ELSEVIER

Prostaglandins & other Lipid Mediators 77 (2005) 35–45

PROSTAGLANDINS & other LIPID MEDIATORS

Review

Targeted lipidomics: fatty acid amides and pain modulation

J. Michael Walker[a,b,*], Jocelyn F. Krey[b], Jay S. Chen[b], Eivind Vefring[c], Jan A. Jahnsen[c], Heather Bradshaw[a,b], Susan M. Huang[b]

[a] *Department of Psychology, Indiana University, 1101 E. 10th St., Bloomington, IN 47405-7007, USA*
[b] *Departments of Psychology and Neuroscience, Brown University, 89 Waterman St. Providence, RI 02912, USA*
[c] *Department of Pharmacology, The Danish University of Pharmaceutical Sciences, Universitetsparken 2, DK 2100, Copenhagen Ø, Denmark*

Received 31 August 2004; accepted 14 September 2004
Available online 24 November 2004

Abstract

Mass spectrometric approaches to the identification and quantification of lipid signalling molecules are reviewed. Fatty acid amides are an important new class of lipid signalling molecules which include oleamide, the endocannabinoid anandamide, the endovanilloid/endocannabinoid *N*-arachidonoyldopamine (NADA) and the endovanilloid *N*-oleoyldopamine (OLDA) among many others. This diverse group of endogenous compounds comprises combinations of acyl backbones coupled by an amide bond to any of a variety of different small polar molecules such as ethanolamine, various amino acids, and catecholamines. Many fatty acid amides appear to play a role in pain and inflammation. Targeted lipidomics of fatty acid amides aims to identify new members of this diverse class of compounds, of which only a few representative molecules have been characterized to date. This effort has been made feasible by advances in chromatography and mass spectrometry, which permits: (1) identification of compounds present in complex mixtures, (2) astronomical increases in sensitivity due to miniaturization of HPLC components,

* Corresponding author. Tel.: +1 812 855 4559; fax: +1 812 855 4691.
E-mail address: walkerjm@indiana.edu (J.M. Walker).

1098-8823/$ – see front matter
doi:10.1016/j.prostaglandins.2004.09.017

and (3) novel scanning modes that permit the identification of compounds exhibiting similar structural components. Insofar as lipid signalling molecules such as prostanoids, leukotrienes and endocannabinoids operate via G-protein coupled receptors (GPCR), it appears likely that many of the numerous lipids awaiting identification may serve as ligands for any of the greater than 150 orphan GPCRs.

Keywords: Mass spectrometry; Fatty acid amide; Lipid; Lipidomics; *N*-Arachidonoyldopamine; *N*-Oleoyldopamine; HPLC

Contents

1. Introduction 36
2. Fatty acid amides as signalling molecules 36
3. Fatty acid amides and pain 37
4. Advances in mass spectrometry enable rapid identification of signalling lipids 38
5. Identification of novel compounds from complex mixtures 38
6. Increased sensitivity by HPLC miniaturization 38
7. Identification of lipids with common features by novel scanning modes 43
8. Summary and conclusions: targeted lipidomics and pain therapeutics 43
References 44

1. Introduction

This review discusses new approaches used for the identification of signalling lipids, with a focus on fatty acid amides. Fatty acid amides (FAAs) are increasingly recognized as an important class of signalling molecules (reviewed by [1]). Examples include ceramides, glycosphingolipids, *N*-acylated lipids, and amides of fatty acids and primary amines (such as ethanolamine, amino acids and catecholamines). Recently, much attention has been given to the latter class of compounds. The origins of this research can be traced to 1957 when Kehul et al. [2] identified *N*-palmitoylethanolamine as an anti-inflammatory factor present in egg yolk, soybeans, and peanuts. Renewed interest in this and similar *N*-acylethanolamines arose with the discovery of *N*-arachidonoylethanolamine (anandamide [3]), an endogenous compound that binds to cannabinoid receptors with high affinity and vanilloid receptors with low affinity [4]. Another important member of this class is oleamide, a FAA that accumulates in the cerebrospinal fluid of sleepy cats [5].

