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Research report

Anandamide and 2-AG are endogenously present within the laterodorsal tegmental nucleus: Functional implications for a role of eCBs in arousal

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ABSTRACT

Previously, we presented electrophysiological evidence for presence in mice brain slices of functional cannabinoid type I receptors (CB1Rs) within the laterodorsal tegmentum (LDT), a brain stem nucleus critical in control of arousal and rapid eye movement (REM) sleep. Further, using pharmacological agents, we provided data suggestive of the endogenous presence of cannabinoids (CBs) acting at LDT CB1Rs. However, in those studies, identification of the type(s) of CB ligands endogenously present in the LDT remained outstanding, and this information has not been provided elsewhere. Accordingly, we used the highly-sensitive liquid chromatography/mass spectrometry (LC–MS) method to determine whether N-arachidonoylethanolamide (Anandamide or AEA) and 2-arachidonyl glycerol (2-AG), which are both endogenous CB ligands acting at CB1Rs, are present in the LDT. Mice brain tissue samples of the LDT were assayed using ion trap LC–MS in selected ion monitoring mode. Chromatographic analysis and product-ion MS scans identified presence of the CBs, AEA and 2-AG, from LDT mouse tissue. Data using the LC–MS method show that AEA and 2-AG are endogenously present within the LDT and when coupled with our electrophysiological findings, lead to the suggestion that AEA and 2-AG act at electropharmacologically-demonstrated CB1Rs in this nucleus. Accordingly, AEA and 2-AG likely play a role in processes governed by the LDT, including control of states of cortical gamma band activity seen in alert, aroused states, as well as cortical and motor activity characteristic of REM sleep.

1. Introduction

The brain cycles between states of wakefulness and sleep (Jones, 2003). Our understanding of the neurobiological mechanisms by which the brain cycles between different behavioral states is incomplete. However, activity within the ascending reticular activating system of the brain stem promotes the gamma band-rich, cortical electroencephalographic (EEG) activity typical of aroused wakefulness and the state of rapid eye movement sleep (REM), which is also characterized by a desynchronized EEG (Moruzzi and Magoun, 1995). A central player in the control of this activity is the laterodorsal tegmentum (LDT) and consistent with a role in control of cortical states exhibiting a high degree of activation, activity of the cholinergic cells in this nucleus is highest during active waking and REM sleep, concurrent with presence of high levels of acetylcholine (ACh) in targets of the LDT (Boucetta et al., 2014; el Mansari et al., 1989; reviewed by Saper et al., 2001; Thakkar et al., 1998; Williams et al., 1994). In order to gain understanding of how aroused cortical states are generated, many studies have identified the actions of classic neurotransmitters and neuroactive peptides within the LDT and have characterized how these agents shape the activity and output of the cholinergic cells of this nucleus.

The endocannabinoid system, which was named after the cannabis plant that led to its discovery, is now well recognized as playing a major function in neuronal modulation, and this system has been found to exert control of various processes including memory, appetite, energy balance and stress responses (Gaoni and Mechoulam, 1964; Mackie, 2006). The neuronal endocannabinoid system is comprised of several different lipid-based agonists which are called endogenous endocannabinoid (eCBs), and two membrane-bound and internalized receptors, although other receptors have been identified outside the CNS (Cadas et al., 1997; Di Marzo et al., 1994; Mechoulam and Golan, 1998). The majority of eCBs are synthesized 'on demand' in neuronal cells and are released immediately as ligands at the two-known CNS eCB receptors: type 1 receptors (CB1R) and type 2 receptors

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(CB2R) (Howlett et al., 2002; Munro et al., 1993; Zheng et al., 2013). N-arachidonoylethanolamine (AEA), also known as anandamide, AEA, was the first endogenously-produced, lipid-based agonist of the CB1R to be recognized (Devane and Axelrod, 1994; Martin et al., 1999). Soon thereafter, 2-arachidonyl glycerol (2-AG) was identified as another eCB, which acts as a full agonist at CB1Rs (Mechoulam et al., 1995; Stella et al., 1997; Sugiura et al., 2006).

