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161	Abstract	<p>The endogenous lipid agent <i>N</i>-arachidonylethanolamine (anandamide), among other effects, has been shown to be involved in nociceptive processing both in the central and peripheral nervous systems. Anandamide is thought to be synthesised by several enzymatic pathways both in a Ca^{2+}-sensitive and Ca^{2+}-insensitive manner, and rat primary sensory neurons produce anandamide. Here, we show for the first time, that cultured rat primary sensory neurons express at least four of the five known Ca^{2+}-insensitive enzymes implicated in the synthesis of anandamide, and that application of 1,2-dioleoyl-<i>sn</i>-glycero-3-phosphoethanolamine-<i>N</i>-arachidonoyl, the common substrate of the anandamide-synthesising pathways, results in anandamide production which is not changed by the removal of extracellular Ca^{2+}. We also show that anandamide, which has been synthesised in primary sensory neurons following the application of 1,2-dioleoyl-<i>sn</i>-glycero-3-phosphoethanolamine-<i>N</i>-arachidonoyl induces transient receptor potential vanilloid type 1 ion channel-mediated excitatory effect that is not inhibited by concomitant activation of the cannabinoid type 1 receptor. Finally, we show that sub-populations of transient receptor potential vanilloid type 1 ion channel-expressing primary sensory neurons also express some of the putative Ca^{2+}-insensitive anandamide-synthesising enzymes. Together, these findings indicate that anandamide synthesised by primary sensory neuron via a Ca^{2+}-insensitive manner has an excitatory rather than an inhibitory role in primary sensory neurons and that excitation is mediated predominantly through autocrine signalling. Regulation of the activity of the Ca^{2+}-insensitive anandamide-synthesising enzymes in these neurons may be</p>

capable of regulating the activity of these cells, with potential relevance to controlling nociceptive processing in these neurons.

162	Keywords separated by ' - '	TRPV1 - CB1 - Nociceptive processing - Pain
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163	Foot note information	A. Varga and A. Jenés contributed equally to this work. The online version of this article (doi:10.1007/s00424-013-1360-7) contains supplementary material, which is available to authorized users.
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Electronic supplementary material

ESM 1
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Anandamide produced by Ca^{2+} -insensitive enzymes induces excitation in primary sensory neurons

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Abstract The endogenous lipid agent *N*-arachidonoyl-ethanolamine (anandamide), among other effects, has been shown to be involved in nociceptive processing both in the central and peripheral nervous systems. Anandamide is thought to be synthesised by several enzymatic pathways both in a Ca^{2+} -sensitive and Ca^{2+} -insensitive manner, and rat primary sensory neurons produce anandamide. Here, we show for the first time, that cultured rat primary sensory neurons express at least four of the five known Ca^{2+} -insensitive enzymes implicated in the synthesis of anandamide, and that application of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-

arachidonoyl, the common substrate of the anandamide-synthesising pathways, results in anandamide production which is not changed by the removal of extracellular Ca^{2+} . We also show that anandamide, which has been synthesised in primary sensory neurons following the application of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-arachidonoyl induces transient receptor potential vanilloid type 1 ion channel-mediated excitatory effect that is not inhibited by concomitant activation of the cannabinoid type 1 receptor. Finally, we show that subpopulations of transient receptor potential vanilloid type 1 ion channel-expressing primary sensory neurons also express some

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 39 synthesised by primary sensory neuron via a Ca²⁺-insensitive
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47 **Keywords** TRPV1 · CB1 · Nociceptive processing · Pain

48 **Introduction**

49 *N*-Arachidonylethanolamine (anandamide) [7] is an endoge-
 50 nous lipid agent implicated in a variety of physiological
 51 functions and pathological processes both within, and outside,
 52 the nervous system [3, 21]. Although anandamide interacts
 53 with a variety of proteins [15], the majority of its actions are
 54 mediated via the cannabinoid type 1 (CB1) receptor and the
 55 transient receptor potential vanilloid type 1 ion channel
 56 (TRPV1) [6, 7, 9, 26, 51]. One of the most prominent func-
 57 tions of anandamide resides in its TRPV1- and CB1 receptor-
 58 mediated regulatory action in nociception in primary sensory
 59 neurons (PSN) [9].

60 Several cell types in various tissues, including PSN, pro-
 61 duce anandamide [5, 8, 10, 44, 45], the synthesis of which is
 62 believed to occur either in a Ca²⁺-sensitive, or Ca²⁺-insensi-
 63 tive manner [22, 23, 32, 36, 41, 43, 47, 48] and through
 64 several enzymatic pathways (Fig. 1). The molecular identities
 65 and biochemical activities of six enzymes implicated in the
 66 synthesis of various *N*-acylethanolamines (NAEA) including
 67 anandamide from corresponding *N*-acylphosphatidyl-etha-
 68 nolamines (NAPE) have now been established (Fig. 1) [22,
 69 23, 32, 36, 41, 43]. We have recently shown that one of these
 70 six enzymes, *N*-acylphosphatidylethanolamine-selective
 71 phospholipase D (NAPE-PLD; Fig. 1), which is the only
 72 known Ca²⁺-sensitive enzyme involved in anandamide syn-
 73 thesis [32, 43, 47, 48], is expressed by a sub-population of
 74 PSN [29]. However, the ability of PSN to produce ananda-
 75 mide in the absence of Ca²⁺ [45] indicates that, in addition to
 76 the Ca²⁺-sensitive pathway (Fig. 1), one or more Ca²⁺-insen-
 77 sitive pathways must also be present in these cells.

78 Anandamide synthesised in a Ca²⁺-sensitive manner in-
 79 duces excitation of PSN through the activation of TRPV1
 80 [44]. However, the effect of anandamide synthesised by
 81 Ca²⁺-insensitive enzymes acting on NAPE in PSN is not
 82 known. Therefore, after having first examined the expression
 83 and function of putative Ca²⁺-insensitive NAPE-converting
 84 enzymes, we also studied the effect of anandamide produced
 85 by these enzymes, in cultured rat PSN.

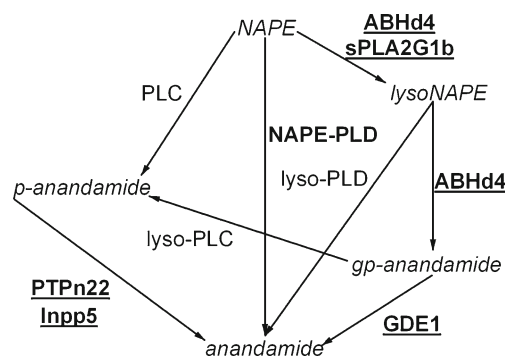


Fig. 1 Putative anandamide-synthesising enzymatic pathways. A schematic representation of the putative anandamide-synthesising enzymatic pathways. The molecular identity of six enzymes has been established so far (*bold*). While NAPE-PLD synthesises anandamide in a Ca²⁺-sensitive manner, other enzymes (*underlined*), the expression of which has been studied in these experiments, act in a Ca²⁺-insensitive manner. Substrates are shown in *italics*. NAPE-PLD *N*-acylphosphatidylethanolamine phospholipase D, GDE1 glycerophosphodiester phosphodiesterase 1, ABHd4 α/β -hydrolase 4, PTPn22 protein tyrosine phosphatase, non-receptor type 22, sPLA2G1b group 1b soluble phospholipase A₂, Inpp5 inositol 5'-phosphatase, NAPE *N*-acylphosphatidylethanolamine, PLC phospholipase C

96 **Materials and methods**

97 All experiments were performed in accordance with the UK
 98 Animals (Scientific Procedures) Act 1986, and the Directive
 99 2010/63/EU of the European Parliament and of the Council on
 100 the Protection of Animals Used for Scientific Purposes. Every
 101 effort was taken to minimize the number of animals used.
 102 Altogether, seventy eight 80–120 g male Sprague–Dawley
 103 rats, five wild type (C57BL/6 x129SvJ; WT) and six
 104 TRPV1^{-/-} mice (with C57BL/6 x129SvJ background; KO)
 105 were used.

106 **Drugs**

107 The following compounds were used: 1,2-dioleoyl-*sn*-
 108 glycerio-3-phosphoethanolamine-*N*-arachidonoyl (20:4-
 109 NAPE; Avanti Polar, dissolved in 1:1 ethanol/Tocrisolve
 110 [Tocris]); capsaicin (Tocris; dissolved in dimethyl sulphoxide
 111 [DMSO], Sigma); capsazepine (Tocris; dissolved in DMSO);
 112 (5Z,8Z,11Z,14Z)-5,8,11,14-eicosatetraenyl-methyl ester
 113 phosphonofluoric acid (MAFP; Tocris, dissolved in
 114 DMSO); rimonabant (Cayman Chemicals, dissolved in
 115 DMSO), mustard oil (Sigma, dissolved in DMSO).

