Bisphenol A induces fatty liver by an endocannabinoid-mediated positive feedback loop

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The xenoestrogen bisphenol A (BPA) is a widespread plasticizer detectable within several ecosystems. BPA is considered a metabolic disruptor affecting different organs; however, little is known about its mechanism of action in the liver, where it triggers triglyceride accumulation. Exposed adult zebrafish (*Danio rerio*) to BPA developed hepatosteatosis, which was associated with an increase in the liver levels of the obesogenic endocannabinoids 2-AG and anandamide and a concomitant decrease in palmitoylethanolamide. These changes were associated with variations in the expression of key endocannabinoid catabolic and metabolic enzymes and an increase in the expression of the endocannabinoid receptor *cnr1*. Acute and chronic *in vitro* treatments with nano and micromolar BPA doses, showed increased anandamide levels in line with decreased activity of FAAH, the main anandamide hydrolytic enzyme, and induced triglyceride accumulation in HHL-5 cells in a CB1-dependent manner. We conclude that BPA is able to produce hepatosteatosis in zebrafish and human hepatocytes by upregulating the endocannabinoid system.

Industrial progress has resulted in massive environmental contamination with anthropogenic chemicals, which until only recently have been identified as endocrine disruptors (EDs) (1). This definition encompasses all the exogenous chemicals that interfere with hormonal responses by blocking or activating hormone receptors (2). Among these, bisphenol A (BPA) appears to have a ubiquitous distribution due to its use as plasticizer in numerous products from food and drink packaging to children's toys. Its capability to leak from plastic matrices into the water, combined with low volatility and high lipophilicity, results in the massive accumulation of this chemical in the environment, suspended solids, soil and sediments and subsequent uptake by aquatic wild life (3). Unconjugated BPA has been frequently detected in human blood with concentrations ranging from 0.3 to 4.4 ng/ml (1- 19.4 nM) (4). Similarly, its conjugated form is commonly found in urines samples with average values that fluctuate from 0.4 to 149 μg/L (1.7- 653 nM) (5). Estimation of BPA intake from leaching of consumer products ranges from 1 μg/kg/d (6) to almost 5 μg/kg/d (0.325 mg/d/adult) (7). In vivo data in treated rats and human samples confirmed the ability of BPA to bioaccumulate especially in adipose tissue and liver (8, 9).

In the last years, a different view of EDs as metabolic disruptors has been introduced (10). Given the high number of chemical obesogens and their negative consequences on the human population and the environment in general, it has been suggested that high-throughput zebrafish screens be utilized to readily characterize these compounds.

Abbreviations:

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At least part of these effects is likely due to increased adipogenesis as BPA acts as an agonist for the proadipogenic nuclear peroxisome proliferator-activate receptor (PPAR) γ (11) but inhibits the release from mature adipocytes of adiponectin, an important insulin sensitizing adipokine (12). Moreover, BPA affects glucose-induced calcium signaling in pancreatic α cells (13) and insulin content and release in β cells (14).

Despite the alterations that BPA produces in adipose and pancreatic tissues, very little is known about its hepatic effects. However, a recent paper showed the ability of BPA to up-regulate the expression of lipogenic genes and increase of de novo fatty acid synthesis in the liver (15). The autocrine/paracrine lipid-based endocannabinoid signaling system (ECS) is a major player in liver lipid metabolism (16). Peripheral pharmacological blockade of the endocannabinoid receptor CB1 (*Cnr1*) in mice with diet induced hepatic steatosis, significantly reduced dislypidemia and steatosis (17). Moreover, liver-specific *Cnr1* knock-out produced similar effects as whole-body *Cnr1* deficiency with respect to insulin resistance, indicating a crucial role for the ECS within the liver (18). ECS tone is also influenced by various hepatosteatotic stimuli; a high fat diet increases hepatic AEA levels thereby upregulating the fatty acid synthase (FAS) pathway and increasing de novo fatty acid production via CB1 activation (19). The ECS also plays a central role in neuronal hypothalamic networks regulating food intake (20). *Cnr1*$^{-/-}$ mice possess increased corticotropin releasing hormone (CRH) and reduced cocaine-amphetamine-related transcript (CART) expression, proving an intimate control of the ECS over peptides involved in the regulation of food intake (21). Furthermore, an inhibitory cross-talk between CB1 and melanocortin receptor-4 (MCR4) has been described (22), while in the arcuate nucleus, the neuropeptide Y/agouti-related protein system does not seem to be directly influenced by endocannabinoid action (23). Finally, two other acylethanolamides: oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), were found to be involved in pathways that, independent of the endocannabinoid system, may modulate lipolysis, food intake and inflammation. OEA mediates peripheral regulation of feeding (24) and together with PEA activates PPARα to modulate lipid metabolism (25). Therefore, given the xenoestrogen effects of BPA on hepatic lipid metabolism leading to triglyceride accumulation, we aimed at studying its influence on nonalcoholic fatty liver disease (NAFLD) in the teleost fish *Danio rerio*, and human hepatocytes, using the ECS as an early biomarker for disease onset.