2. Fatty acid amides as signalling molecules

At one time, various criteria for the identification of a neurotransmitter were dogmatically enumerated in textbooks, but as the complexity of signalling became apparent, most of these criteria have gone by the wayside. For example, we know that lipid signalling molecules are

not stored in vesicles; that endocannabinoid signalling may operate in the 'wrong' direction, i.e. release of the endocannabinoid from postsynaptic elements acting on the presynaptic terminals [6,7]; and some lipids (e.g. sphingosine-1-phosphate) serve both trans-cellular and second messenger functions (reviewed by [8]). These aspects of lipid signalling have added important new dimensions to how we think about neuronal and immune signalling in general.

Much of our understanding of FAA signalling derives from studies of anandamide. Evidence for signalling by endocannabinoids may be found in experiments with cultured cells, which exhibit increased membrane levels of anandamide following stimulation with calcium ionophores (reviewed by [4]). Perhaps the best evidence to date that anandamide is released from neurons stems from two in vivo microdialysis studies. Giuffrida et al. [9] found increased levels of anandamide in striatal dialysates following depolarization with KCl. We found increased extracellular levels of anandamide in the periaqueductal gray (PAG) following either electrical stimulation of the PAG or injection of a chemical irritant (formalin) in the hindpaws [10]. It is important to note that others, including Richardson et al. [11], Strangman et al. [12], Wilson and Nicoll [6], and Kreitzer and Regehr [7], have made a strong case for the existence of endocannabinoid signalling using receptor antagonists, although this type of experiment does not identify the particular molecule(s) involved.

3. Fatty acid amides and pain

It is perhaps unsurprising that many of the recently discovered FAAs affect pain and inflammation, since many of the compounds are derived from arachidonic acid, a key metabolic precursor for numerous pro-inflammatory/pro-nociceptive compounds including the prostanoids, and leukotrienes. The evidence that endocannabinoids function naturally to suppress pain has been reviewed [13]. Other FAAs also suppress pain. *N*-Arachidonoylglycine (NAGly), the first compound identified in swhat appears to be a large family of lipoamino acids, suppresses edema produced by arachidonic acid, suppresses pain behavior induced by intradermal injections of dilute formalin, and inhibits nuclear factor κB [14]. A similar suppression of pain is produced by *N*-arachidonoyl-γ-amino-butyric acid (NAGABA). By contrast, the endogenous FAAs *N*-arachidonoyldopamine (NADA) and *N*-oleoyldopamine (OLDA) possess activity at capsaicin (TRPV1) receptors with a potency similar to that of capsaicin, and produce thermal hyperalgesia upon intradermal injections in rats [15–17].

An important current direction in the study of FAAs stems from the recognition that some FAAs that contain arachidonic acid, are susceptible to metabolism by cyclooxygenase-2 (COX2), the inducible form of COX. Anandamide and NAGly are converted by COX2 to prostaglandin E2 ethanolamide (PGE2E) and prostaglandin E2 glycine, respectively. Ross et al. [18] showed that PGE2E is active at prostaglandin receptors suggesting that COX2 converts anandamide to a pro-nociceptive prostanoid. An intriguing recent finding reported by Guhring et al. [19] is the blockade of the antinociceptive effects of the COX2 inhibitor indomethacin by the cannabinoid antagonist SR141716A. The most obvious interpretation of this finding is that non-steroidal anti-inflammatory agents produce their effects at least

in part by preventing the COX-mediated conversion of antinociceptive/anti-inflammatory endocannabinoids to pro-nociceptive/pro-inflammatory prostanoids.

4. Advances in mass spectrometry enable rapid identification of signalling lipids

While there is strong evidence for the existence of numerous FAAs in the brain and other tissues, only a few have been identified to date. This is because of the technical challenges historically associated with the chemical analysis of lipids. First, the traditional methods required purification to homogeneity prior to structural analysis. Second, the difficulties associated with purification of tissue extracts were magnified by the low abundance of lipid signalling molecules. Recent advances in mass spectrometry and liquid chromatography have greatly enhanced the enterprise of identifying signalling lipids. These advances have made possible the field of lipidomics, which aims to identify all endogenous lipids. Targeted lipidomics of signalling molecules promises to enhance the armamentarium of biologists seeking to identify novel targets for treating important diseases such as chronic pain, mental illness, and drug addiction. The advances that have led to the field of lipidomics are outlined below.