Behavioral actions of eCBs found in cannabis include reductions in cortical activation (Chait and Perry, 1994; Heishman et al., 1997; Perez-Reyes et al., 1988) and injections of ANA in the brain stem induce slow wave sleep (SWS) and REM sleep (Murillo-Rodriguez et al., 1998), which suggest the possibility that the eCB system plays an endogenous role in LDT functioning. However, to the best of our knowledge, our pharmacological data, which showed that a CB1R antagonist exerts membrane actions on LDT cells, is the only evidence to date that indicates presence of an endogenous agent active at CB1Rs within the LDT (Soni et al., 2014; Soni and Kohlmeier, 2016). Acquiring more direct evidence suggestive of presence of CB1R agonist(s) in the LDT would further our elucidation of mechanisms which control the activity of these neurons. Further, knowledge of the identity of specific CB1R-acting agents present in the LDT would be useful in order to understand the role played by individual eCBs in state control. LC–MS is a powerful technique which exhibits high selectivity and sensitivity well-suited to detection of natural, lipid-based products found in mixtures. Accordingly, we used LC–MS to determine the presence of AEA and 2-AG in the mouse LDT. Our results, when taken in combination with our electrophysiological and pharmacological data, support our hypothesis that the eCB system plays an endogenous role in processes governed by this nucleus. In addition, when taken together, our data provide information relevant for considerations of pharmacological management of arousal disorders via targeting of the eCB system.

2. Results

Detection of AEA and 2-AG utilizing LC–MS instrumentation has been well established in the literature (Chen et al., 2009; Gong et al., 2015; Liput et al., 2014; Mukhopadhyay et al., 2011; Zoerner et al., 2011). The breakdown products for AEA and 2-AG vary depending upon the type of mass spectrometer used. Ion trap LC–MS produces the main fragmented ion at m/z 287.2 for AEA and 2-AG (Han et al., 2013; Lehtonen et al., 2008). We used ion trap LC–MS which gave us m/z 348→m/z 287.2 and m/z 379→m/z 287.2 as the major product ion when 200 μM AEA or 2-AG standards were injected, respectively (Fig. 1A and B and insert). The presence of more minor peaks at m/z 330.2 for AEA, and at m/z 269.5 and m/z 361.2 for 2-AG were apparent (Fig. 1A and B, asterisks) and most likely reflected the breakdown products resulting from loss of water ($[(M+H) - H_2O]^+$), which is consistent with findings from other work using ion trap mass spectrometry to detect these lipids (Lehtonen et al., 2008).

From LDT samples, the m/z 348 for AEA and 379 for 2-AG were isolated and fragments under selected reaction monitoring (SRM) method were followed for further analysis (Chen et al., 2009; Gachet et al., 2015; Liput et al., 2014). The major product ion of m/z 287.2 was monitored for AEA (Fig. 1C). A peak at m/z 330 was not detectable above noise, likely due to presence of tissue matrix, which is absent in standards. The major product ion of m/z 287.2 was monitored for 2-AG (Fig. 1D1). While a peak at m/z 361.2 was not noted above noise, a product ion of m/z 269.5 was also detected (Fig. 1D2). When taken together, as shown in Fig. 1C and D1, analysis of the major product ions indicates presence of AEA and 2-AG in LDT samples.

The cerebellum, which is a region of the brain known to contain AEA and 2-AG (for review, see Chevaleyre et al., 2006), was treated as a positive control to detect lipids and as expected, m/z values consistent with presence of AEA and 2-AG were obtained (Fig. 1E and F). The concentrations of tested eCBs were not quantified in either brain area, as only signature studies were intended for the present report. Consistent with other studies (Chen et al., 2009), we found that very little, or no amount, of AEA and 2-AG was detected in LDT samples more than 30 days post extraction (data not shown). In pilot experiments using mass spectrometry imaging (MSI) of LDT-containing brain slices, we were not successful in detecting AEA and 2-AG from the LDT cryosections. Regardless, while we were not able to characterise their distribution at this time using MSI, use of analytical chemistry shows that AEA and 2-AG are present in the LDT.