Materials and Methods

Maintenance and treatment of adult fish. Adult zebrafish were kept in aquaria at 28°C in oxygenated water and fed twice daily with commercial food (Vipagram, Sera, Italia) and with Artemia salina. Six month old zebrafish females were exposed for 48 hrs to BPA[Sigma-Aldrich] with a final concentration of 100 μg/L (438.6 nM), EE2 [Sigma-Aldrich] 200 ng/L (0.887 nM) or vehicle (EtOH). At the end of the treatment fish were lethally anesthetized adding in the water 500 mg/l of MS-222 (3-aminobenzoic acid ethyl ester, Sigma-Aldrich, Milano, Italy) buffered to pH 7.4 and tissues were sampled for further analysis. Procedures were performed in accordance with the Guidelines on the handling and Training of Laboratory Animals by the Universities Federation for Animal Welfare (UFAW) and with the Italian animal welfare legislation (D.L. 116/92).

Liver morphology. Animals were fixed in neutral 4% paraformaldehyde prepared in phosphate-buffered saline (PBS) (PBS, 0.1 M, pH 7.4) at 4°C overnight, washed in PBS, dehydrated through a graded series of ethanol and embedded in paraffin. Consecutive sections were cut at a thickness of 4 μm using a microtome. All sections were stained with Mayer's hematoxylin and eosin, dehydrated, mounted in Eukitt and examined under an Olympus Vanox photomicroscope. In order to ascertain the degree of steatosis in liver, we estimated (visually and semiquantitatively) the area of the section occupied by fat vacuoles.

Cell Culture and triglyceride analysis. HHL-5 cells are an immortalized human hepatocyte line with a stable primary hepatocyte phenotype and were a gift from Dr. A.H. Patel (26). HHL-5 cells were cultured in standard growth media (DMEM [Lonza] supplemented with 10% FBS [Lonza], NEAA [Gibco] and Pen.Strep [Gibco]). Cells were plated in 96-well plates so that they would be 95% confluent at the time of the initiation of the experiments. Cells were washed at least three times with phenol-red free DMEM (Gibco; 11 880–028) supplemented with 10% FBS, Pen/Strep and NEAA and then treated as indicated. Media and drugs were refreshed every one day during the chronic BPA treatments. After the indicated exposure times, cells were washed with PBS, stained with AdipoRed [Lonza] and read with a Genios Pro [Tecan] or Envision multilabel [Perkin Elmer] plate reader according to the manufacturer's instructions. All the cannabinoid drugs, except for NESS 0327 [Sigma-Aldrich], were purchased from Tocris Bioscience. Drug powders were suspended in 100% DMSO and successively diluted in growth media with a final dilution of 1: 1000.

Measurement of endocannabinoids AEA, 2-AG and endocannabinoids-like PEA, OEA from adult zebrafish brain and liver. The extraction, purification, and quantification of endocannabinoids (EC) has been performed as previously described (23). The amounts of endocannabinoids in zebrafish brain and liver were quantified by isotope dilution with deuterated standards and data are expressed as pmol per mg of tissue weight.

RT and qPCR analysis. Total RNA was isolated from zebrafish liver, brain or HHL-5 cells using Trizol (Invitrogen) and treated with Dnase I (Ambion), and reverse transcribed with the

SuperScript III RT reaction kit (Invitrogen) according to the manufacturer's instructions. 10 to 20 ng of starting RNA was then used for qPCR analysis using IQ SybrGreen Supermix (Bio-Rad). on a CFX 384 optical thermal cycler (Bio-Rad). Data analysis was performed using is CFX Manager software (Bio-Rad) using *elfa* (for *Danio rerio*) and *RNAP* (for HHL-5 cells) as a reference genes, and data are expressed as relative mRNA levels with standard errors of the mean of triplicate reactions. Statistical significance was determined with the REST 2009 software. Primers were designed by AlleleID software (Premier Biosoft).

Silencing experiments. Human hepatocytes were plated at 70% confluence and reverse transfected utilizing the Lipofectamine RNAi transfection system according to the manufacturer's (Thermo Fisher). Predesigned siRNAs were used for negative control (Thermo Fisher, Cat. # 4 390 843) and *cnr1* (Thermo Fisher, Cat. # 4 392 420) knockdown group. Once treated with siRNAs, cells were then assayed at the indicated time points for gene expression and triglyceride levels as above.

FAAH enzymatic activity. HHL-5 cells were homogenized at 4°C in 50 mM Tris-HCl buffer, pH 7.0, centrifuged at 800 g and then the supernatant was centrifuged at 10 000 g. Protein concentrations were measured by Bradford assay (Biorad), 7 μg of membranes from either naïve cells, which were subsequently exposed to BPA (from 11.25 to 90 μM) in the assay buffer, or BPA-treated cells were then incubated with [^{14}C] AEA (10,000 cpm, 1.8 μM) in 50 mM Tris- HCl, pH 9, for 30 minutes at 37°C. [^{14}C] Ethanolamine produced from [^{14}C]AEA hydrolysis was then extracted from the incubation mixture with 2 volumes of CHCl3/CH3OH (1:1 by volume) and the subsequent aqueous phase measured by scintillation counting in order to calculate FAAH activity.