5. Identification of novel compounds from complex mixtures

Lipids obtained from tissue extracts were traditionally identified by purification to homogeneity followed by mass spectrometry and sometimes nuclear magnetic resonance. In recent times, the development of liquid chromatography combined with tandem mass spectrometry have made possible the identification of lipids in complex mixtures. Tandem mass spectrometers include triple quadrupole, ion trap, and quadrupole/time-of-flight instruments, among others. These instruments typically use quadrupole technology to isolate a compound based upon its molecular weight prior to collisional activation (fragmentation) and mass analysis of the fragmented components. This means that the mixture must be purified to the only to the point that the sample applied to the mass spectrometer is free of other compounds that have the same mass (typically within 1 amu). This can often be accomplished with a liquid–liquid extraction from the tissue followed by solid phase extraction methods (e.g. [14,15], Fig. 1). For FAAs, LC/MS/MS methods have used a reversed phase separation during analysis, which separates the desired compound from the interfering (similar mass) compounds. While quadrupole technology provides approximately 1 amu resolution, improved isolation within the mass spectrometer is now being accomplished using TOF/TOF instruments, which permits much finer resolution. To the authors' knowledge, these instruments have not been used for lipid analysis to date.

6. Increased sensitivity by HPLC miniaturization

A major advance in mass spectrometry that led to markedly increased sensitivity and simpler sample preparation for lipids was the development of electrospray ionization which

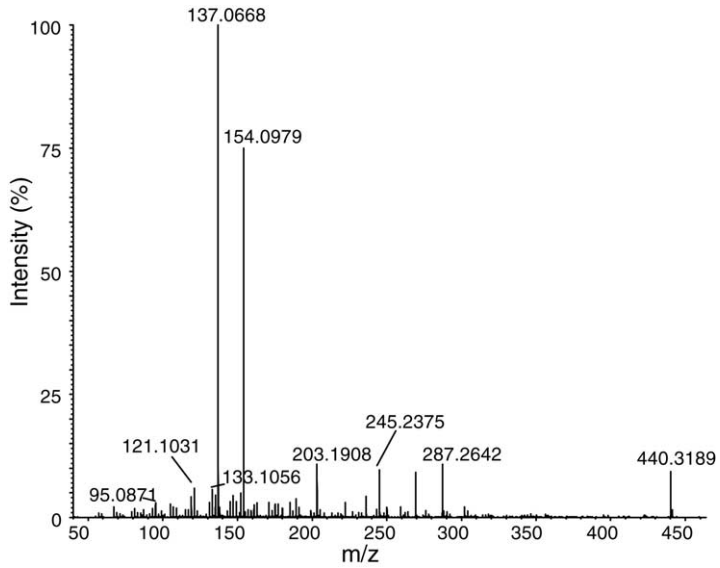

Fig. 1. Identification of *N*-arachidonoyldopamine (NADA) from a complex mixture. Following extraction in methanol, lipids from bovine striata were partially purified using phenylboronic acid and reversed phase solid phase cartridges and subjected to reversed phase LC/MS/MS using an Applied Bioystems/MDS Sciex quadrupole-time-of-flight mass spectrometer. As shown, analysis of material in the brain extract in positive ion mode yielded a mass estimate of 440.3189, which is within 4.6 ppm of the mass of $[NADA + H]^+$. Exact masses of fragment ions permitted structural reconstruction of NADA. Redrawn from Huang et al. [15].