3. Discussion

Using LC–MS, we provide for the first time direct and non-pharmacologically-based evidence supporting the hypotheses that the two eCB ligands, AEA and 2-AG, are endogenously present in the LDT. Our conclusions are based on the generation of mass spectra and chromatograms from LDT tissue, which identified major break down products identical to those of the AEA and 2-AG reference standards, and those reported in literature (Lehtonen et al., 2008). Our previous electrophysiology-based studies suggested the presence of functional CB1Rs within the LDT and endogenous activation of these receptors by an agonist (Soni et al., 2014; Soni and Kohlmeier, 2016). Our present study takes knowledge of eCB transmission in the LDT further by explicitly identifying the nature of the CB1R agonists present in this nucleus.

In other regions where it has been studied, presence of both CB1R agonists examined in this study has been noted and their levels have exhibited a dynamism determined in part by behavioral and circadian states (Vaughn et al., 2010). While the necessity for two molecules acting at the same receptor is not well understood, it is believed that differences in their metabolism, and in their pharmacology, allow a very fine control over neuronal functioning. Given its slower mobilization, relatively shorter half-life and partial agonism of the CB1R (Felder et al., 1993; Fride and Mechoulam, 1993), AEA is thought to function as a tonic eCB signal (Alger, 2012; Ohno-Shosaku and Kano, 2014), although data has emerged that it can be involved in phasic events as well (Lee et al., 2015; Ohno-Shosaku and Kano, 2014). Regardless, as it may be a regulator of tonic transmission, AEA may have been responsible for the presence of AM251-sensitive tonic inhibition directed to cholinergic LDT neurons noted in our earlier electrophysiological work (Soni et al., 2014; Soni and Kohlmeier, 2016). 2-AG, which relies on rises in calcium for its production, is a full agonist at the CB1R (Savinainen et al., 2001; Stella et al., 1997; Sugiura et al., 1997), and is present at relatively high levels in the CNS. It is thought to underlie phasic activity directed to neurons, which is substantiated by several functional studies (Azad et al., 2004; Cachope, 2012; Castillo et al., 2012; Kim and Alger, 2010). In our earlier work, pharmacological stimulation of LDT neurons resulted in rises in calcium and changes in phasic synaptic activity, which may have resulted from the calcium-dependent production of 2-AG (Soni and Kohlmeier, 2016).

Presence of eCBs in the LDT indicates these lipids could act in concert with the other neuromodulators sourcing from the LDT, which have been shown or suggested to play a role in arousal. Cholinergic LDT neurons are major players in the reticular activating system where they have been shown to modulate states of arousal and sleep by release of their signature neurotransmitter, ACh. The state-dependent release of ACh from these cells to thalamic targets