Data analysis

Data are expressed as means ± SEM of the reported number of experiments (n). Statistical significance was calculated using the unpaired Student's *t* test or one-way ANOVA, as appropriate.

Results

BPA increases lipid content in zebrafish liver. We first established whether BPA modulates zebrafish liver lipid content utilizing histological analysis. Fish treated with BPA for 48hr had severe lipid accumulation in livers as compared to controls (Figure 1A). Similarly, the positive control exposed to ethinyl estradiol (EE2), a potent synthetic estrogen, produced a comparable, though less dramatic, hepatosteatotic liver. We also noted a slight level of hepatic steatosis in the control group, which may be explained by the high energetic content of the commercial food generally used for breeding zebrafish.

A dysregulated endocannabinoid system is present in liver and brain of zebrafish treated with BPA. We went on to determine if BPA-induced hepatosteatosis is associated with changes in central and peripheral endocannabinoid levels. Brain endocannabinoid levels only showed modest variations, with 2-AG showing a trend toward an increase and AEA displaying a reduction only in the EE2 treated fish (Figure 1B). However, OEA, a potent anorexigenic mediator (24), was decreased in BPA and EE2-treated animals, whereas PEA did not change (Figure 1B). In contrast, livers of BPA-treated fish exhibited significant increases of AEA and 2-AG content, similarly to those of EE2-exposed fish (Figure 1C), whereas no changes were observed for OEA. Finally, PEA decreased in the livers of BPA and EE2-treated fish as compared to controls (Figure 1C).

BPA modulates central signals mediating energy homeostasis. In line with the endocannabinoid quantifications described above; we observed moderate variations in the expression of endocannabinoid catabolic and anabolic enzymes in the brain of BPA-treated fish. The 2-AG metabolic enzymes, *dagla* and *mgll*, did not show any significant change. However, the synthetic enzymes of AEA, *abdh4* and *nape-pld*, exhibited an opposite trend, with the former being slightly decreased with BPA and significantly down regulated by EE2 and the latter slightly increased by BPA and EE2 exposure (Figure 2A). In contrast, the AEA catabolic enzyme *faah2a*, was significantly down-regulated in response to BPA and EE2. *Cnr1* presented a clear increase in the BPA-treated group while EE2 elicited no change (Figure 2A). Examining the expression of appetite-regulating genes revealed that the orexigenic neuropeptides Neuropeptide Y (*npy*) and agouti related peptide (*agrp*) showed no significant changes in response to BPA, although they were consistently down-regulated in EE2-treated animals (Figure 2B). In line with this, melacortin receptor 4 (*mc4r*), also displayed a slight but nonsignificant decrease in the EE2 group (Figure 2C). BPA however, significantly down-regulated the anorexigenic signals leptin (*lep*a) and cocaine- and amphetamine-regulated transcript 4 (*cart4*), similar to EE2 (Figure 2C).

BPA induces de novo hepatic fatty acid synthesis by modulation of the ECS, and lipogenic and lipolytic gene expression. As seen above, the liver is significantly influenced by BPA with respect to changes in endocannabinoid levels and fat content. There is a discrepancy between the observed increase in 2-AG levels and the expression of regulatory genes. The synthetic enzyme *dagla* did not show any significant change, while the catabolic enzyme *mgll* increased in expression in zebrafish exposed to BPA (Figure 3A). The AEA synthetic enzymes *abdh4* and *nape-pld*, showed a massive up-regulation in the livers of BPA-

treated animals (Figure 3A). In contrast, EE2 only modestly increased *abdh4*. No changes were found for the catabolic enzyme *faah2a* in response to BPA, while the EE2 group exhibited a strong down-regulation (Figure 3A). Most interestingly, *cnr1* which is a key regulator during conditions of steatosis (18), was also strongly induced by BPA.

Subsequently, we analyzed the expression level of *srebf2* a key lipogenic marker (27), which positively responds to *cnr1* activation (19), adiponectin (*acrp30*), ad-