116 Chloroform, in which 20:4-NAPE is delivered, was evap-
 117 orated in a N₂ atmosphere. Dried 20:4-NAPE was stored at
 118 -80 °C and re-dissolved in ethanol (100 mM) on the day of
 119 the experiment and stored at -20 °C for a maximum of a
 120 month. The final stock solution (50 mM) of 20:4-NAPE was
 121 prepared by adding equal volume of Tocrisolve to the 100 mM
 122 20:4 NAPE and vortexed. This final stock was used on the day
 123 of the preparation. The maximum final concentration of

114	Tocrisolve and ethanol were 0.05 %. The maximum final	Immunofluorescence staining	161
115	concentration of DMSO was also 0.05 %.		
116	Cultures of dorsal root ganglia	Cells attached to the coverslips were washed with PBS and	162
117	Dorsal root ganglion (DRG) cultures were prepared as de-	fixed by 4 % paraformaldehyde in 0.1 M PBS (pH 7.4).	163
118	scribed [31]. Briefly, DRG from the first cervical to the sixth	Permeabilisation (0.3 % Triton-X 100), and blocking (10 %	164
119	lumbar segments were collected in Ham's nutrient F12 culture	normal donkey serum) were followed by overnight incubation	165
120	medium (Sigma) supplemented with 2 % Ultrosor G	at 4 °C with primary antibodies at dilutions listed in Table 2,	166
121	(BioSeptra SA), 1 mM glutamine (Invitrogen), 50 IU/ml pen-	and visualisation with secondary antibodies. Coverslips were	167
122	icillin (Invitrogen) and 50 µg/ml streptomycin (Invitrogen).	mounted on glass slides with Vectashield containing DAPI	168
123	Following incubation in 2,000 U/ml collagenase type IV	(Vector Laboratories) and examined using a Leica DMR	169
124	(Worthington Biochemical Corporation) for 3 h, DRG were	Fluorescence microscope. Images were captured on a Hama-	170
125	trituated and the cells were plated on poly-DL-ornithine	matsu CCD camera using the QWin software package (Leica).	171
126	(Sigma)-coated glass coverslips. Cells were grown for 24–	On some images the brightness and contrast were digitally	172
127	48 h in the supplemented medium to which nerve growth	modified. For control, the primary antisera were omitted, or	173
128	factor (NGF, 50 ng/ml; Promega), was added. For Ca ²⁺ -im-	the immunoreactions were performed on sections of tissues in	174
129	aging experiments, after trituration, the cell suspension was	which the expression of the various anandamide-synthesising	175
130	centrifuged through 15 % bovine serum albumin (Sigma) and	enzymes had been shown previously. While no staining in	176
131	then also supplemented with 10 µM cytosine arabinoside	PSN neurons was seen when the primary antibodies were	177
132	(Sigma).	omitted, enzyme expression was seen in various tissues with	178
133	Isolation of total RNA	already demonstrated expression of the anandamide-	179
134	The total RNA was isolated using QIA shredder and RNeasy	synthesising enzymes (data not shown).	180
135	Mini or RNeasy Plus Mini Kits (Qiagen), as described [31].		
136	Reverse transcriptase polymerase chain reaction (RT-PCR)	Anandamide release	181
137	Extracted RNA (1 µg) was reverse-transcribed using Super-	Cultures were washed twice with HBSS–HEPES buffer (in	182
138	Script II cDNA synthesis reagents (Invitrogen). Primers	mM: NaCl 111, KCl 3, MgCl ₂ 0.49, CaCl ₂ 1.27, glucose 5.5,	183
139	designed to amplify rat glycerophosphodiester phosphodies-	MgSO ₄ 0.4, KH ₂ PO ₄ 0.44, Na ₂ HPO ₄ 0.33, HEPES 10, 100	184
140	terase 1 (GDE1, NM_198779.1), α/β-hydrolase 4 (ABHD4,	nM MAFP, pH 7.4) then incubated for 5 min in HBSS–	185
141	NM_001108866.1), protein tyrosine phosphatase, non-	HEPES buffer or HBSS–HEPES buffer containing 20:4-	186
142	receptor type 22 (PTPn22, NM_001106460.1), group 1b se-	NAPE (30 nM, 100 nM, 300 nM, 1 µM, 3 µM, 10 µM,	187
143	cretory phospholipase A ₂ (sPLA2G1b, NM_031585.1), ino-	30 µM, 100 µM). In some experiments, CaCl ₂ was omitted	188
144	sitol 5'-phosphatase (Inpp5, NM_019311.1) and the house-	from, and 10 mM ethylene glycol tetraacetic acid (EGTA,	189
145	keeping gene, glyceraldehyde-3-phosphate dehydrogenase	Sigma) was added to the buffer. All the washes and incuba-	190
146	(GAPDH, NC_005103), were purchased from Eurofins. The	tions were carried out at 37 °C unless stated otherwise.	191
147	sequences of the primers, annealing temperatures and the	The superfusate was collected on ice and either processed	192
148	predicted product sizes are shown in Table 1.	immediately for lipid extraction or stored at –80 °C for further	193
149	The PCR reaction mixture contained cDNA, forward and	processing. Cells were immediately scraped into ice-cold cell-	194
150	reverse primers, 1.5 mM MgCl ₂ , 1× Green Go-Taq Reaction	lysis buffer, used for protein quantification using BCA Protein	195
151	buffer (Promega), 0.2 mM deoxynucleotide mix (Promega)	Assay Reagent. For some anandamide measurements, only	196
152	and 1.25 U Go-Taq DNA polymerase (Promega). The ampli-	10 % of the cells were used for protein quantification. The	197
153	fication reaction consisted of 30 cycles with 0.5 min of dena-	remaining 90 % of the cells were stored in methanol, and then	198
154	turation at 95 °C, 1 min annealing at the given temperatures	added to the buffer for anandamide measurements.	199
155	(Table 1), and followed by 1 min final extension at 72 °C in		
156	a thermal cycler (Eppendorf-Mastercycler Personal;	Anandamide measurements	200
157	Eppendorf). For GAPDH, the amplification reaction consisted	The 20:4-NAPE application-induced anandamide synthesis was	201
158	of only 25 cycles. PCR products were separated on 2 %	measured using three slightly different methods (Fig. S1). For	202
159	agarose gels by electrophoresis and visualized with ethidium	protocol A (Fig. S1a), lipids were extracted from the buffer into	203
160	bromide.	ethyl acetate. Following evaporation of ethyl acetate, lipids were	204
		reconstituted in acetonitrile prior to quantification of ananda-	205
		amide by ultra-high performance liquid chromatography-	206
		electrospray-tandem mass spectrometry (UHPLC-ESI-MS/	207
		MS) using an Acquity UPLC BEH C ₁₈ (2.1×30 mm) column	208

Table 1 Sequences, annealing temperature and expected product size of primers used for RT-PCR

	Primer	Sequence	Temperature (°C)	Expected product size (bp)
t1.1	GDE1	F: TGCAGAAGGATTTGTCTCC	57	298
t1.2		R: TTGGGGTAAACTGTGGCTA		
t1.3	ABHd4	F: CAAAGCCATGATGGAGTCCT	57	244
t1.4		R: GATCTCCTCCACCACAGCAT		
t1.5	PTPn22	F: CATTGAAGACCCCAAGAAAA	61	202
t1.6		R: CAAGTTGATGGTGGGTTTCTT		
t1.7	sPLA2G1b	F: ACTGCTACAATCAGGCCAAG	57	292
t1.8		R: GTGCGGTGCAGAAATAAGAC		
	Inpp5	F: TACACACCTCTCACCCACCCA	57	160
		R: GCTGGTGAGGTTCTTCAAGC		
	GAPDH	F: ACCCATCACCATCTTCCA	57	380
		R: CATCACGCCACAGCTTTCC		

(Waters) with an Acquity UPLC BEH C₁₈ VanGuard Pre column (Waters) maintained at 60°C and a Shimadzu Nexera UHPLC (Shimadzu Scientific Instruments). Mobile phases comprised A (water containing 0.1 % formic acid) and B (acetonitrile containing 0.1 % formic acid) and the gradient conditions were: 0 min, 2 % B; 1.8 min, 95 % B; 2.3 min, 95 % B, 2.31 min, 2 % B then re-equilibrated at 2 % B until 3.0 min. Samples were maintained at 4°C throughout. Analytes were detected using tandem electrospray mass spectrometry in positive ion mode (API 4000, ABSciex) and multiple reaction monitoring with transitions of *m/z* 348.5>62.0 and *m/z* 348.5>91.052 for anandamide and *m/z* 260.420>116.200 for propranolol, used as internal standard (Fig. S1a). Anandamide

concentrations were derived from the relative response against authentic standards using Analyst 1.5.1 (ABSciex). The threshold of quantitation was 0.01 nM.

For protocol B (Fig. S1b), lipids were extracted from the superfusate using solid-phase extraction as described [19]. Briefly, superfusates, buffers and cell-free controls were supplemented with octa-deuterated anandamide (Cayman Chemicals, 2.5 pmol/ml) and loaded onto a pre-conditioned Oasis HLB 1 cm³, 30 mg cartridge (Waters). Cartridges were washed with 40 % aqueous methanol and then eluted with acetonitrile (1 ml, Fisher Scientific). Eluants were dried under constant N₂ stream and then re-suspended in acetonitrile (80 µl). Quantification of anandamide was done by UHPLC-ESI-MS/MS as described [25] using an Acquity UPLC BEH C₁₈ (2.1×50 mm) column (Waters) maintained at 40 °C and an Acquity UHPLC system (Waters). Mobile phases comprised A (2 mM ammonium acetate containing 0.1 % formic acid and 5 % acetonitrile) and B (acetonitrile containing 0.1 % formic acid) and the gradient conditions were as follows: 0–0.5 min, 20 % B; 2.5 min, 100 % B; 3.5 min, 20 % B then re-equilibrated at 20 % B until 4.0 min. Samples were maintained at 4 °C all throughout. Analytes were detected using tandem ESI-MS/MS in positive ion mode (Quattro Premier tandem mass spectrometer, Waters) and multiple reaction monitoring with transitions of *m/z* 348.25>61.85 and *m/z* 356.4>63.0 for anandamide and deuterated anandamide internal standard, respectively (Fig. S1b). Anandamide concentrations were derived from the relative response against authentic standards using Quanlynx (Waters). The threshold of quantitation was 0.005 nM.