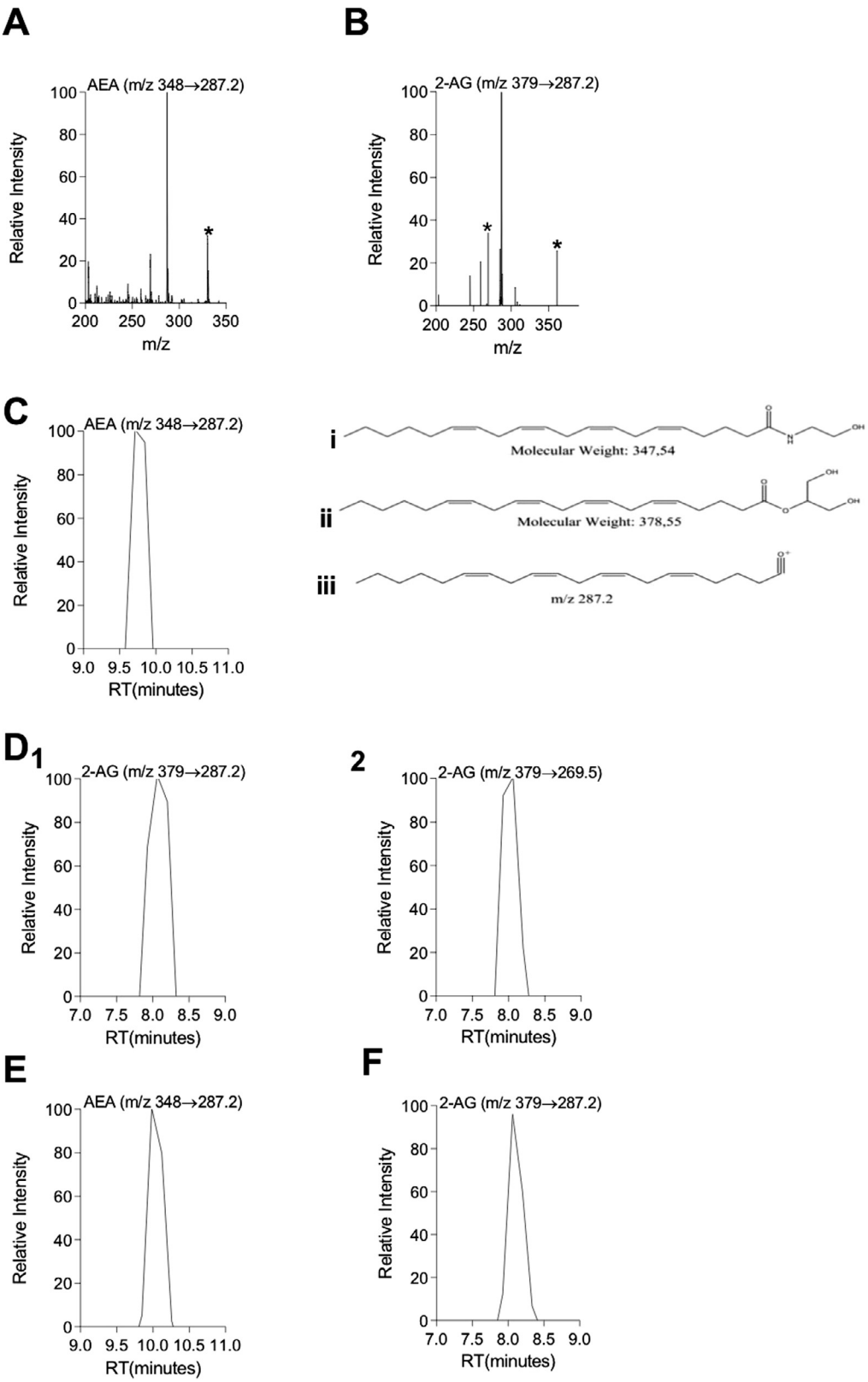

Fig. 1. Mass spectra and chromatographs indicate presence of AEA and 2-AG in the LDT. A) Mass spectrum of 200 μM of an AEA standard indicating a major peak at *m*/*z* 287.2, representative of the major product ion. (Asterisk) A minor peak is noted at *m*/*z* 330.2, likely representative of loss of water. B) Mass spectrum of a 2-AG standard (200 μM) denoting a major peak at *m*/*z* of 287.2, representative of the major product ion, and more minor peaks at *m*/*z* 269.5 and *m*/*z* 361.2 (asterisks), putatively reflective of loss of water. Insert: i. Structure of AEA (MW 347.55) and ii. 2-AG (MW 378.55) and its major break down product iii. *m*/*z* 287.2 (modified from, Lehtonen et al. (2008)). C) Representative mass chromatogram (SRM mode) with *m*/*z* of 287.2 for the detection of AEA from LDT samples. D1) Representative mass chromatogram (SRM mode) with *m*/*z* of 287.2 for the detection of 2-AG from LDT samples. D2) Representative mass chromatogram (SRM mode) with *m*/*z* of 269.5 for the detection of a minor breakdown product of 2-AG from LDT samples. E) Representative mass chromatogram (SRM mode) with *m*/*z* of 287.2 for the detection of AEA from cerebellum, which served as control tissue. F) Representative mass chromatogram (SRM mode) with *m*/*z* of 287.2 for the detection of 2-AG from cerebellum samples.