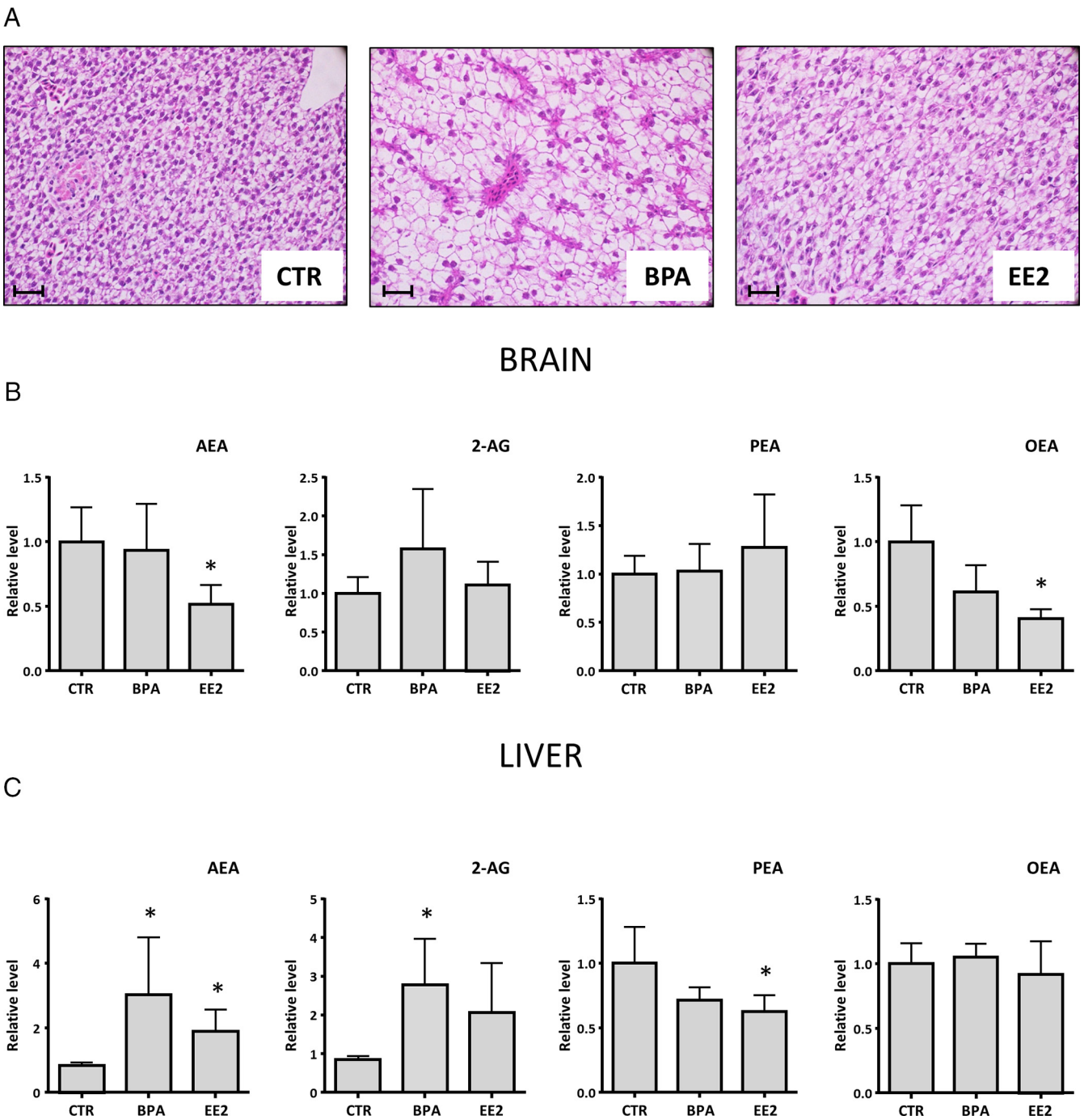

Figure 1. Histological slices and endocannabinoids quantification revealed intense hepatic steatotic state in adult zebrafish brain and liver exposed to BPA and EE2 for 48 hrs. Representative pictures of zebrafish liver slices exposed for 48 hrs to BPA and EE2 (**A**), marked with hematoxilin/eosin stain, unstained structures are composed by lipids and each picture was taken at 20x magnification, scale bar = 20 μm. LC-MS quantification of the main endocannabinoids and N-acylethanolamines in zebrafish brain (**B**) and liver (**C**) treated for 48 hrs with BPA and EE2. Data are expressed in relative units since every point is the mean of three independent experiments and each one of it was compose by five animals per group. Values are represented relative to the control group with mean and standard error and mean. (* $P < .05$ vs EtOH control). Control values were expressed in pmol/mg and normalized with total lipid extract from zebrafish tissues.

ipsin (*cfdl*) and leptin (*lepa*) which are overexpressed in hepatosteatotic livers (28). Both treated groups displayed higher expression of *srebf2*, and a similar trend was found for *acrp30*, but not for *cfdl* and *lepa*, which showed significant increases only in the BPA group (Figure 3B). Analysis of *ppara* mRNA levels revealed an intense down-regulation in the BPA and EE2 treated groups indicating a reduced potential for triglyceride hydrolysis (Figure 3C).

BPA increases neutral lipid content in a Human hepatocyte cell line in a dose dependent manner. In order to confirm the ability of BPA to induce hepatosteatosis through ECS activation, immortalized human hepatocytes (HHL-5) were utilized as an in vitro model. BPA did not affect HHL-5 cell viability at the tested doses (Supplemental Figure 1A), and oleic acid (OA), a well-known steatogenic compound, at low doses, induced a massive production of lipid droplets after only 24 hrs (Supplemental Figure 1B). BPA dose-dependently triggered fatty acid accumulation in HHL-5 cells in 24 hrs, similar to the estrogenic control EE2 (Figure 4A), and lower doses ($\leq$ of 12 μM) then selected, were found ineffective in our acute model (Supplemental Figure 1C).