Protocol C (Fig. S1c) was used in experiments in which the anandamide content of the superfusate and cells were measured together. Here, the samples were treated with 5 pmol octa-deuterated anandamide (Cayman Chemicals) in 1 vol. of chloroform/methanol (2:1 by vol.) and sonicated for 1 min in a sonication bath. The aqueous phase was then extracted twice with chloroform (1 vol.). The three, pooled organic phases were lyophilized and purified on silica mini columns as previously described [25]. The silica column fractions containing

Table 2 List of primary and secondary antibodies used for immunostaining

	Primary Antibodies (supplier)	Dilution of primary antibodies	Secondary antibodies and their dilutions
t2.1	GDE1 (Santa Cruz)	1:500	AF-568 donkey anti-rabbit IgG (1:1,000)
t2.2	ABHd4 (Santa Cruz)	1:200	AF-568 donkey anti-goat IgG (1:3,000)
t2.3	Inpp5 (Santa Cruz)	1:150	AF-568 donkey anti-mouse IgG (1:1,000)
t2.4	PTPn22 (ProteinTech Group)	1:500	AF-568 donkey anti-rabbit IgG (1:1,000)
t2.5	sPLA2G1b (Millipore)	1:150	AF-568 donkey anti-mouse IgG (1:1,000)
t2.6	NeuN (Millipore)	1:1,000	AF-488 donkey anti-mouse IgG (1:1,000)
t2.7	TRPV1 (different suppliers ^a)	1:2,000	AF-488 donkey anti-goat IgG (1:10,000)

^a Either guinea pig anti-TRPV1 antibody (NeuroMics, Edina, MN, USA) or rabbit anti-TRPV1 antibody was used. The latter one was a kind gift from Dr. Antonio Avelino (Institute of Histology and Embryology, Faculty of Medicine, University of Porto, Porto, Portugal) and tested intensively including on dorsal root ganglia of TRPV1^{-/-} mice

260	anandamide were analysed by liquid chromatography-	CaCl ₂ , 1; MgCl ₂ , 2; HEPES, 10; glucose, 10; pH 7.3) in a	307
261	atmospheric pressure-mass spectrometry (LC-APCI-MS)	laminar flow perfusion chamber (Warner Instrument Corpo-	308
262	conducted as described [25]. Analyses were carried out in the	ration). The following test solutions were applied subsequent-	309
263	selected ion-monitoring mode using <i>m/z</i> values of 356 and 348	ly: 20:4-NAPE (50 μM), capsaicin (1 μM) and KCl (50 mM)	310
264	(molecular ions +1 for deuterated and undeuterated ananda-	(timing is indicated in the corresponding figures). For study-	311
265	amide), using a Shimadzu HPLC apparatus (LC-10ADVP;	ing the involvement of TRPV1 or the CB1 receptor in the	312
266	Shimadzu Scientific Instruments) coupled to a Shimadzu	20:4-NAPE-evoked responses, either capsazepine (5 μM), or	313
267	LCMS-2020) quadrupole MS via a Shimadzu APCI interface	rimonabant (200 nM) was used to inhibit TRPV1 or the CB1	314
268	(Shimadzu Scientific Instruments; Fig. S1c). The threshold of	receptor, respectively. The antagonists were applied for 60 s	315
269	quantitation was 0.005 nM.	prior the application of 20:4-NAPE together with capsazepine	316
270	Cobalt uptake	or rimonabant. For control purposes, following the application	317
271	Cobalt uptake was assessed as described [24, 34, 38, 39].	of 20:4-NAPE together with capsazepine or rimonabant, the	318
272	Briefly, cells attached to the coverslips were washed in a buffer	antagonists were not removed from the bath and capsaicin	319
273	containing (in mM): NaCl 57.5, KCl 5, MgCl ₂ 2, HEPES 10,	with capsazepine or rimonabant was applied. Only KCl-	320
274	glucose 12, sucrose 139 (pH 7.4) then incubated in the presence	sensitive cells (neurons) were involved in subsequent analy-	321
275	cobalt (5 mM) put into the buffer with or without 20:4-NAPE	ses. At the end of some experiments, mustard oil (50 μM;	322
276	(100 nM, 1 μM, 10 μM, 100 μM), for 5 min at 37 °C. The	Sigma) or/and ionomycin (5 μM, Sigma) was also applied.	323
277	cobalt taken up by the cells was precipitated by 2.5 % β-	Application of drugs was controlled manually with one visual	324
278	mercaptoethanol (Sigma). Cells were then fixed in 70 % etha-	field being tested per coverslip. Experiments were performed	325
279	nol and the coverslips were mounted on glass slides with	at 37 °C, except where otherwise indicated.	326
280	glycerol. The mean gray value of more than 100 cells which	Images were captured with a Peltier element-cooled slow	327
281	were chosen randomly but systemically was established by a	scan charge-coupled camera system (PTI). Following subtrac-	328
282	Leica light microscope attached to a PC running the QWin	tion of the background fluorescence, the ratio of fluorescence	329
283	software package (Leica), and analysed with the ImageJ soft-	intensity at the two wavelengths as a function of time (rate 1	330
284	ware package (NIH, USA) as described [24, 34, 38, 39].	Hz) was automatically calculated ($R = F_{340}/F_{380}$) and the	331
285	Whole-cell voltage-clamp recordings	resultant graphs analysed using the ImageMaster 5.0 software	332
286	An Axopatch 200B amplifier and a Digidata 1200 digitizer	package (PTI) and the Microsoft Excel software package.	333
287	(Molecular Devices, UK) were used to record whole-cell	Only recordings with a stable baseline before 20:4-NAPE	334
288	currents from cultured PSN as described [31]. Borosilicate	application were included in the analyses. First, the noise	335
289	glass micropipettes (4–6 MΩ) were pulled on a DMZ puller	and baseline before the first drug application were established.	336
290	(DMZ), and filled with the following solution (concentrations	Then, the maximum amplitude of the 340/380 ratio was	337
291	in mM): NaCl 5, KCl 150, MgCl ₂ 2, HEPES 10, EGTA 1;	established in defined sections of recordings. Each of these	338
292	pH 7.4. The extracellular buffer contained (in mM): NaCl 150,	defined sections started at the beginning of a drug application	339
293	KCl 5, MgCl ₂ 2, CaCl ₂ 2, HEPES 10, glucose 10; pH 7.4. All	and lasted until the beginning of the next drug application. An	340
294	recordings were done at 37 °C and collected with the pClamp	increase of more than 10% in the 340/380 ratio was then	341
295	8 software package (Molecular Devices) with 1 kHz sampling	regarded as a response, if, during visual verification, the	342
296	rate and 5 kHz filtering. The holding potential was –60 mV.	change was clearly associated with the drug application.	343
297	Recordings were analysed offline by the ClampFit 8.0 soft-	Based on our pilot data, 2.5 times of the standard deviation	344
298	ware package (Molecular Devices).	of the amplitude of the baseline noise equals with a maximum	345
299	Ca ²⁺ imaging	of 9.5–10 % increase in the 340/380 ratio.	346
300	Cells were loaded with Fura-2 acetoxymethyl ester (Fura-2	Statistics	347
301	AM, 5 μM; Molecular Probes) in the presence of 2 mM	Data of repeated measurements were averaged. Differences	348
302	probenecid (Molecular Probes) for 60 min at 37 °C in a	between the averaged values were analysed by Student's <i>t</i> -test,	349
303	HEPES-buffered saline (in mM): NaCl, 122; KCl, 3.3; CaCl ₂ ,	one-way analysis of variance (ANOVA) or repeated measure	350
304	1.3; MgSO ₄ , 0.4; KH ₂ PO ₄ 1.2; HEPES, 25; glucose, 10;	multivariate ANOVA as appropriate. In ANOVA, following	351
305	adjusted with NaOH to pH 7.3. Coverslips were superfused	checking the normality of data, post hoc analysis for the statis-	352
306	with extracellular solution (in mM: NaCl, 160; KCl, 2.5;	tically significant differences among the treatment groups was	353
		performed by the Fischer's test. Significant differences in the	354
		proportion of neurons responding to various drugs were	355
		assessed by Fisher's exact test. Data are shown as <i>t</i> mean ±	356
		standard error of mean. A difference between two values was	357

358 considered to be significant if $p < 0.05$. The n values refer to the
 359 number of repeated experiments (number of cultures used for
 360 immunohistochemistry, anandamide release measurements and
 361 cobalt uptake, and the number of cells measured in whole-cell
 362 recordings and calcium imaging) and p values are given as
 363 < 0.0001 if the actual value was smaller than that.

364 **Results**

365 Several Ca^{2+} -insensitive enzymes, which are implicated
 366 in anandamide synthesis, are expressed in rat cultured primary
 367 sensory neurons

368 RT-PCR analysis showed gene expression for all five Ca^{2+} -
 369 insensitive enzymes that are known to be implicated in the
 370 synthesis of NAEA, including anandamide, from the corre-
 371 sponding NAPEs in cultures prepared from rat DRG (Figs. 1
 372 and 2a). In order to study the expression of the enzymes at a
 373 cellular level, immunostaining was performed on cultured rat
 374 PSN using antibodies raised against the enzymes together with
 375 an anti-NeuN-antibody, which identifies neurons. By analysing
 376 the staining in at least 100 neurons in each culture, we found
 377 that sub-populations of PSN express ABHD4, GDE1, Inpp5,
 378 and PTPn22 (Fig. 2b; for the proportions of cells expressing
 379 these enzymes please see Table 3). The analysis also revealed
 380 that while only neurons express ABHD4, Inpp5 and PTPn22,
 381 neurons and some non-neuronal cells express GDE1 (Fig. 2b).

382 The antibody raised against sPLA2G1b did not produce
 383 staining in the cultures. However, the same antibody produced
 384 staining in the pancreas (data not shown). The anti-ABHD4-,
 385 anti-Inpp5-, anti-PTPn22- and anti-GDE1 antibodies also pro-
 386 duced staining in tissues in which the expression of these
 387 enzymes have been reported previously (data not shown).
 388 When the primary antibodies were replaced with normal
 389 serum, no staining was seen either in cultured PSN or in any
 390 tissues which we processed (data not shown).