is believed to be responsible for gamma band activity underlying alert, aroused states and the desynchronized EEG activity characteristic of REM sleep (Moruzzi and Magoun, 1995; Boucetta et al., 2014; el Mansari et al., 1989; Thakkar et al., 1998; Williams et al., 1994; Steriade and Glenn, 1982; Steriade et al., 1993; Steriade, 1996; reviewed by Saper et al. (2001)), and the eCB system could play a role in controlling this release as has been shown in other brain regions such as the hippocampus (Gifford and Ashby, 1996; Gessa et al., 1997).

In addition to enzymes for production of ACh, cholinergic LDT neurons contain high levels of neuronal nitric oxide synthase (nNOS), which has been shown to add production and release of nitric oxide (NO) to the signaling repertoire of these cells (Vincent et al., 1983; Vincent and Kimura, 1992). Presence of NOS in these cells has led to the suggestion that NO plays a role in state control, and consistent with this interpretation, rises in NO have been detected in LDT targets within the thalamus during aroused states and during REM sleep (Williams et al., 1997). When taken together with data showing that NO can be detected in the LDT when cholinergic LDT neurons were electrically or chemically activated (Leonard et al., 2001), it has been suggested that NO may modulate synaptic transmission or alter cellular excitability (Garthwaite and Boulton, 1995) during the states of cortical arousal and REM sleep, when the firing of cholinergic LDT neurons is highest (Boucetta et al., 2014; el Mansari et al., 1989; Saper et al., 2001; Thakkar et al., 1998). Interestingly, in other brain regions where it has been studied, eCBR agonists stimulate the production and release of NO by a CB1R-mediated, nNOS-dependent mechanism (Fimiani et al., 1999). In the electrophysiological studies in which NO was stimulated within the LDT (Leonard et al., 2001), it was not determined whether CB1Rs played a role in the activity-dependent rises in NO, in part, because knowledge of the presence of the eCB system in the LDT was lacking. While early studies revealed the presence of mRNA for CB1Rs in many brain regions, including the cortex and hypothalamus, they failed to detect mRNA for the CB1R in the human brainstem (Mailleux et al., 1992). However, follow-up studies in the rat brain revealed a wider-distributed presence of CB1R, including within the pons, (Mailleux and Vanderhaeghen, 1992; Matsuda et al., 1993; for review, see Howlett et al., 2002). Low levels of mRNA for the CB1R were noted in the LDT, however, data were not shown, and this point was only briefly presented in the discussion making it difficult to evaluate (Matsuda et al., 1993). When our current LC–MS data is taken together with our previously-reported electrophysiological findings which indicated that functional CB1Rs exist in the LDT (Soni et al., 2014; Soni and Kohlmeier, 2016), we are tempted to speculate that eCBs interact with other signaling systems in the LDT, as has been seen in other brain regions. Therefore, it would be interesting to examine if endogenous eCBs interact with ACh and NO-based signaling, and if such an interaction influences the control the LDT plays in arousal and REM sleep.

