesthesia with 100% isoflurane. A coronal block of the brain ~1 mm in thickness containing the LDT was isolated. Blocks containing the LDT were either placed in oxygenated artificial cerebral spinal fluid (aCSF; composition: NaCl (124 mM), KCl (5 mM), NaHCO3 (26 mM), Na_2HPO_4 (1.2 mM), glucose (10 mM), $CaCl_2$ (2.7 mM) and Mg_2SO_4 (1.2 mM)), or in oxygenated aCSF which contained OTMK (Sigma-Aldrich, O9887 Merck, DK). OTMK was dissolved in DMSO at a concentration of 25 mM and frozen until use. On the day of the experiment, aliquots were added to aCSF at a final concentration of 25 μM, and the brains containing the LDT were incubated in aCSF or aCSF with OTMK at room temperature for 1 h with constant exposure to 95%/5% oxygen/carbon dioxide and protection from light. Following incubation, the LDT was isolated bilaterally from the block with caution being taken to reduce extraction of nearby regions. The LDT was visualized from coronal brainstem sections located by known anatomical markers that had been used similarly in electrophysiology experiments focused on eCB functionality [19] and excised for subsequent processing using a fine surgical blade. The LDT was then placed on dry ice to rapidly cool.

2.3. Sample preparation for LC-MS based proteomics and metabolomic studies

The LDT samples were placed in 15 mL Falcon tubes prefilled with 5 mL of extraction cocktail [methanol:chloroform:water (5:1:4)] and vortexed for 30 s. The LDT sections were homogenized in the cocktail using a handheld Stuart SHM1 grinder at low intensity (5000 rpm) for 2 min until a suspension was formed. The suspension was kept on ice for 10 min followed by high intensity vortexing (15000 rpm) for 2 min. Following 10 min more on ice, the suspension was centrifuged at 15000 rpm at 4 °C for 15 min. There was a phase separation and upper and lower layers were carefully removed and stored in prelabeled eppendorf tubes without disturbing the protein pellet formed at the interface. The lower fractions (chloroform and methanol) were dried to evaporation under nitrogen and reconstituted in LC-MS grade 100% acetonitrile (0.1% formic acid) and centrifuged at 14000 rpm for 10 min and supernatants were subjected to LC-MS analysis. The protein pellets for proteome analysis were lyophilized and resuspended in preheated 99 °C lysis buffer (6 M Guanidine hydrochloride, 100 mM Tris-HCl pH 8.5, 5 mM tris(2-carboxyethyl)-phosphine (TCEP) and 10 mM chloroacetamide (CAA)). The samples were then incubated for 10 min at 99 °C and sonicated for 1 min. The samples were diluted x10 and digested with trypsin (ThermoScientific PierceTM) in ratio 1/50 (trypsin/protein, w/w) at 37 °C and placed overnight on a shaking table. After digestion, 10% trifluoroacetic acid (TFA) was added to a final concentration of ~1%, to stop the reaction and the samples were centrifuged for 5 min at 14000 rpm. The samples were then cleaned using Sep-Pak C18 columns (Water Corporation, Milford, MA) and eluted with 40 and 60% acetonitrile with 0.1% TFA. Speedvac concentrator was used to concentrate the peptide samples.

2.4. LC-MS analysis for proteomics and metabolomic studies

Metabolomic analysis was carried out on Waters 2695 HPLC coupled to Waters QTOF premier mass spectrometer. The composition of mobile phases was: A: 95% MQ water, 5% acetonitrile, 0.1% formic acid and B: 95% acetonitrile, 5% MQ water, 0.1% formic acid. An amount of 5 μL of the sample was injected into HPLC and was separated on a phenomenex C18 column (50 mm × 4.6 mm; 100 Å, 3 μm) at 40 °C. The temperature in the autosampler was maintained at 4 °C. A linear gradient was applied for separation (0–100% B) in 20 min and then the gradient was maintained for the next 5 min at a flow rate of 0.5 mL/min. Total run time was 25 min. The injected analyte after passing through the LC was sprayed using electrospray ionization (ESI) into the mass spectrometer, with a capillary voltage of 3 kV and collision energy of 5. The gas temperature was set at 180 °C at a flow rate of 80 L/h within a scan range of m/z 100–500. The raw files were exported to mzmL files using MSconvert 3.2 [26]. The converted files were uploaded onto the XCMS server [27] and analyzed using pairwise analysis and parameters HPLC-QTOF. An XCMS online server is a useful tool for conducting metabolomics analysis as an investigator without much experience in coding can independently

perform most of the functions [28]. The metabolomic features are characterized by a unique mass to charge ratio (*m/z*) and retention time. The entire deconvolution, alignment and systems biology analyses were carried out with the online tool itself [28–32]. The results table was exported to KNIME workflow to perform PCA analysis [33], and then, the matrix and volcanic plots were generated in Graphpad Prism 9.1.