permitted direct coupling of mass spectrometers to the effluent of an HPLC (see [20]). This then permitted the use of capillary HPLC, which enhances sensitivity by miniaturization of the HPLC components. By reducing the diameter of the HPLC column (to ∼75 μm) with a concomitant reduction of flowrate (∼200 nl/min), increases in sensitivity occur due to the greater concentration of the analyte. Theoretically, the increase in sensitivity that occurs is the square of the proportion of the column diameters. Hence, if 20 pmol is required to obtain a reasonable mass spectrum using a 2 mm column, the expected amount required using a 100 μm column would be a 50 fmol (a 400-fold decrease). These levels of sensitivity are routinely achieved in the field of proteomics, and are now being realized with lipid signalling molecules. An example from our laboratory is provided in Fig. 2, which shows the mass spectrum of *N*-palmitoyl-γ-aminobutyric acid (PALGABA), an endogenous FAA obtained from a rat brain extract partially purified using ion exchange, reversed phase, and normal phase extraction columns. Data from experiments conducted on a triple quadrupole mass spectrometer with this extract indicated that <100 fmol was applied to the column for this experiment. This increase in sensitivity permits identification of numerous low abundance lipids from one or a few rat brains.

HPLC miniaturization has also been applied to increase the sensitivity for quantification of known compounds. As shown in Fig. 3, quantification of the levels of the FAA OLDA, a potent endovanilloid [17], was markedly increased using nano-HPLC combined with multiple reactions monitoring (MRM) on a triple quadrupole mass spectrometer. Using this