Based on the LC–MS results, we attempted to characterise the spatial distribution of both AEA and 2-AG using MSI, which allows molecular localization of products in flat tissue sections. Our inability to detect the lipids was likely due to several suboptimal analysis conditions imposed by this technique. MSI requires very long scan times of the sections precluding the tissue from remaining frozen and allowing solvents to evaporate, which likely allows the lipids to degrade. Further, MSI requires quite thin tissue slices to be utilized (≈10-μm thick), which means while lipids with a high abundance may be detected, detection of lipid products with a low expression, such as AEA and 2-AG, can be challenging (Murphy et al., 2009). Development of MSI technology is ongoing and improvements should be possible which are likely to allow suitable strategies in the future to characterise the spatial distribution of eCBs within the LDT (Harkewicz and Dennis, 2011).

Our study does contain several caveats, which suggest some caution in interpretation of our data. We used mice that were restricted to the age range of 15–20 days, which was a range of ages selected in order to parallel the range used in our previous eCB electrophysiological studies. However, it is possible that expression of AEA and 2-AG varies across ontogeny in the LDT, and therefore, it remains to be determined whether younger, or older mice also express these lipids in the LDT. Another caveat of our study is that while we did verify the detection sensitivity of our LC–MS technology and proper handling of brain tissue by confirmation of detection of presence of lipids in the cerebellum, we did not examine another brain region for absence of AEA or 2-AG. Further, MSI studies, which would have examined areas surrounding the LDT, were not successful. Therefore, we can't rule out that other brain areas would have shown similar presence of AEA and/or 2-AG, nor can we provide negative data to indicate any presence of nuclei specificity. Regardless, while we could not show regional specificity, and presence of these lipids could be due to diffusion (Castillo et al., 2012), our LC–MS data do indicate AEA and 2-AG are present in the LDT, and therefore we believe these lipids can act at LDT-located targets. Finally, while a circadian pattern of release of these lipids has been reported in other regions (Vaughn et al., 2010), our data do not test whether circadian differences in presence of AEA and 2-AG in the LDT exist. All of our brain samples were collected at the same time in the morning, during the rest phase of the nocturnally-active mouse, which is the circadian time of the lowest degree of arousal. Determination of whether variability across the circadian cycle exists would be very interesting in order to evaluate whether eCBs, possibly in interactions with other transmitter systems, play a role in control of behavioral states which exhibit a circadian pattern.

Now that distinctly different methodological approaches, analytical chemistry (this report) and pharmacologically-based electrophysiology (Soni et al., 2014; Soni and Kohlmeier, 2016), have been utilized to collect data which strongly support the conclusion that there is presence within the LDT of endogenously-produced eCBS and functional CB1Rs, future studies should be directed at elucidating the role in behavioral state control played by AEA and 2-AG in the LDT and the mechanisms involved in that control. While use of selective CB1R agonists or antagonists has proved problematic, pharmacologic targeting of the eCB system by altering metabolic enzymes in other brain regions has been suggested to be a potentially realistic strategy (Freund et al., 2003; Koppel and Davies, 2008; Stella et al., 1997; Walter and Stella, 2004), and therefore, compounds which alter the production or catabolism of eCBs within the LDT could be a viable way to manage arousal-based disorders.

4. Methods and materials

4.1. Brain sample preparation

All animal studies complied with the European Communities Council Directive of 24 November 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. Brain samples were taken from 15 to 20 days old NMRI mice (Harlan Mice laboratories, Denmark) that were housed in a temperature-controlled room on a 12:12 light and dark schedule (lights on 7:00–19:00). 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.