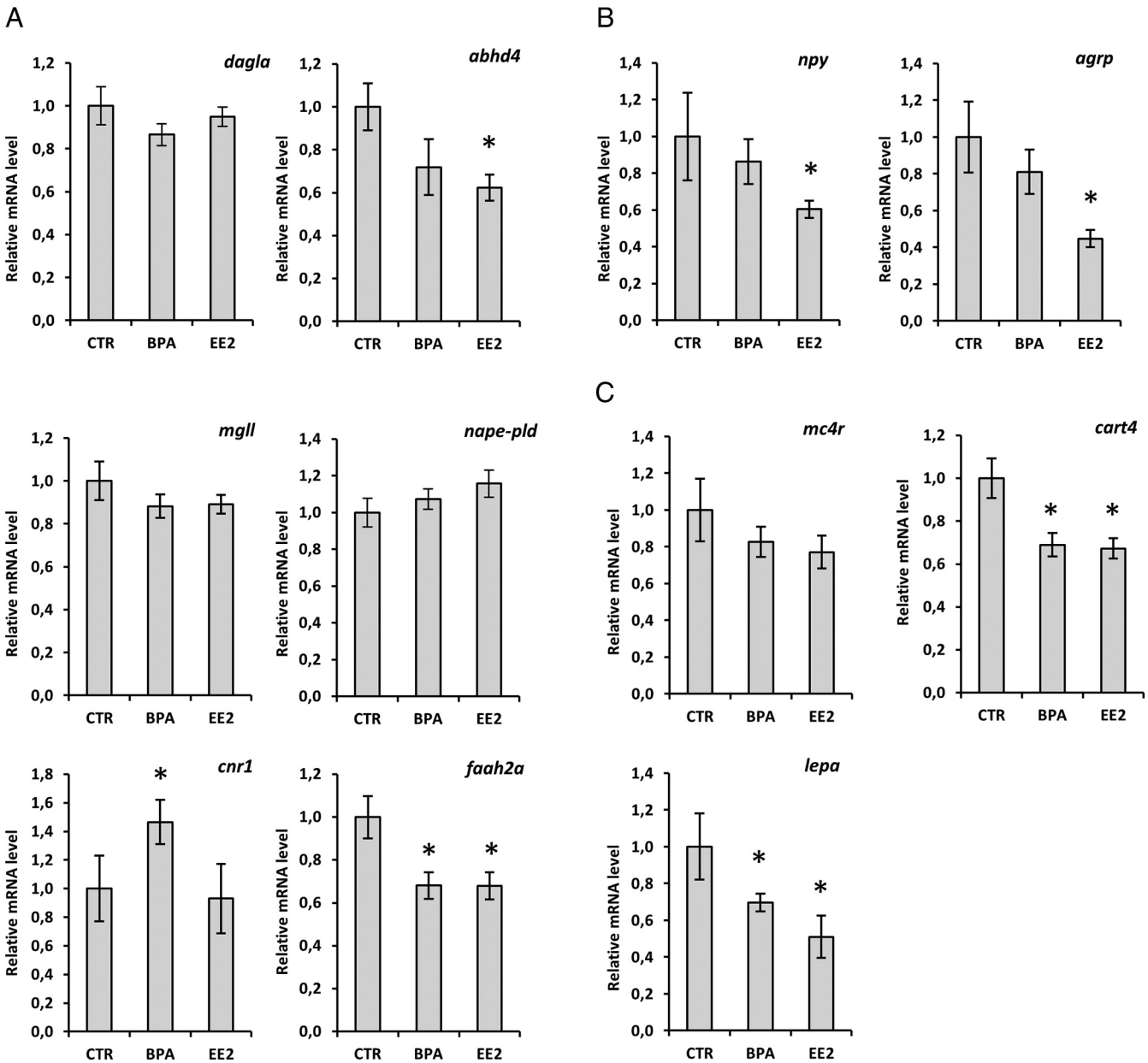

Figure 2. Hypothalamic signals and ECS genes expression in the brain of zebrafish exposed to BPA and EE2. qPCR analysis of genes codifying enzymes for AEA and 2-AG metabolism (**A**), anabolic effectors (**B**) and anorexic signals (**C**) expressed in zebrafish CNS after 48hrs to BPA and EE2 estrogen control. Each single point consist of five livers expressed relative to the control. (Relative expression ± SEM as determined by BioRad CFX Manager Software).

BPA-mediated steatogenesis is attenuated by CB1 selective antagonism. Considering ECS regulation of liver lipid metabolism, and the changes in the ECS observed in the livers of BPA-treated fish, we investigated if BPA acted on hepatocytes through this signaling system. We focused our attention on the CB1 receptor by performing a coincubation of an effective dose (ED) of BPA with two concentrations of the CB1 antagonist AM251, in HHL-5 cells for 24 hrs. The BPA-mediated increase of neutral lipids was dose-dependently blocked by AM251, which was inactive per se (Figure 4B).

BPA exposure enhances AEA production through CB1 activation in HHL-5 cells. Consistent with data from zebrafish livers, BPA induced a significant increase of AEA and reduction of PEA in HHL-5 cells (Figure 4C). Notably, AM251 blocked BPA-mediated overproduction of AEA (Figure 4C). In contrast, AM251 was not able to reverse BPA effects on PEA and OEA reduction, and indeed exacerbated them, while producing a decrease on its own (Figure 4C). No significant differences in 2-AG were noticed among the groups (Figure 4C).