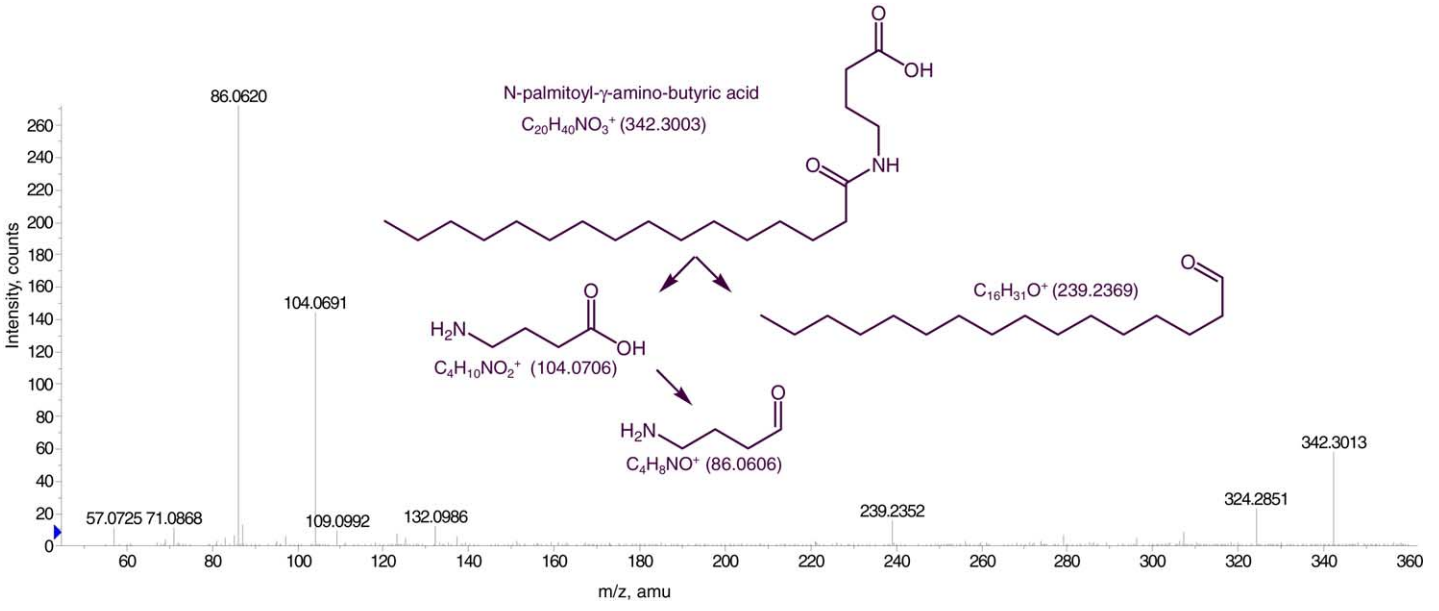

Fig. 2. Identification of *N*-palmitoyl-γ-amino-butyric acid (PALGABA) using capillary HPLC/nano-electrospray MS/MS. Lipoamino acids were extracted from rat brain using a method modified from that of Folch et al. [27] and purified by ion exchange, reversed phase, and normal phase extraction columns, concentrated, reconstituted and subjected to gradient capillary HPLC on a 75 μm C18 column flowing at 200 nl/min. The effluent was subjected to nano-electrospray on an Applied Biosystesms/MDS Sciex QStar quadrupole/time-of-flight mass spectrometer in product ion scan mode (filtering ions of 342 amu). The marked increase in sensitivity provided by these methods compared to small bore HPLC/electrospray provided the mass spectrum shown obtained from <100 fmol of PALGABA on column. The measured mass of the precursor ion was within 1.4 ppm of the exact mass of $[PALGABA + H]^+$. Mass estimates of the fragment ions allowed reconstruction of the molecule as PALGABA.

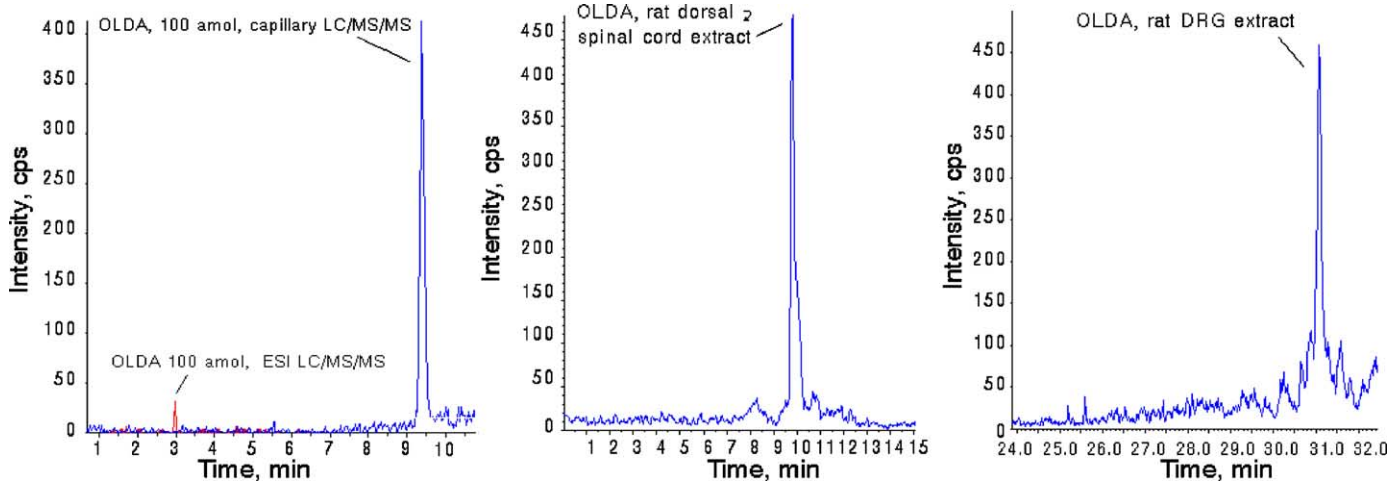

Fig. 3. Capillary HPLC/MS/MS permits marked increases in sensitivity for quantification of analyte levels in small tissue samples. Left panel: 100 amol of synthetic OLDA was applied to an Applied Biosystems/MDS Sciex triple quadrupole mass spectrometer either by standard narrowbore HPLC (2 mm column, 200 μl/min) or capillary HPLC (75 μm column, 200 nl/min). As shown, the detection limit (2:1, signal:noise) for capillary LC/MS/MS is approximately 10 amol. These methods permitted the detection of OLDA in dorsal spinal cord (middle panel) and an extract from the three dorsal root ganglia that subserve the primary afferent neurons of the sciatic nerve (approximate wet weight, 6 mg).