391 Application of 20:4-NAPE induces anandamide production
 392 in cultured PSN

393 20:4-NAPE is the substrate for anandamide synthesis used by
 394 all the known enzymatic pathways (Fig. 1) [20, 22, 23, 32, 36,
 395 37, 41, 43]. Therefore, to ascertain whether the enzymes found
 396 to be expressed in cultured rat PSN form any functional
 397 anandamide-synthesising pathways, the level of 20:4-NAPE
 398 application-induced anandamide synthesis was measured.

399 Exposure of the cells to 100 μ M 20:4-NAPE significantly
 400 increased the anandamide content of the buffer from a level
 401 below the quantitation threshold in the control to 8.86 ± 3.44
 402 pmol/ml ($n=4$; Student's t -test, $p=0.01$; Protocol B) at 37°C.
 403 After normalisation to the protein content, the anandamide
 404 concentration was 13.25 ± 4.39 ng/mg protein ($n=4$; Fig. 3a).

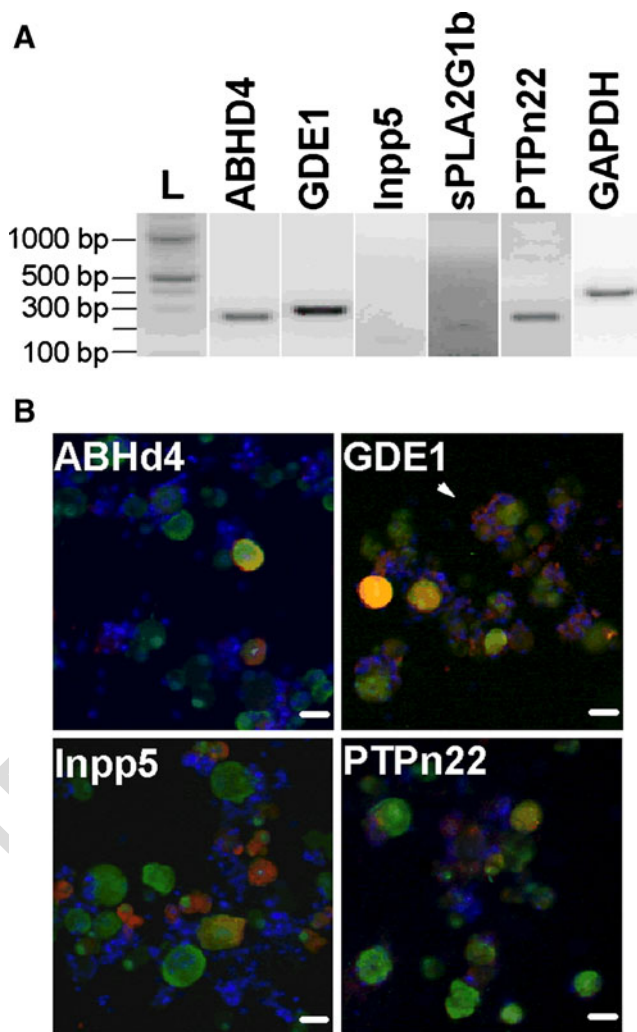


Fig. 2 Expression of enzymes implicated in Ca^{2+} -insensitive anandamide synthesis in cultured rat primary sensory neurons. **a** Representative gel images of reverse transcriptase polymerase chain reaction products. The products synthesised by specific primers to cDNA prepared by reverse transcribing the total RNA isolated from cultured PSN. While mRNA of all the five known Ca^{2+} -insensitive enzymes are expressed in rat cultured PSN, the level of their expression varies; GDE1 shows the highest level of expression (please see also Fig. S2), while PTPn22 shows the lowest level of expression. L indicates ladder. The predicted size of the PCR products is shown in Table 1. **b** Representative images of cultured PSN immunostained with antibodies raised against the putative Ca^{2+} -insensitive anandamide-synthesising enzymes with known molecular identity (red) in combination with an antibody which selectively and specifically recognises neurons (NeuN; green). The mounting medium contains DAPI (blue). The anti-ABHD4, anti-GDE1, anti-Inpp5 and anti-PTPn22 antibodies produce staining in various sub-populations of cells. The anti-sPLA2G1b antibody does not produce staining. The anti-GDE1 antibody, in addition to neurons, also stains some non-neuronal cells (arrow). Quantitative analysis of the immunostaining is shown in Table 3. Bars=25 μ m

405 Incubation of the cells in the vehicle resulted in an anandamide
 406 content of 0.01 ± 0.01 ng/mg ($n=3$) protein in the superfusate
 407 (data not shown). This value was not significantly different
 408 from that measured in the control of this experiment ($0.04 \pm$
 409 0.04 ng/mg protein, $n=3$; $p=0.5$, Student's t -test). Incubation

t3.1 **Table 3** Relative number of cultured rat primary sensory neurons exhibiting immunopositivity for various enzymes, which are implicated in Ca²⁺-insensitive anandamide synthesis, and for TRPV1

t3.2		ABHd4	GDE1	Inpp5	PTPn22	sPLA2G1b
t3.3	Relative number of cells exhibiting immunopositivity	29.8±1.9 <i>n</i> =3	40.27±9.94 <i>n</i> =3	44.1±1.9 <i>n</i> =3	48.53±10.04 <i>n</i> =3	<i>n. d.</i>
t3.4	Relative number of TRPV1-expressing cells	50.63±3.84 <i>n</i> =3	46.6±3.46 <i>n</i> =3	56.2±4.77 <i>n</i> =3	42.4±2.37 <i>n</i> =3	<i>n. q.</i>
t3.5	Relative number of TRPV1-expressing neurons which also exhibit enzyme-immunopositivity	23.11±8.55 <i>n</i> =3	25.9±2.68 <i>n</i> =3	64.93±10.9 <i>n</i> =3	17.93±7.58 <i>n</i> =3	<i>n. d.</i>
t3.6	Relative number of enzyme-expressing neurons which also express TRPV1	13.25±4.5 <i>n</i> =3	21.8±4.21 <i>n</i> =3	51.27±8.33 <i>n</i> =3	16.4±5.49 <i>n</i> =3	<i>n. d.</i>

n number of cultures, *n.q.* not quantified, *n.d.* not detected

410 of the cells in 100 μM 20:4-NAPE at room temperature also
 411 significantly increased the anandamide concentration in the
 412 buffer (from 0.06±0.03 ng/mg protein to 1.17±0.03 ng/mg
 413 protein, *n*=3; Student's *t*-test, *p*=0.009; Fig. 3b). However,
 414 the 20:4-NAPE application-induced increase in the anandamide
 415 content of the buffer at room temperature was significantly less
 416 than that measured at 37°C (Student's *t*-test, *p*=0.03) indicating
 417 that the production of anandamide depends on enzyme activity.
 418 To make sure that no 20:4-NAPE hydrolysing activity in the
 419 buffer contributed to the increase in the anandamide levels, we
 420 also quantified the anandamide content of cell-free superfusate
 421 containing 100 μM 20:4-NAPE. The buffer was kept either at
 422 room temperature or 37°C up to 4 h. We measured the lowest

and highest anandamide concentration at 2.5 h at 37°C (0.023
 423 ng/ml) and at 5 min at 37°C (0.15 ng/ml), respectively. We
 424 found no apparent relationship between the anandamide con-
 425 tent and the time or temperature of incubation (data not shown).
 426 These data indicated that cultured rat PSN has the ability to
 427 convert 20:4-NAPE into anandamide.
 428

The 20:4-NAPE application-evoked increase in the
 429 anandamide content of the supernatant at 37°C was
 430 concentration-dependent (Fig. 3c; Protocol A). The lowest
 431 20:4-NAPE concentration at which the anandamide content
 432 of the superfusate was significantly different from that of
 433 the control was 10 μM (ANOVA followed by Fisher's
 434 test, *p*=0.03).
 435

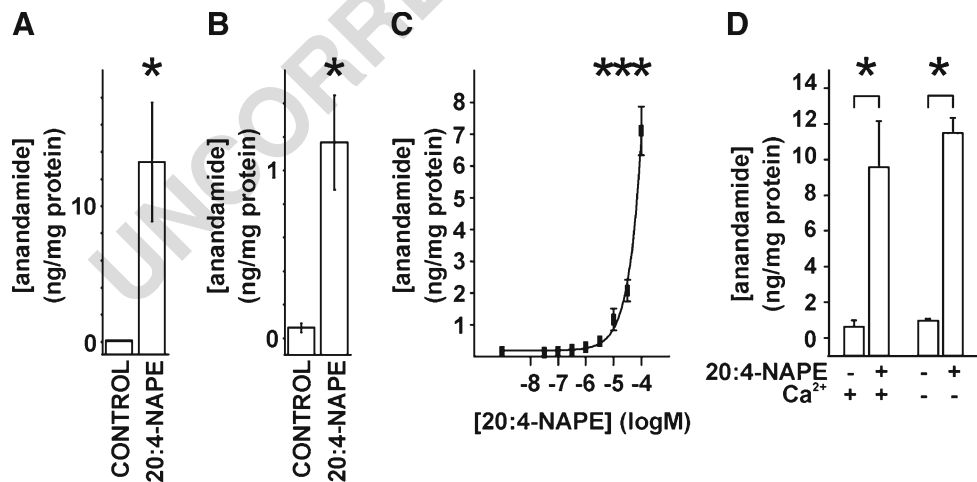


Fig. 3 20:4-NAPE application induces anandamide production in cultured rat primary sensory neurons. **a** Incubation of rat cultured PSN in the presence of 100 μM 20:4-NAPE for 5 min at 37 °C results in a significant increase in the anandamide content of the superfusate when compared to the basal anandamide level. The measurement was done by ultra-high performance liquid chromatography-electrospray-tandem mass spectrometry following the extraction of lipids from the superfusate. Spectrograms of anandamide analysis are shown in Fig. S1. (*n*=4, *asterisk* indicates significant difference.) **b** Incubation of rat cultured PSN in the presence of 100 μM 20:4-NAPE for 5 min at room temperature also increases the anandamide content of the superfusate. However, the anandamide content following incubation of the neurons at room temperature is significantly smaller than following incubation of the neurons at 37 °C