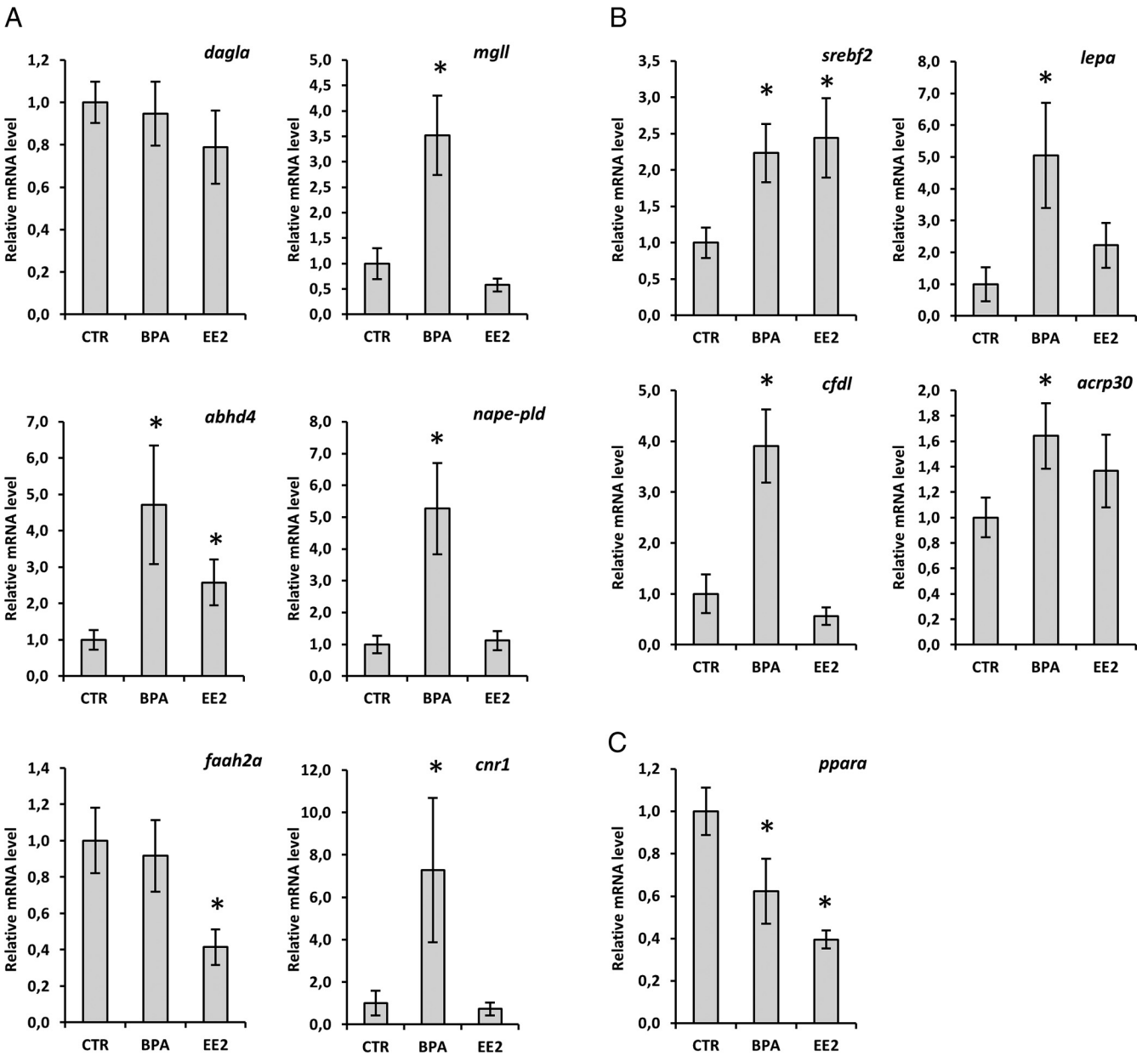

Figure 3. ECS and liver metabolism genes expression of adult zebrafish exposed to BPA and EE2. Expression analysis of genes codifying for AEA and 2-AG catabolic and anabolic enzymes (**A**), lipogenic markers (**B**) and lipolytic genes (**C**) in response to BPA and EE2 exposure for 48 hours. Each single point consist of five livers expressed relative to the control. (Relative expression ± SEM as determined by BioRad CFX Manager Software).

Table 1. Summary table of the primers used for the qPCR analysis with their corresponding gene target name and accession numbers.