For the proteomics analysis 1 μg were loaded onto the LC-MS/MS instrument (LTQ Velos Orbitrap). Peptides were trapped on a C18 column (5 μm, 5 mm, 0.3 mm) and separated on a 15 cm fused silica column (75 μm inner diameter), pulled and packed in-house with 1.9 μm C18 beads (Reprosil-AQ Pur, Dr. Maisch) on an Ultimate 3000 system connected to a LTQ Velos Orbitrap (Thermo Scientific, San Jose, US). The peptides were separated with a 110 min gradient with increasing buffer B (90% ACN and 0.1% formic acid), going from 5 to 30% in 70 min, 30–50% in 15 min, 50–95% in 20 min followed by a 5 min wash and re-equilibrating step. All steps were performed at a flow rate of 250 nL/min. The LTQ Velos Orbitrap was operated in data-dependent top 15 mode. Full scan mass spectra were recorded in the orbitrap at a resolution of 60,000 at m/z 200 over the *m/z* range 375–1600 with a target value of 1×10^6 and a maximum injection time of 500 ms. CID-generated product ions were recorded in the iontrap with a maximum ion injection time set to 100 ms and a target value set to 1×10^4. Spray voltage was set to 2.2 kV, *S*-lens RF level at 50, and heated capillary at 300 °C. Normalized collision energy was set at 35 and the isolation window was 2 *m/z*.

Raw data were processed with MaxQuant (1.6.17.0). The data were then further analyzed using Perseus (1.6.14.0). A total of 1609 proteins were identified across all the samples. The data analyses were performed on 851 proteins (valid values across all replicates, 3 biological replicates per sample). A principal component analysis (PCA, 5 components, BH-FDR of 0.05) was performed to determine the quality of the biological samples as well as to visualize sample separation based on the protein abundance variations.

3. Results and discussion

The mice were subjected to the new *ex vivo* sample preparation, and metabolomics and proteomics were used to identify whether it was feasible to observe OTMK-associated changes in the LDT. Three different age groups of mice (16, 26 and 66 days) were tested to check differences in the global metabolome and proteome profiles at different stages of brain development.

3.1. Proteomics analysis of LDT

Samples were processed to obtain a global proteomic profile. As FAAH degrades lipids and not proteins, we did not expect to see a difference between OTMK treatment and non-treatment. Consistent with this, the profiles observed in the two youngest groups at the earlier stages of brain development were found to be aligned with one another (Fig. 1), indicating that there was not much of a difference in the set of proteins in the LDT between OTMK treated and untreated groups. However, we did note a difference in the oldest age group. The OTMK treated mice in this adolescent group aligned with the younger mice as compared to the untreated matched age group (Fig. 1). Interestingly, loss of activity of FAAH-4 in C. elegans resulted in elevated levels of 2AG, which were associated with an extension in life span [34]. Similarly, administration of the FAAH inhibitor URB597 reduced the age-related reduction in hippocampal long-term potentiation [35]. When taken together with previous findings, our data strongly indicate that a better understanding of FAAH inhibition could open avenues to treat age-related, neural-based disorders. Furthermore, the alteration in protein profiles of adolescent mice, and effects of OTMK treatment on this ontogenic change has, to the best of our knowledge, never been reported within LDT and is relevant to age-related changes in attention and arousal.

3.2. Metabolomic analysis on methanol extracts from LDT

We did observe an effect of OTMK treatment on metabolite levels as we were able to differentiate between treated and untreated samples based on PCA analysis (Fig. 2). When control LDT was compared to that exposed to OMTK treatment (across all time points), a total of approximately 97 features were identified that significantly changed. The volcano plot (Fig. 3) shows features (defined as exhibiting an unique *m/z* and retention time) that were significantly upregulated in green, significantly downregulated in red or statistically insignificant features in black (Fig. 3), and the degree to which the features were either up or down regulated is plotted as a log fold change. More features were upregulated when compared to the number of features which showed a downregulation. These results are consistent with our expectations that inhibiting the activity of FAAH activity enhances the relative amounts of fatty acids within the LDT. Although we have not definitively shown that we were successful in FAAH inhibition in the LDT, as we did observe features which were upregulated in this nucleus in the OTMK treated groups, the most parsimonious explanation for our data is that treatment