(please note the difference in the scale of the *y*-axis in **a** and **b**). Spectrograms of anandamide analysis are shown in Fig. S1. (*n*=3, *asterisk* indicates significant difference from the basal anandamide level measured at room temperature.) **c** Incubation of rat cultured PSN in 30 nM–100 μM 20:4-NAPE for 5 min at 37 °C results in a concentration-dependent increase in the anandamide content of the superfusate. The lowest 20:4-NAPE concentration that induces significant increase of the anandamide content of the superfusate is 10 μM. (*n*=3 for each data point, * indicates significant difference.) **(D)** Withdrawal of Ca²⁺ from the superfusate does not change either the basal anandamide content of the superfusate or the anandamide content of the superfusate following the addition of 100 μM 20:4-NAPE for 5 min (*n*=3 for each data point, *asterisk* indicates significant difference)

436 Among the known anandamide-synthesising enzymes,
 437 NAPE-PLD activity is significantly enhanced in the pres- 488
 438 ence of Ca^{2+} [32, 42, 43, 47, 48]. To ascertain whether or 489
 439 not during 20:4-NAPE application, any Ca^{2+} influx in- 490
 440 creases the intracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_i$ to an 491
 441 extent which is enough to contribute NAPE-PLD to anan- 492
 442 damide synthesis, we applied 20:4-NAPE to cultured PSN 493
 443 either with Ca^{2+} present or absent in the buffer. In the 494
 444 presence of Ca^{2+} , the anandamide content of the 495
 445 superfusate of cells incubated with vehicle was $0.62 \pm$ 496
 446 0.35 ng/mg protein ($n=3$), whereas in the absence of 497
 447 Ca^{2+} , it was 0.98 ± 0.12 ng/mg protein ($n=3$; Protocol 498
 448 A). These values did not differ significantly from each 499
 449 other (Student's t -test, $p=0.4$; Fig. 3d). Application of
 450 20:4-NAPE ($100 \mu\text{M}$) resulted in a significant increase
 451 in the anandamide content of the superfusate in both the
 452 presence and absence of Ca^{2+} (9.57 ± 2.58 ng/mg protein
 453 ($n=3$) and 11.47 ± 0.92 ng/mg protein ($n=3$); Student's t -
 454 test, $p=0.01$ in the presence and $p=0.0001$ in the absence
 455 of Ca^{2+} ; Fig. 3d). Thus, the anandamide content of the
 456 Ca^{2+} -containing, and Ca^{2+} -free, superfusates did not differ
 457 significantly from each other after the application of this
 458 substrate (Student's t -test, $p=0.5$).

459 We also compared the anandamide content of the
 460 superfusate to that of 90 % of the cells together with the
 461 superfusate (the other 10 % of the cells was used for protein
 462 measurements). The anandamide content of the cells and
 463 superfusate together under control conditions (incubation with
 464 vehicle) was 0.09 ng/mg protein ($n=2$; Protocol C). Follow-
 465 ing $100 \mu\text{M}$ 20:4-NAPE application for 5 min, the ananda-
 466 mide content of the cells and superfusate increased to
 467 28.03 ng/mg protein ($n=2$; data not shown).

468 Together, these data indicate that the 20:4-NAPE applica-
 469 tion-evoked anandamide synthesis in cultured rat PSN is not affect-
 470 ed by the removal of Ca^{2+} from the extracellular buffer. These
 471 data also show that about 2/3 of anandamide produced in PSN
 472 following 20:4-NAPE application, similar to anandamide pro-
 473 duced by increasing the $[\text{Ca}^{2+}]_i$ in central neurons [8], is
 474 retained in the cells.

475 20:4-NAPE application induces cobalt influx
 476 into a sub-population of cultured rat PSN

477 Anandamide synthesised in PSN by increasing the $[\text{Ca}^{2+}]_i$
 478 induces TRPV1-mediated excitation [44]. TRPV1 activa-
 479 tion by anandamide results in cobalt accumulation in a
 480 sub-population of PSN [24, 34, 38, 39]. Therefore, to test
 481 whether endogenous anandamide synthesised following the
 482 application of 20:4-NAPE has similar effect to that
 483 synthesised in a Ca^{2+} -sensitive manner, we studied cobalt
 484 accumulation during 20:4-NAPE application. The efficacy
 485 of this technique for assessing TRPV1-activity has been
 486 consistently demonstrated [24, 30, 34, 38, 39, 49, 50].

487 While application of the vehicle did not ($2.63 \pm$ 487
 488 0.34% , $n=3$; $p=0.44$, ANOVA followed by Fisher's 488
 489 test), application of 20:4-NAPE significantly increased 489
 490 the proportion of labelled neurons and this effect was 490
 491 concentration-dependent (Fig. 4a-c). The lowest concen- 491
 492 tration of 20:4-NAPE which produced a significant in- 492
 493 crease in the proportion of labelled cells was $0.1 \mu\text{M}$ 493
 494 ($6.44 \pm 1.16\%$, $n=4$ cultures; ANOVA followed by 494
 495 Fisher's test, $p=0.01$; Fig. 4c). The EC_{50} of 20:4-NAPE 495
 496 was $7.41 \pm 1.4 \mu\text{M}$. The majority of the cobalt-labelled cells 496
 497 were small diameter neurons (Fig. 4d). These observations 497
 498 suggest that the cobalt influx occurred in nociceptive neurons, 498
 499 the great majority of which express TRPV1 [6, 31]. 499

20:4-NAPE application induces inward currents 500
 in a sub-population of cultured rat PSN 501

502 To confirm the excitatory effect of 20:4-NAPE applica- 502
 503 tion, we recorded whole cell currents from cultured rat 503
 504 PSN. In total, 22 cells were found that responded with 504
 505 inward currents to $50 \mu\text{M}$ 20:4-NAPE which is the near 505
 506 maximal concentration for this agent to induce cobalt 506
 507 uptake (Fig. 5a). The peak amplitude of the response 507
 508 was -0.41 ± 0.06 nA ($n=22$). 508

509 All nine of the 20:4-NAPE-responsive neurons that we 509
 510 tested also responded to 500 nM capsaicin (Fig. 5a and b). 510
 511 The average amplitude of the 20:4-NAPE-evoked currents 511
 512 (-0.35 ± 0.08 nA, $n=9$) was significantly smaller than that 512
 513 of the capsaicin-evoked currents (-2.36 ± 0.55 , $n=9$; Stu- 513
 514 dent's t -test, $p=0.002$; Fig. 5c). However, in addition to the 514
 515 double-responsive cells, 27 neurons which responded to 515
 516 capsaicin but not to 20:4-NAPE application were also 516
 517 found (data not shown). In the control experiments, no 517
 518 neurons produced any noticeable responses to the vehicle 518
 519 ($n=11$, data not shown). 519

20:4-NAPE application increases the $[\text{Ca}^{2+}]_i$ in a capsazepine- 520
 and rimonabant-sensitive manner 521

522 In order to study the pharmacological properties of the 20:4- 522
 523 NAPE application-evoked excitatory responses, we assessed 523
 524 changes in $[\text{Ca}^{2+}]_i$ produced by the application of $50 \mu\text{M}$ 20:4- 524
 525 NAPE. Application of the vehicle for a minute did not pro- 525
 526 duce any increase in the $[\text{Ca}^{2+}]_i$ in any of the 384 KCl- 526
 527 responding neurons, whereas 234 of these 384 responded to 527
 528 $1 \mu\text{M}$ capsaicin application (data not shown). Application of 528
 529 20:4-NAPE, however, increased $[\text{Ca}^{2+}]_i$ in 189 of 546 KCl- 529
 530 responding neurons (34.6% ; Fig. 6a and b). All the neurons 530
 531 that responded to 20:4-NAPE application were sensitive to 531
 532 capsaicin (Fig. 6a and b). In addition to the double-responsive 532
 533 neurons, 273 of the 546 neurons (50%) responded only to 533
 534 capsaicin (Fig. 6a and b). Hence, the total number of 534
 535 capsaicin-responsive neurons was 462 of 546 (84.6% ; 535

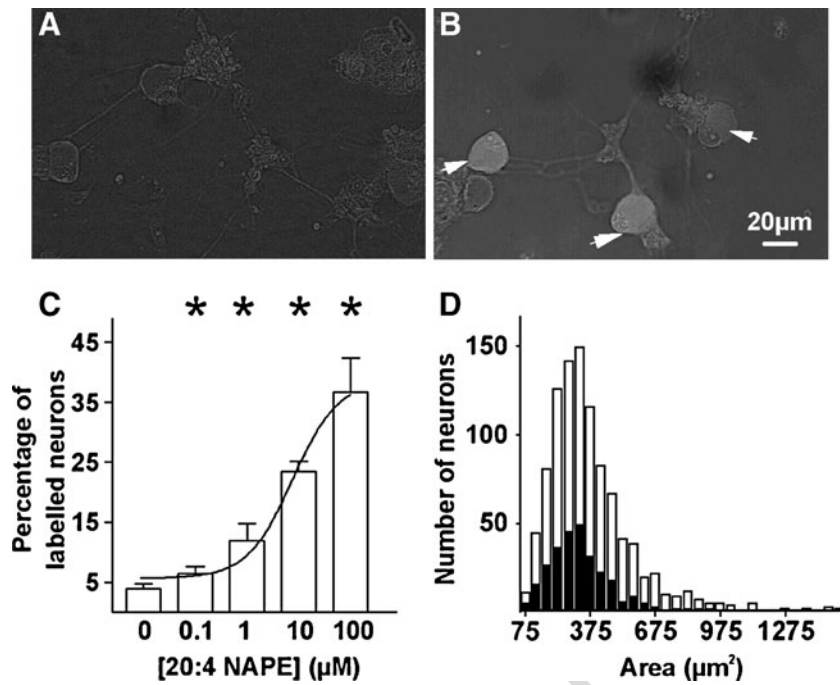


Fig. 4 20:4-NAPE application induces cobalt uptake in a sub-population of cultured rat primary sensory neurons. Representative images of cultured rat PSN following the incubation of cells in a 5-mM CoCl₂-containing buffer for 5 min without (a) or with 20:4-NAPE (100 μM) (b). The accumulated cobalt in the cells was precipitated by 2.5 % β-mercaptoethanol. The images are inverted hence labelled cells appear as bright cells which are indicated by arrows. When no 20:4-NAPE is added to the CoCl₂-containing buffer very few cells, if any at all, accumulate

cobalt (a). However, when 20:4-NAPE is added, a significant number of neurons accumulate Co²⁺ (b). c Incubation of cultured rat PSN in a CoCl₂-containing buffer in the presence of 20:4-NAPE (100 nM-100 μM) for 5 min results in a concentration-dependent increase in the number of cobalt-labelled cells. (n=3-9 for various data points, asterisk indicates significant difference.) d The overwhelming majority of neurons labelled by 20:4-NAPE application-induced cobalt influx (black bars) are small diameter neurons. Empty bars depict non-labelled cells