Accession Number	Gene name	Forward primer	Reverse primer
AY422992	*elfa*	CTTCTCAGGCTGACTGTGC	CCGCTAGCATTACCCTCC
XM 692781	*dagla*	GAGGGTTTCCGTCGTCAC	TGTTCCTCCAGCAATGATCC
NM 200297	*mgll*	AAGTGAAGGTGAGAGGAT	AATGTCCAACGATGAAGA
NM 001017613	*abdh4*	GCGTCACTCTTATTGAAG	TTAGTCCACCGTATTACA
NM 001080613	*nape-pld*	CTCAAGGACATGCACTCA	GAGCACAATCTTCAAGACAAT
NM 001002700	*faah2a*	AACAACGATGCTTGAACA	TCAGAATGCCCTCACTAT
NM 212820.1	*cnr1*	TCTGTGGGAAGCCTGTTTC	ACCGAGTTGAGCCGTTTG
NM 131074.2	*npy*	GGGGACTCTCACAGAAGGGT	TTTCCCATACCTCTGCCTTGTT
XM 001923904.3	*agrp*	ATCTCATCCACACCTGAGACG	ATTTCAGGCAGTGAGTCTGTGTC
NM 173278.1	*mc4r*	GGTGGACCGCTACATCACAA	GCGCCAGCATGGTAAAGAAC
NM 001082932.1	*cart4*	GGCTGAGGCACTCGATGAA	CCCTACGTCACACCTGGGAAT
NM 001128576	*lepa*	CATCATCGTCAGAATCAG	GGAATCTCTGGATAATGTC
NM 001089466	*srebf2*	ACCATGTCCCAGCAAGTG	TTGGTGGTCAGAAGCAGAG
NM 001045425.1	*acrp30*	AGTCCACCTGATGACAGACAGCC	GCCTTTCTCACCTGCTTCACCTTG
NM 001020532.1	*cfdl*	GCTAAAGCACACTCTCGCCCGT	CACCAGATGTCCTCCCATCCTGAA
NM 001161333	*ppara*	TCTTCAGGAGAACCATTC	ATCGGCAGTATTGACATT
Z27113	*RNAP*	AACCAGAAGCGAATCACC	AACGGCGAATGATGATGG
XM 011523999	*SREBP*	GAAGACTGAGGTGGAGGAC	CAGGACAGGCAGAGGAAG
NM 004104	*FASN*	ACGATGACCGTCGCTGGAAGG	GGTTGATGCCTCCGTCCACGAT
AY237919	*ACACA*	TCCAACCTCAACCACTAT	TGGAGTGAATGAGTTGTC
NM 016083	*CNR1*	TCTGTTCATCGTGTATGC	CTTGGCTAACCTAATGTCC

BPA produces steatosis in hepatocytes via a CB1/AEA positive feed-back loop. In order to rule out the possibility that the effects observed with AM251 were due to its CB1 inverse agonism, we tried to block BPA lipogenic effects with a CB1 neutral antagonist, NESS0327. Both tested doses of this compound significantly inhibited neutral lipid increases produced by BPA (Figure 5A). Similarly, qPCR analysis of key genes involved in de novo hepatic fatty acid synthesis (*srebp-1c*, *fasn* and *acaca*) confirmed a strong up-regulation in response to BPA exposure (Figure 5B), which was partially or totally blocked by coincubation with NESS0327. Given the above, we hypothesized that exogenous AEA, the endogenous levels of which are elevated by BPA, could induce hepatosteatosis, and that CB1 blockade would inhibit this effect. In line with this, we found that several doses of AEA increased neutral lipid content, and this was blocked by NESS0327 (Figure 5C). BPA did not exhibit any noticeable affinity for human recombinant CB1 receptors in a displacement/binding assay up to 100 μM (Supplemental Figure 1D), indicating that it activates CB1 only via its effect on AEA levels.

BPA requires CNR1 to induce steatosis and impairs human FAAH activity. To further investigate the positive feedback loop described above, we carried out knockdown experiments to better evaluate the role of the *cnr1* gene in BPA-induced steatosis and assayed BPA effects on FAAH activity to gain an understanding of how it increases AEA levels. In HHL-5 cells in which *CNR1* gene expression was silenced by 80%, BPA was unable to induce lipid accumulation, unlike mock transfected cells (Figure 5D). In FAAH activity assays, HHL-5 cell membrane fractions incubated with BPA had 40% reduced FAAH activity with respect to controls (Figure 5E). Moreover, treating HHL-5 cells with BPA for 24 hrs prior to membrane isolation also resulted in the reduction of FAAH activity at both concentrations tested, ie, 45 μM (15.16% $\pm$1.3, n = 3) and 90 μM (20.93% $\pm$2.01, n = 3).

Chronic exposure to low BPA doses produces transient FAAH inhibition and steatosis in HHL-5 cells. In view of the daily BPA human exposure scenario, we wanted to extend our in vitro experiments also to chronic studies with nanomolar doses of BPA. Starting from 1 nM, we tested three log units of BPA concentrations in a six-day exposure protocol, using EE2 as an estrogen control. Except for the lowest concentration (1 nM), all the BPA treated groups, showed a significant increase in neutral lipid content with respect to the control (Figure 6A). Interestingly, the chronic BPA treatment produced a comparable steatotic induction (roughly a 25% increase with respect to the control) as that observed with acute treatments. In parallel, we also assessed FAAH activity in HHL-5 cells treated with 10 and 100 nM BPA. No differences were found in FAAH activity after six days of treatment (Figure 6B). Similarly, nanomolar doses of BPA were unable to inhibit FAAH activity after 24 hrs treat-

ment. On the other hand, both tested doses of BPA after 48 hrs exposure produced a significant reduction in FAAH activity (Figure 6B), ie, 10 nM (20% ±4.6, n = 3) and 100 nM (29% ±2.4, n = 3).