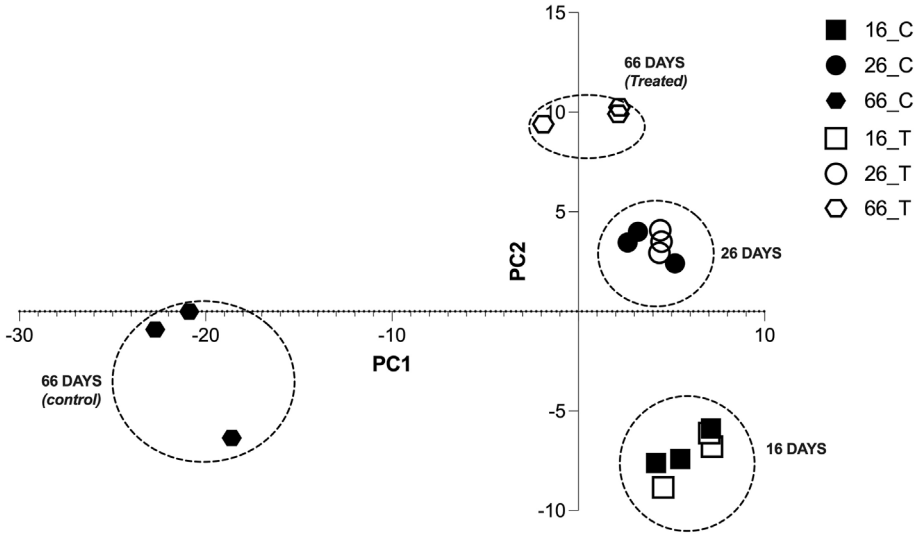

Fig. 1. PCA plot of proteomic data from the LDT collected from three different age groups (16-, 26 and 66 days) of untreated and OTMK treated animals. The plot represents data from 851 proteins from 3 biological replicate samples from the laterodorsal tegmental nucleus (LDT). Open and closed symbols represent treatment and control groups. Squares, circles and hexagons represent mice with ages 16, 26 and 66 days.

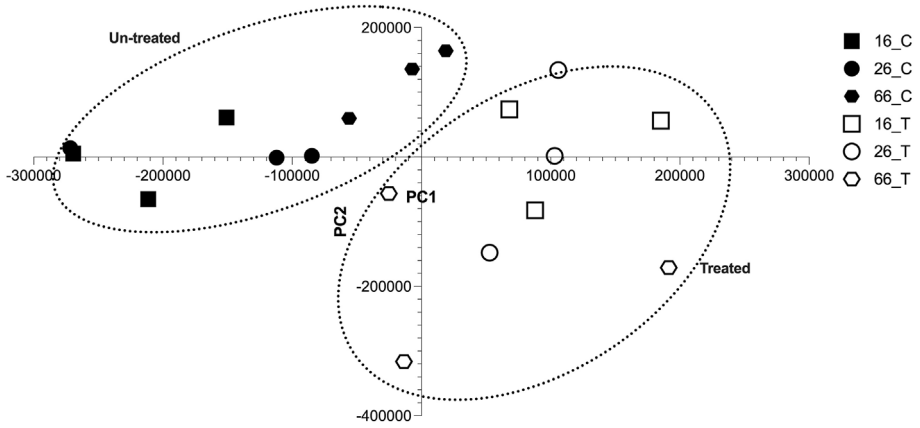

Fig. 2. Principal Component Analysis (PCA) plot of metabolomics data in OTMK treated (open) and untreated control (closed) groups. Squares, circles, and hexagons represent mice in three different age groups, 16, 26, and 66 days, respectively.