536 Fig. 6b), which was significantly higher than the proportion of
 537 capsaicin-sensitive neurons measured when only the vehicle
 538 ($p < 0.0001$; Fischer's exact test) or the buffer (224 of
 539 343 cells) was applied before capsaicin application. The
 540 20:4-NAPE application-evoked changes in $[Ca^{2+}]_i$ activated
 541 more slowly than those evoked by capsaicin
 542 (Fig. 6a). Furthermore, the amplitude of the 20:4-NAPE
 543 application-evoked responses were significantly smaller
 544 than those evoked by capsaicin when normalised to the
 545 KCl-evoked responses (20:4-NAPE/KCl = 0.15 ± 0.01 , $n =$
 546 189; capsaicin/KCl = 0.78 ± 0.02 , $n = 462$; Student's t -test,
 547 $p < 0.0001$; Fig. 6a). Interestingly, the great majority of
 548 neurons, which generated responses to 20:4-NAPE ap-
 549 plication, exhibited sustained/very slowly inactivating
 550 Ca^{2+} transients to capsaicin application. On the other
 551 hand, the great majority of capsaicin-responsive neu-
 552 rons, which did not respond to 20:4-NAPE application,
 553 exhibited fast inactivation of responses during capsaicin
 554 application (Fig. 6a).

555 In the presence of capsazepine (5 μM), 403 KCl-
 556 responding neurons exhibited a stable baseline before
 557 20:4-NAPE application. Of these, 26 responded to
 558 20:4-NAPE and capsaicin (6.45 %; Fig. 6b). In addition

559 to these neurons, 16 cells responded only to 20:4-NAPE
 560 application, and 21 cells responded only to capsaicin.
 561 Thus, the overall proportions of both the 20:4-NAPE-
 562 (42 of 403, 10.4 %) and capsaicin-responding neurons
 563 (47 of 403, 11.7 %) in the presence of capsazepine were
 564 significantly smaller than that measured in the absence
 565 of capsazepine (Fisher's exact test, $p < 0.0001$ for both
 566 20:4-NAPE and capsaicin; Fig. 6b).

567 In the presence of rimonabant, 20:4-NAPE application in-
 568 creased $[Ca^{2+}]_i$ in 105 of 498 KCl-responding neurons with a
 569 stable base line before 20:4-NAPE application (21.08 %;
 570 Fig. 6b). This proportion was significantly smaller from that
 571 measured in the control (Fisher's exact test; $p < 0.0001$).
 572 Rimonabant, however, had no significant effect on the overall
 573 number of capsaicin-responding neurons (421 of 498 cells,
 574 84.54 %, $p = 1$, Fisher's exact test; Fig. 6b). Rimonabant signifi-
 575 cantly reduced the amplitude of both the 20:4-NAPE
 576 application-evoked (to 20:4-NAPE/KCl = 0.12 ± 0.009 , Student's
 577 t -test, $p = 0.02$), and the amplitude of the capsaicin-induced
 578 responses (to capsaicin/KCl = 0.61 ± 0.018 , Student's t -test,
 579 $p < 0.0001$). These data suggest that the 20:4-NAPE
 580 application-evoked excitatory effect is mediated by TRPV1,
 581 and that 20:4-NAPE application, when assessed average

Fig. 5 20:4-NAPE application induces inward currents in a sub-population of cultured rat primary sensory neurons. **a** A typical current trace recorded from a PSN during the application of 20:4-NAPE (50 μ M) for 60 s. Bar above recording indicates 20:4-NAPE application. **b** The neuron shown in **a** also responds to 500 nM capsaicin. Bar above recording indicates capsaicin application. Note the difference in the kinetics of the 20:4-NAPE- and capsaicin-induced currents. **c** Application of capsaicin (C) (500 nM) induces a significantly greater current than application of 50 μ M 20:4-NAPE (N). ($n=9$ for each data point, asterisk indicates significant difference)

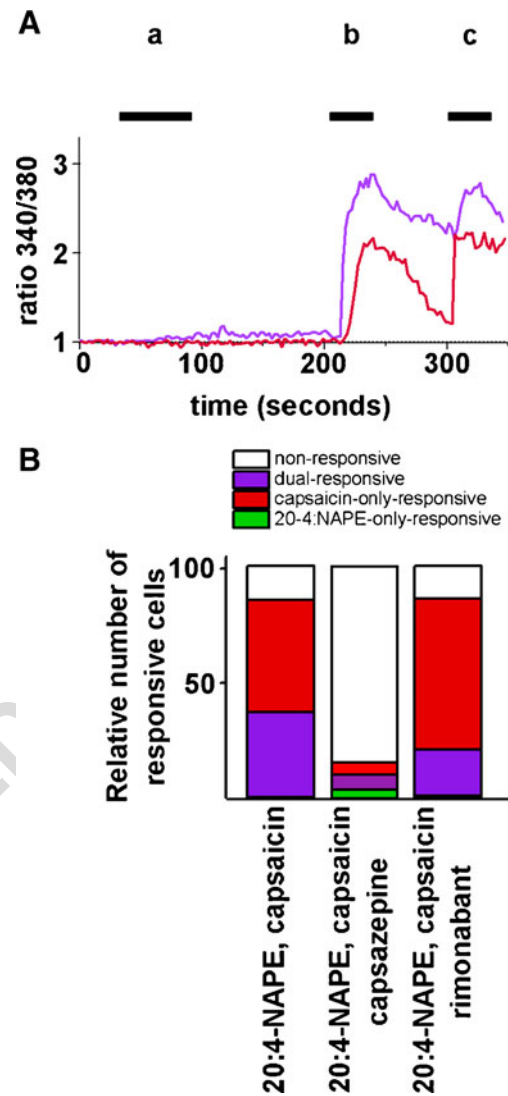
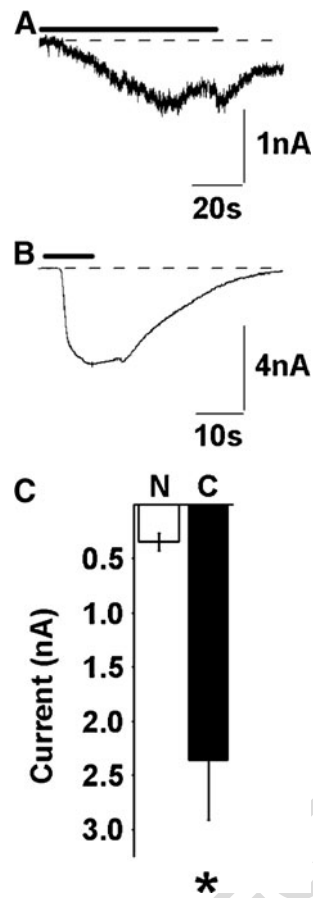


Fig. 6 20:4-NAPE application induces increase in $[Ca^{2+}]_i$ in a sub-population of cultured rat primary sensory neurons, which is significantly reduced by the inhibition of both the transient receptor potential vanilloid type 1 ion channel and the cannabinoid 1 receptor. **a** Representative recordings of changes in $[Ca^{2+}]_i$ in cultured rat PSN superfused by 20:4-NAPE (50 μ M) (a), capsaicin (1 μ M) (b) and KCl (50 mM) (c) as indicated. A proportion of neurons responds to 60-s application of 50 μ M 20:4-NAPE (a) by a slow increase in $[Ca^{2+}]_i$ (purple trace). All the 20:4-NAPE-responding neurons also respond to 1 μ M capsaicin (b) application (purple trace). However, not all capsaicin-sensitive neurons (red trace) respond to 20:4-NAPE application. **b** The bar chart shows the proportion of neurons responding to various activators in the absence or presence of the transient receptor potential vanilloid type 1 ion channel antagonist capsazepine (5 μ M) or the cannabinoid 1 receptor antagonist rimonabant (200 nM). Both capsazepine and rimonabant significantly reduces the proportion of the 20:4-NAPE-responding cells

582 responses, does not result in a CB1 receptor mediated
583 inhibitory effect.

584 The 20:4-NAPE application-evoked increase in $[Ca^{2+}]_i$
585 in mouse PSN is mediated by TRPV1

586 In order to confirm the role of TRPV1 in 20:4-NAPE
587 application-evoked responses, we also studied the effect of
588 this agent on PSN isolated from WT and TRPV1^{-/-} (KO)
589 mice. Ten of 68 KCl-responsive neurons (14.71 %) from
590 WT mice were regarded as responsive to 20:4-NAPE
591 (50 μ M) application (Fig. 7a). As in experiments using rat
592 PSN cultures, all the 20:4-NAPE-responsive neurons were
593 responsive to capsaicin (1 μ M; Fig. 7a) and the 20:4-NAPE
594 application-evoked responses activated more slowly than the
595 capsaicin-evoked responses (Fig. 7a). As with cultures pre-
596 pared from rat DRG, cultures prepared from WT mouse DRG
597 also had neurons, which responded only to capsaicin (32 of 68
598 [48.53 %]; Fig. 7a).