4.2. Isolation of LDT

15–20 days old NMRI mice (n = 6) (Harlan Mice laboratories, Denmark) were anesthetized with 100% isofluorane and once reflex to a pinch of the paw was absent, the mice were rapidly decapitated. Whole brains were isolated, and quickly put into dry ice to rapidly cool. After freezing 20 min in dry ice, brains were placed on tissue paper and coronal sections of ~1 mm in thickness with a total size of ~1–2 mm^2 were made using a fine surgical blade. The laterodorsal tegmental nucleus (LDT) was visualized from coronal brainstem sections located by known anatomical markers used similarly in electrophysiology experiments focused on eCB functionality (Soni et al., 2014). The LDT was isolated bilaterally with caution being taken to reduce extraction of nearby regions. Two different batches were prepared. One batch was immediately used for the extraction of AEA and 2-AG and the second batch of LDT brain tissue was stored at −80 °C until further processing. Each isolated LDT was divided into 3 samples in order to monitor the efficiency of lipid extraction. As the cerebellum is a demonstrated robust source of AEA and 2-AG, cerebellar tissue was also harvested from two other animals in which ~2 mm^2 of the posterior lobe was collected and prepared as above to serve as control for proper tissue handling and correct operation of the LC–MS devices.

4.3. Extraction of Anandamide (AEA) and 2-Arachidononyl glycerol (2-AG)

Examination of whether AEA and 2-AG could be extracted from the mice brain tissues was conducted using a well established, protein precipitation protocol (Liput et al., 2014). Historically, long analyses times have posed difficulties in detecting eCBs using MS and therefore much effort has been exerted to decrease the analysis time, as reduced analysis time can improve the stability of lipids in the auto sampler (Chen et al., 2009). Accordingly, we took care to ensure that AEA and 2-AG from LDT samples were extracted immediately after the mice brain was sliced. Utmost care was taken with handling of the samples, including use of light-sensitive Eppendorf tubes for extraction and storage, and protecting of the LC–MS vials with aluminum foil for LC–MS analysis (Zoerner et al., 2011).

Samples were weighed out as frozen tissue. A mixture of 500 μl of PBS and pre-chilled acetonitrile was added to the fresh tissue sample in a 2 ml Eppendorf tube which was briefly vortexed (2–3 s). Tissue samples were then homogenized by sonication (Bandelin, Germany, UW 2070) at intensity of 50% in 0.5 s bursts for 30 s on and 30 s off for a total of 10 cycles. The homogenate was then centrifuged at 15000*g* for 20 min at 4 °C. The supernatant was removed and analyzed by mass spectrometry. Samples were kept on dry ice at all times and extraction was carried out under light-sensitive conditions. (Zoerner et al., 2011). Samples were thawed only once and discarded immediately after the analysis.

4.4. LC–MS analysis

HP-LC was performed on an Agilent 1100 series coupled to an Esquire 3000 ion trap mass spectrometer. Degassed mobile phases A and B contained 0.5% HCOOH in water and 80% Acetonitrile. An amount of 20 μL of sample was injected into the LC and separated on an Aeris peptide XB C-18 (phenomenex) column (3.6 μm, 2.1 × 100 mm, column temperature 40 °C). A linear acetonitrile gradient of 10–90% in 8 min was used to separate lipids with a total runtime of 12 min and at a flow rate of 0.2 mL/min. Electro spray ionization (positive mode) was utilized, and the ion trap was filled with 50,000 ions at a speed of 13,000 m/z per second. The scan range was set at 50–500 m/z (mass-to-charge ratio). The SRM method for AEA (m/z 348 – m/z 287.2) and 2-AG (m/z 379 – m/z 287.2) was followed. Each sample was run on two different SRM methods to detect the presence of AEA and 2-AG. The data analysis was performed with Bruker Daltonics 5.3 software. Only the intensities with a signal to noise ratio >1.5 were used. The extracted ion chromatograms of peaks of interest were exported to file format .cdf and processed in MATLAB. All the chromatograms were exported to excel and figures were plotted using graph pad prism 7.0

4.5. Drugs

HPLC grade acetonitrile was used for sample extraction. Standards for AEA, and 2-AG, (Tocris biosciences) were utilized. Sterile phosphate buffered saline (PBS) was obtained from Life Technologies.

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All of the authors declare they have no conflicts of interest, financial or otherwise, regarding this manuscript.

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