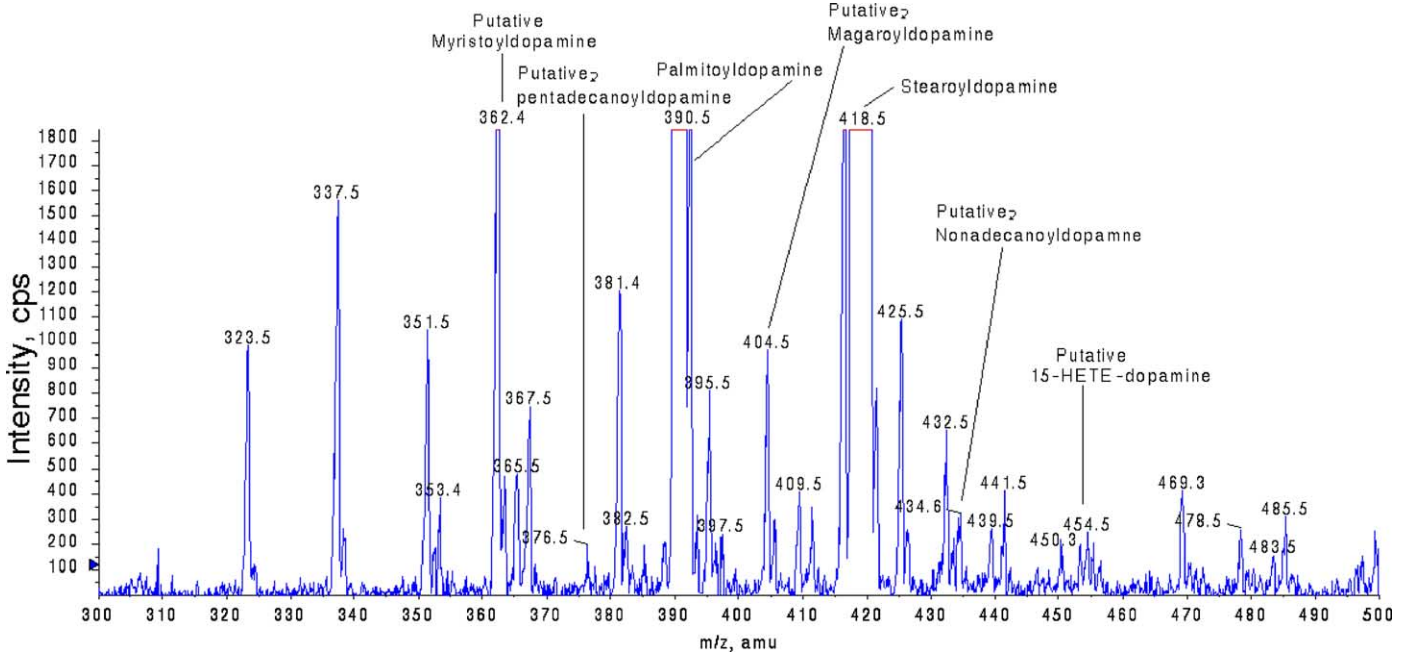

Fig. 4. Precursor ion scan of a rat striatal extract reveals the existence of novel acyldopamines. Using methods similar to those described in Figs. 2 and 3, an extract from striatal tissue obtained from a single rat (approximate wet weight, 50 mg) was subjected to analysis in precursor ion scanning mode on a triple quadrupole mass spectrometer. The spectrum provides masses of the precursors (parent masses) of ions that yield a fragment of 123 amu, the mass of a fragment of dopamine commonly produced by acyl-dopamides in the negative ion mode. As shown, the spectrum suggests the occurrence in striatum of novel dopamides of a variety of fatty acids.

method, it was possible to achieve low attomole detection limits and the measurement of OLDA in small tissue samples such as the rat dorsal root ganglion (wet weight ∼ 2 mg).