599 In contrast, none of the KCl-responsive neurons ($n=59$)
600 responded to 20:4-NAPE application in cultures prepared from
601 DRG of KO mice (Fig. 7b). As expected, none of the neurons
602 in the cultures prepared from DRG of KO mice responded to

603 capsaicin (Fig. 7b). However, 22 of the 59 KCl-responding
604 cells responded to mustard oil, showing that the cells were
605 healthy and responsive to agents for which they expressed
606 receptors (Fig. 7b). Therefore, our findings on neurons of

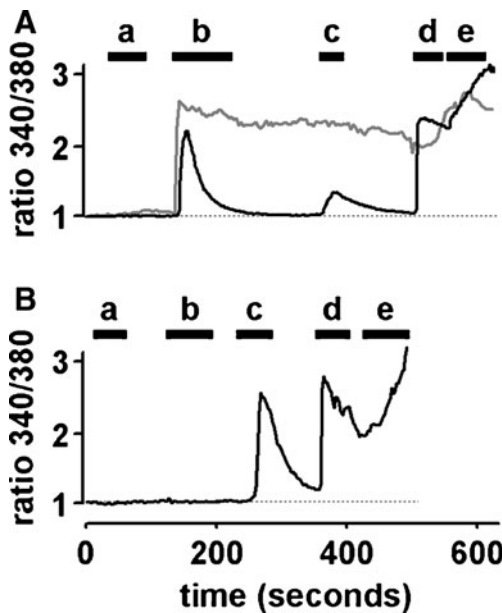


Fig. 7 20:4-NAPE induces Ca^{2+} influx into cultured primary sensory neurons isolated from wild type mice but not into those which are isolated from $\text{TRPV1}^{-/-}$ mice. (a and b) Representative recordings of changes in $[\text{Ca}^{2+}]_i$ in cultured mouse PSN superfused by 20:4-NAPE (50 μM) (a), capsaicin (1 μM) (b), mustard oil (50 μM) (c), KCl (50 mM) (d) and ionomycin (5 μM) (e) as indicated. Recordings in panel a are from cells dissected from a wild type mouse, whereas the recording shown in b is from a cell isolated from a $\text{TRPV1}^{-/-}$ mouse. A sub-population of neurons isolated from wild type mice (a) responds to 50 μM 20:4-NAPE application (gray trace). All these 20:4-NAPE-responding neurons also respond to capsaicin (gray trace). However, not all capsaicin-responding neurons respond to 20:4-NAPE application (black trace). While a sub-population of neurons isolated from $\text{TRPV1}^{-/-}$ mice respond to mustard oil (b), none of the cells respond either to 20:4-NAPE or capsaicin

607 $\text{TRPV1}^{-/-}$ mice confirm that the 20:4-NAPE application-
608 evoked excitatory effects are mediated through TRPV1.

609 Sub-populations of cultured rat primary sensory neurons
610 co-express TRPV1 and enzymes implicated
611 in Ca^{2+} -insensitive anandamide syntheses

612 The concentration of anandamide found in the superfusate
613 (~10 nM) is significantly lower than the concentration of
614 exogenous anandamide needed to induce TRPV1-mediated
615 excitatory effects [1, 12, 14, 38, 51]. Therefore, we
616 hypothesised that the excitatory effect of anandamide of
617 PSN origin is mediated through autocrine signalling. To find
618 out whether there is an anatomical basis for such autocrine
619 signalling, we used combined immunostaining for studying
620 the co-expression of TRPV1 with the Ca^{2+} -insensitive
621 anandamide-synthesising enzymes. Analysis of this double
622 immunofluorescent staining showed that a significant propor-
623 tion of cultured rat PSN exhibit co-expression for TRPV1 and
624 the putative Ca^{2+} -insensitive anandamide-synthesising en-
625 zymes (Fig. 8, Table 3).

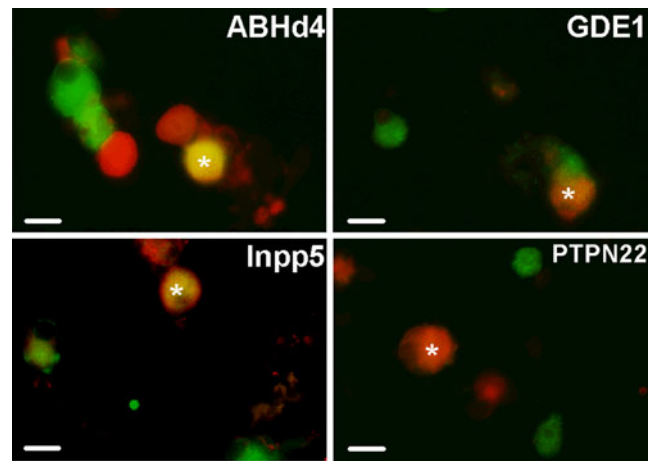


Fig. 8 Sub-populations of cultured rat primary sensory neurons co-express TRPV1 and enzymes implicated in Ca^{2+} -insensitive anandamide synthesis. Cultured rat PSN were incubated in antibodies raised against TRPV1 (green) and ABHd4, GDE1, Inpp5 or PTPn22. For quantitative data, please see Table 3. Scale bar=25 μm

Discussion

626

We have shown in the present study that four of the five
627 enzymes (ABHd4, GDE1, Inpp5 and PTPn22) which have
628 been implicated previously in Ca^{2+} -insensitive anandamide
629 synthesis from 20:4-NAPE [22, 23, 32, 36, 41, 43], are found
630 at transcript level (please see also Fig. S2) in cultures prepared
631 from rat DRG, and at protein level in cultured rat (and mouse,
632 see Fig. S3) PSN. The GDE1 protein is also expressed by non-
633 neuronal cells as well as by PSN. The anti-sPLA2G1b anti-
634 body, which we used in this study, does not produce any
635 staining in rat PSN cultures, although it labels cells in acini
636 in the pancreas. RT-PCR shows weak expression of
637 sPLA2G1b mRNA in PSN cultures. Hence, one cannot ex-
638 clude the possibility that this enzyme may be expressed under
639 the detection threshold of the immunostaining technique in
640 some neurons or non-neuronal cells.

20:4-NAPE application to PSN cultures increases the anan-
642 damide concentration in the cells and the superfusate. The
643 finding that application of 20:4-NAPE to cell-free superfusate
644 does not increase the anandamide content of the buffer, to-
645 gether with the finding that the anandamide content of the
646 cells and superfusate is greater than that of the superfusate
647 alone indicates that 20:4-NAPE passes the cells membrane
648 and anandamide is synthesised intracellularly. Indeed, all
649 NAPE-converting enzymes are intracellular and transiently
650 associated with membranes to act on NAPEs embedded in
651 those [4, 22, 23, 32, 36, 41, 43]. Therefore, the conversion of
652 20:4-NAPE to anandamide in an enzymatic way, as we have
653 shown here, can be only through the rapid incorporation of
654 this phospholipid into the membranes and its hydrolysis by
655 some of the enzymes we investigated here. The rate of anan-
656 damide production induced by 100 μM 20:4-NAPE 657

658 application (~38 pmol/5 min/mg protein) is well within the
659 range of 20:4-NAPE hydrolysis (up to ~40 pmol/min/mg
660 protein) measured in brain homogenates [20, 36, 37]. The
661 distribution of enzymes across PSN and non-neuronal cells
662 indicates that, while non-neuronal cells may produce some
663 anandamide, the greatest amount of this endocannabinoid/
664 endovanilloid is likely to be synthesised in PSN.

665 Calcium regulates the synthesis of anandamide mostly
666 through the NAPE-synthesising enzyme known as Ca^{2+} -
667 dependent *N*-acyltransferase, which catalyses the formation of
668 20:4-NAPE [4, 8]. However, when this rate-limiting step in
669 anandamide and NAEA biosynthesis is bypassed, as in our
670 case, the only way for anandamide to be produced in a Ca^{2+} -
671 sensitive manner is via NAPE-PLD action on 20:4-NAPE [4,
672 22, 23, 32, 36, 41–43] (Fig. 1). Indeed, in the absence of Ca^{2+} ,
673 NAPE-PLD activity is negligible even in the presence of the
674 substrate [42, 47], whereas increasing the $[\text{Ca}^{2+}]_i$ alone results
675 in anandamide production [8, 44]. Although, application of
676 20:4-NAPE induces some increase in the $[\text{Ca}^{2+}]_i$, the finding
677 that removal of Ca^{2+} from the extracellular buffer does not
678 change the anandamide concentration in the superfusate sug-
679 gests that Ca^{2+} influx during 20:4-NAPE application is not
680 sufficiently high to induce NAPE-PLD activity. Hence, follow-
681 ing 20:4-NAPE application, at least the great majority of anan-
682 damide is synthesised by PSN by Ca^{2+} -insensitive pathways.
683 Consequently, some of the Ca^{2+} -insensitive anandamide-
684 synthesising enzymes present in the cultures prepared from
685 rat DRG form functional pathways, which may also include
686 the Ca^{2+} -independent NAPE biosynthesising enzyme previ-
687 ously identified by Jin and colleagues [17]. However, the exact
688 identity of the pathway(s) is not known and attempts to dissect
689 them would produce ambiguous results at present because of
690 the unavailability of selective and specific blockers of the
691 enzymes.