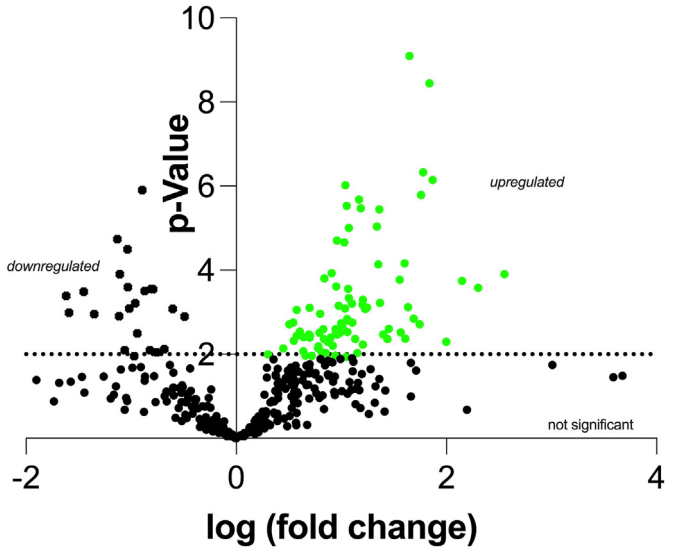

Fig. 3. Volcano plot of differentially regulated metabolomic features within treated and untreated LDT tissues. Features in green circles are upregulated while those in red are downregulated. The features that are not statistically relevant are in black. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with OTMK in our *ex vivo* preparation inhibits the activity of FAAH within LDT.

A recent metabolomic study in mice reported changes with age in 10 studied brain regions [36]; however, reports within LDT are sparse. Herein, we did not see age-related differences in the metabolome. However, we cannot rule out age-related differences as our technical approach may require optimization to study the effect of age in the LDT on the metabolome. Additionally, mice older than 66 days of age could show differences in the metabolome. Moreover, our samples were analyzed on a relatively low-resolution instrument, which could have led to an improper mass-based separation of lipids; and therefore, several features might have been clustered making individual identification impossible. Another limitation of our study is that we did not distinguish the sex of our mice. While we used matched tissue in each of the age groups, it is possible that differences in the metabolome between the sexes exist which would not be detectable under our experimental protocols [37]. Future studies should be conducted in which males and females are examined separately, older animals are also included, the OTMK treatment is further optimized by varying i.e. incubation times and concentrations, and a high-resolution, state of the art instrument is utilized to enhance the resolution in order to address the issue more definitively as to whether metabolites differ across age and sex within the LDT.

As expected, the number of metabolomic features that were upregulated were greater than those which were downregulated (Fig. 3). While we could speculate which metabolites were altered by OTMK treatment, we cannot identify these metabolites due to isomeric lipid forms as well as formation of adducts. In future analysis, we wish to isolate single features and identify them by subjecting them to MS^n analysis, which was beyond the scope of the present work. Furthermore, it should be noted that we were mainly interested in the lipids of less than $m/z = 500$ as most of the FAAH substrates fall within this mass range [38–40]. But there may be interesting lipids above m/z 500 which also could have been affected, and thus, lipids of larger sizes should also be investigated. Despite the analytical limitations, we conclude from the metabolomic analysis that it is possible to detect changes in metabolite features as a function of OTMK treatment in brain slices, indicating that an *ex vivo* preparation can be suitable for study of LDT small molecular lipid profiles, which should substantially assist further characterization of the molecules within the LDT. Interestingly, while age-related differences were seen in proteomic profiles in the control group of adolescent mice (66 days), OTMK treatment in the adolescent mice was associated with a phenotype more like that seen in the younger group of mice (16- and 26-days old group) indicating that FAAH inhibitors should also be examined in aging related studies.

In conclusion, we observed a connection between FAAH inhibitors and aging in the global proteomic profiles in the LDT while no significant differences between treatment was observed in the younger groups. Secondly, the effect of treatment was clearly observed in the metabolomic profiles indicating that our *ex vivo* preparation can be utilized to study LDT pathophysiology. Additionally, as it allows characterization of lipid presence in the LDT, this *ex vivo* preparation could offer accelerated screening of different FAAH inhibitors. Thus, we conclude that our preparation technique is useful for examinations related to therapeutics targeting age-related neuronal alterations with a focus on the role of FAAH.

Author contributions

Conceptualization, BKP, JC, NI, NS, HJ, KAK ; methodology, BKP, AG, NI; formal analysis, HJ, RRJ, NI, BM; investigation, BM, BKP, KAK; resources, KAK, BKP, BM, RRJ; data curation, BKP, BM, RRJ; writing,BKP, BM, RRJ, KAK, RRJ, NI, AG, JC; review and editing, BKP, BM, RRJ, KAK, RRJ, NI, AG, JC; visualization, BM, RRJ, JC, NI. All authors have read and agreed to the published version of the manuscript.

Institutional review board statement

Animals: All protocols for preparation of animal tissue used in this study were approved by the European Communities Council Directive (86/609/EEC).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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