7. Identification of lipids with common features by novel scanning modes

Tandem mass spectrometry provides novel scanning modes that permit rapid examination of molecules that have common structural features. This is particularly important for studies of FAAs because they typically occur in families that contain a variety of different acyl chains and polar moieties. For example, shortly after the identification of the endocannabinoid anandamide, which is the ethanolamide of arachidonic acid, Hanus et al. [21] identified structurally related ethanolamides – dihomo-γ-linolenoylethanolamide (HEA; 20:3 (*n*-6)) and docosatetraenylethanolamide (DEA, 22:4 (*n*-6)). In fact, there are many different acyl-ethanolamides derived from *N*-acylethanolamine phosopholipids (NAPEs), of which anandamide represents a minute fraction [22]. This pattern has held for other FAAs, an example being the *N*-acyl-dopamine family for which the arachidonic, stearic, palmitic, and oleic dopamides have been identified [17]. Further study using a method called precursor ion scanning on a triple quadrupole mass spectrometer permitted tentative identification of many additional fatty acyl dopamides. In this experiment, a striatal extract was presented to the MS/MS following reversed phase capillary HPLC. The first (filtering quadrupole) scanned different masses, the third quadrupole was fixed to pass only ions of mass 123 amu, representative of a common negative ion fragment of acyl-dopamines. The mass spectrum provides the masses of the precursors (or parents in the old nomenclature) of the 123 amu dopamine fragment. As shown in Fig. 4, the scan provided evidence for several novel fatty acyl dopamides. This extract used for this experiment was obtained from the striata of a single rat (approximate wet weight, 50 mg). This type of scan provided the initial evidence for the existence of OLDA, PALDA, and STEARDA, which were identified using a variety of MS/MS methods including a derivatization of the dopamine moiety [17].

8. Summary and conclusions: targeted lipidomics and pain therapeutics

With its origins in the terms genomics and proteomics, lipidomics refers to research aimed at identifying the full complement of endogenous lipids. The research discussed here may be considered targeted lipidomics because it focuses on FAAs involved in pain, rather than the indiscriminate identification of all endogenous lipids. Targeted lipidomics of signalling molecules is important because only a fraction of endogenous signalling lipids have been identified and characterized. Note for example that of the >250 known G-protein coupled receptors (GPCRs), >150 are orphan receptors, and lipids are likely to be endogenous ligands for many of these orphans [23]. These observations highlight the need to identify heretofore unknown signalling lipids, an undertaking of special significance to pain research in light of the pro- and antinociceptive actions of known signalling lipids (e.g. lysophosphatidic acid, prostanoids, leukotrienes, endocannabinoids, and acyl-amino acids [13,14,24–26]).

Much current research attempts to understand pain by integrating knowledge of neural circuits and signalling events. The reductionistic approach explaining pain on the basis of neural circuits and their signalling molecules cannot be realized until we have identified all of the molecules that modulate pain signalling. Hence, the lack of information about the chemical identity of many signalling molecules and their associated molecular mechanisms represents a major roadblock to this effort. Progress in the chemical identification of lipid signalling molecules has lagged behind that of peptides and small polar neurotransmitters due to the difficulties traditionally associated with the chemistry of lipids—their hydrophobicity, rapid metabolism (often to numerous bioactive species), and difficulties with separation and identification. Advances in liquid chromatography/mass spectrometry have largely overcome these problems. These innovations gave rise to the field of lipidomics, which was not a feasible undertaking until recently. The identification and characterization of novel signalling lipids will not only lead to an increased understanding of pain but may also provide new targets for pain pharmacotherapy.