692 Application of 20:4-NAPE results in cobalt-influx in a sub-
693 population of small diameter cultured PSN, the majority of
694 which express TRPV1 [6, 28, 31]. While the lowest concentra-
695 tion of 20:4-NAPE which induces a significant increase in the
696 number of cobalt-labelled neurons is 0.1 μM , the lowest con-
697 centration of 20:4-NAPE which results in a significant increase
698 in the anandamide content of the superfusate is 10 μM . We
699 have shown here, however, that PSN — similar to central
700 neurons [8] — retain the majority of anandamide. Hence, the
701 concentration of anandamide at the intracellular anandamide-
702 binding site of TRPV1 [18], is probably considerably higher
703 than that in the superfusate. This difference renders any direct
704 comparison between minimally effective concentrations of
705 20:4-NAPE at increasing the anandamide concentration in the
706 superfusate and at evoking Co^{2+} uptake very difficult.

707 In addition to inducing cobalt accumulation, application of
708 20:4-NAPE also increases the $[\text{Ca}^{2+}]_i$, and induces whole-cell
709 currents. All the cells which respond to 20:4-NAPE applica-
710 tion with increased $[\text{Ca}^{2+}]_i$, or inward currents, in control

711 conditions, also respond to the archetypical TRPV1 agonist,
712 capsaicin. Taken together, these data suggest that the 20:4-
713 NAPE application-evoked excitatory effect is mediated
714 through TRPV1 in PSN. Indeed, the TRPV1 antagonist
715 capsazepine, which we applied at a concentration known to
716 have little effect on other molecules than TRPV1 [11], signif-
717 icantly reduces the proportion of neurons responding to
718 20:4-NAPE application. However, the inhibitory effect of
719 capsazepine might be different in species other than rat [46].
720 Nevertheless, the finding that cultured PSN prepared from
721 TRPV1^{-/-} mouse DRG do not, whereas a sub-population of
722 cultured PSN prepared from WT mouse DRG does, respond
723 to 20:4-NAPE application provides evidence that, in PSN, the
724 20:4-NAPE application-evoked excitatory effect is indeed
725 mediated through TRPV1.

726 Several findings indicate that the 20:4-NAPE application-
727 induced excitatory effects are exerted indirectly, through anan-
728 damide production. First, both the anandamide production,
729 and the 20:4-NAPE application-induced cobalt accumulation,
730 depend on the concentration of the substrate. Second, if 20:4-
731 NAPE activated TRPV1 directly all capsaicin-sensitive neu-
732 rons would be expected to respond. However, we found that
733 only ~40 % of capsaicin-responsive neurons exhibit responses
734 to 20:4-NAPE application. Third, if 20:4-NAPE activated
735 TRPV1 directly, a similar pattern of temperature-dependent
736 change in the responsiveness of neurons to 20:4-NAPE and
737 capsaicin is expected to occur. However, while application of
738 20:4-NAPE, which induces responses in about a third of the
739 neurons at 37 °C, fails to produce responses in any neurons at
740 room temperature (Fig. S4a and b). At the same time, the
741 proportion of capsaicin-responsive neurons is the same at
742 37 °C and room temperature (Fig. 4b). Fourth, the lack of
743 responsiveness to 20:4-NAPE at room temperature co-occurs
744 with a 10-fold reduction in the anandamide content of the
745 superfusate following the application of 20:4-NAPE to the
746 cells. Fifth, direct TRPV1 activators, such as capsaicin and
747 anandamide [18], activate TRPV1 with fast kinetics. Howev-
748 er, the kinetics of 20:4-NAPE-induced responses are signifi-
749 cantly slower than those of the capsaicin-evoked responses.
750 Sixth, the amplitude of whole-cell currents produced by 20:4-
751 NAPE application, which results in about 30 nM anandamide
752 when the concentration is measured in the cells and
753 superfusate together, is compatible with the amplitude of
754 whole-cell currents evoked by 100 nM anandamide included
755 into the recording electrode [12]. These findings collectively
756 provide evidence that the 20:4-NAPE-evoked effects depend
757 on enzyme activity and at least the majority, if not all, of the
758 20:4-NAPE application-induced TRPV1-mediated excitation
759 is produced indirectly, *via* the conversion of 20:4-NAPE to
760 anandamide. The involvement of 20:4-NAPE-derived metab-
761 olites other than anandamide is highly unlikely because thin
762 layer chromatography shows the presence of no other lipids
763 than anandamide and 20:4-NAPE in the superfusate (Fig. S5).

764 The limited responsiveness of capsaicin-sensitive neurons to
 765 20:4-NAPE may appear in contrast with the co-expression
 766 pattern of TRPV1 and the four enzymes we examined in this
 767 study. However, each of these four enzymes constitutes only a
 768 member of specific enzymatic pathways (Fig. 1), none of which
 769 is selective for the synthesis of anandamide [22, 23, 32, 36, 41,
 770 43]. Due to the unknown molecular identity of some enzymes,
 771 at present, it is impossible to establish the proportion of neurons
 772 which express (or co-express) a complete anandamide-
 773 synthesising pathway(s). Nevertheless, our findings suggest
 774 that less than half of TRPV1-expressing neurons may express
 775 pathways, which are able to produce anandamide in a Ca^{2+} -
 776 insensitive manner. These findings, together with the concentra-
 777 tion of anandamide in the superfusate, following 20:4-NAPE
 778 application, being well below the minimum concentration of
 779 exogenous anandamide needed for TRPV1 activation [1, 12,
 780 14, 38, 51] also suggest that anandamide synthesised in a Ca^{2+} -
 781 insensitive manner, similar to anandamide produced through a
 782 Ca^{2+} -sensitive pathway [44], activates TRPV1 through auto-
 783 crine signalling. Notably, the overall proportion of capsaicin-
 784 responsive cells is increased from ~60 % to above 80 % when
 785 20:4-NAPE is applied before capsaicin. Furthermore, more
 786 dual-responsive than capsaicin-only-responsive neurons exhibit
 787 sustained/slowly inactivating capsaicin-induced increase in
 788 the $[\text{Ca}^{2+}]_i$. Hence, the autocrine signalling by anandamide
 789 synthesised in a Ca^{2+} -insensitive manner by PSN seems to
 790 have some sensitising effect on TRPV1-mediated responses,
 791 which results in exhibiting such responses above the detection
 792 threshold in neurons which do not produce detectable responses
 793 to capsaicin in control conditions.

794 While exogenous anandamide induces a CB1 receptor-
 795 mediated inhibition on TRPV1-mediated responses in PSN
 796 [2, 14, 24, 27, 33], the selective and specific CB1 receptor
 797 antagonist, rimonabant, reduces the proportion of neurons
 798 responding to, and the amplitude of responses induced by,
 799 20:4-NAPE application. These data may suggest that exoge-
 800 nous anandamide, and anandamide produced from 20:4-
 801 NAPE via Ca^{2+} -insensitive pathways may exert their actions
 802 through not completely overlapping mechanisms (i.e., while
 803 exogenous anandamide induces a CB1 receptor-mediated in-
 804 hibitory effect, endogenous anandamide does not induce such
 805 effect). However, the effect of exogenous anandamide on
 806 TRPV1 activity depends on its concentration and the avail-
 807 ability of the CB1 receptor. Hence, exogenous anandamide
 808 above 1 μM induces larger responses in TRPV1-CB1 receptor
 809 co-expressing human embryonic kidney 293 cells than in such
 810 cells when they express TRPV1 alone [16]. Consistently, the
 811 exogenous anandamide-evoked excitatory effect is reduced by
 812 rimonabant in cultured rat PSN [35]. Furthermore, like the
 813 effect of anandamide on TRPV1, the effect of capsaicin is also
 814 reduced when the CB1 receptor is not available either due to
 815 the blocking CB1 receptor activity with rimonabant [35] or to
 816 the deletion of the CB1 receptor [13]. In agreement with

findings in $\text{CB1}^{-/-}$ mice [13], we found here that rimonabant 817
 reduces the amplitude of capsaicin-evoked responses, but not 818
 the proportion of neurons responding to capsaicin. Therefore, 819
 the inhibitory effect of rimonabant on the proportion of neuron 820
 responding to 20:4-NAPE by increased $[\text{Ca}^{2+}]_i$ is consistent 821
 with the proposed constitutive sensitising action of the CB1 822
 receptor on TRPV1, which may occur under certain condi- 823
 tions [13, 16]. Hence, the effects of exogenous anandamide 824
 above 1 μM and the effects of endogenous anandamide 825
 synthesised from 50 μM 20:4-NAPE in a Ca^{2+} -insensitive 826
 manner, at least as far as activating TRPV1 and not producing 827
 CB1 receptor-mediated inhibitory effects are concerned, are 828
 similar in PSN. Together these findings indicate that ananda- 829
 mide that is synthesised in a Ca^{2+} -insensitive manner from 830
 50 μM 20:4-NAPE has an excitatory rather than an inhibitory 831
 effect in PSN. 832

In conclusion, results of the present study show that: (a) a 833
 sub-population of PSN has at least one enzymatic pathway 834
 which synthesises anandamide from 20:4-NAPE in a Ca^{2+} - 835
 insensitive manner; (b) the synthesis of anandamide from 836
 exogenous 20:4-NAPE primarily, if not exclusively, occurs 837
 via Ca^{2+} -insensitive pathways; and (c) anandamide of PSN 838
 origin synthesised from 20:4-NAPE in a Ca^{2+} -insensitive 839
 manner produces an autocrine TRPV1-mediated excitation, 840
 which may complement the previously reported excitatory 841
 effects on TRPV1 of anandamide synthesised in a Ca^{2+} -sen- 842
 sitive manner in PSN [13]. The effect of anandamide of PSN 843
 origin together with the effects of exogenous anandamide on 844
 PSN [1, 12, 14, 24, 33] emphasises the high degree of flexi- 845
 bility of action of this important lipid mediator, and its multi- 846
 faceted role in controlling nociceptive processing [40]. There- 847
 fore, a better understanding of the expression and function of 848
 the anandamide-synthesising enzymes in PSN may allow us 849
 to control the activity of those cells, and hence develop more 850
 effective treatments of somato- and viscerosensory distur- 851
 bances outside the central nervous system. 852

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