References

[1] Schmid HHO, Berdyshev EV. Cannabinoid receptor-inactive *N*-acylethanolamines and other fatty acid amides: metabolism and function. Prostaglandins Leukot Essent Fatty Acids 2002;66:363–76.
[2] Kehul Jr FA, Jacob TA, Ganley OH, Ormond RE, Meisinger MAP. The identification of *N*-2(hydroxyethly)-palmitamide as a naturally occurring anti-inflammatory agent. J Am Chem Soc 1957;79:5577–8.
[3] Devane WA, Hanus L, Breuer A, et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 1992;258:1946–9.
[4] Di Marzo V, DePetrocellis L, Fezza F, Ligresti A, Bisogno T. Anandamide receptors. Prostaglandins Leukot Essent Fatty Acids 2002;66:377–91.
[5] Cravatt BF, Prospero-Garcia O, Siuzdak G, et al. Chemical characterization of a family of brain lipids that induce sleep. Science 1995;268:1506–9.
[6] Wilson RI, Nicoll RA. Endocannabinoid signaling in the brain. Science 2002;29:678–82.
[7] Kreitzer AC, Regehr WG. Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells. Neuron 2001;29:717–27.
[8] Gilmour RS, Mitchell MD. Nuclear lipid signaling: novel role of eicosanoids. Exp Biol Med 2001;226:1–4.
[9] Giuffrida A, Parsons LH, Kerr TM, Rodriguez de Fonseca F, Navarro M, Piomelli D. Dopamine activation of endogenous cannabinoid signaling in dorsal striatum. Nat Neurosci 1999;2:358–63.
[10] Walker JM, Huang SM, Strangman NM, Tsou K, Sanudo-Pena MC. Pain modulation by release of the endogenous cannabinoid anandamide. Proc Natl Acad Sci USA 1999;96:12198–203.
[11] Richardson JD, Aanonsen L, Hargreaves KM. Hypoactivity of the spinal cannabinoid system results in NMDA-dependent hyperalgesia. J Neurosci 1998;18:451–7.
[12] Strangman NM, Patrick SL, Hohmann AG, Tsou K, Walker JM. Evidence for a role of endogenous cannabinoids in the modulation of acute and tonic pain sensitivity. Brain Res 1998;813:323–8.
[13] Walker JM, Krey JF, Chu CJ, Huang SM. Endocannabinoids and related fatty acid derivatives in pain modulation. Chem Phys Lipids 2002;121:159–72.
[14] Huang SM, Bisogno T, Petros TJ, et al. Identification of a new class of molecules, the arachidonyl amino acids, and characterization of one member that inhibits pain. J Biol Chem 2001;276:42639–44.
[15] Huang SM, Bisogno T, Marcello T, et al. An endogenous capsaicin-like substance with high potency at recombinant and native vanilloid VR1 receptors. Proc Natl Acad Sci USA 2002;99:8400–5.
[16] Bisogno T, Melck D, Bobtov MY, et al. *N*-acyl-dopamines: novel synthetic CB1 cannabinoid-receptor ligands and inhibitors of anandamide inactivation with cannabimimetic activity in vitro and in vivo. Biochem J 2000;351:817–24.
[17] Chu CJ, Huang SM, De Petrocellis L, et al. *N*-oleoyldopamine: a novel endogenous capsaicin-like lipid that produces hyperalgesia. J Biol Chem 2003;276:13633–9.

[18] Ross RA, Craib SJ, Stevenson LA, et al. Pharmacological characterization of the anandamide cyclooxygenase metabolite: prostaglandin E2 ethanolamide. J Pharmacol Exp Ther 2002;301:900–7.
[19] Guhring H, Hamza M, Sergejeva M, et al. A role for endocannabinoids in indomethacin-induced spinal antinociception. Eur J Pharmacol 2002;454:153–63.
[20] Cho A, Normile D. Nobel prize in chemistry. Mastering macromolecules. Science 2002;298:527–8.
[21] Hanus L, Gopher A, Almog S, Mechoulam R. Two new unsaturated fatty acid ethanolamides in brain that bind to the cannabinoid receptor. J Med Chem 1993;36:3032–4.
[22] Hansen HH, Hansen SH, Schousboe A, Hansen HS. Determination of the phospholipid precursor of anandamide and other *N*-acylethanolamine phospholipids before and after sodium azide-induced toxicity in cultured neocortical neurons. J Neurochem 2000;75:861–71.
[23] Kroeze WK, Sheffler DJ, Roth BL. G-protein-coupled receptors at a glance. J Cell Sci 2003;116:4867–9.
[24] Inoue M, Rashid MH, Fujita R, Contos JJ, Chun J, Ueda H. Initiation of neuropathic pain requires lysophosphatidic acid receptor signaling. Nat Med 2004;10:712–8.
[25] Lawrence T, Willoughby DA, Gilroy DW. Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nat Rev 2002;2:787–95.
[26] Bisgaard H, Kristensen JK. Leukotriene B4 produces hyperalgesia in humans. Prostaglandins 1985;30:791–7.
[27] Folch J, Lees M, Sloane SGH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497–507.