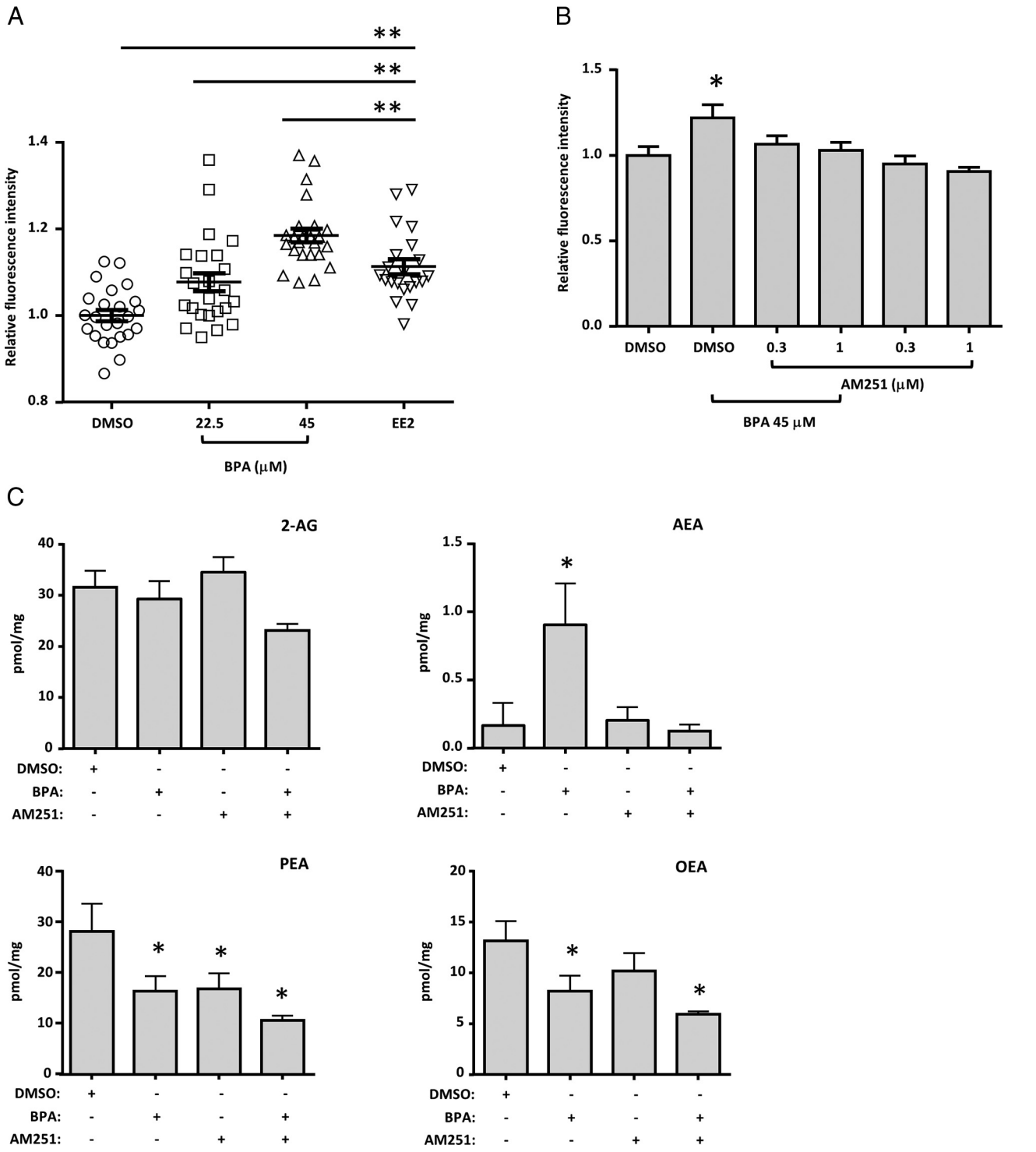

Figure 4. Adipored assay and endocannabinoid levels in HHL-5 treated with BPA and CB1 antagonist. Adipored staining quantification of HHL-5 cells treated with BPA using DMSO as vehicle at the indicated concentrations and EE2 (2 μM) as an estrogen control for 24 hrs (**A**). Combinatory experiment with one ED of BPA (45 μM) and two doses of CB1 antagonist AM251 at 1 and 0.3 μM, exposed for 24 hrs (**B**). LC-MS quantification of levels of the main endocannabinoids and N-acylethanolamines in HHL-5 cell line after 72 hrs exposure with BPA and AM251 (**C**). Individual well fluorescence was measured from nine separate points in a 3 × 3 grid with a Genios Pro plate reader. Data are expressed as the mean and standard error of the mean of signal from 96 wells and LC-MS quantifications are the mean of five single 100 mm cell plate. (* $P < .05$ vs DMSO control, ** $P < .01$).

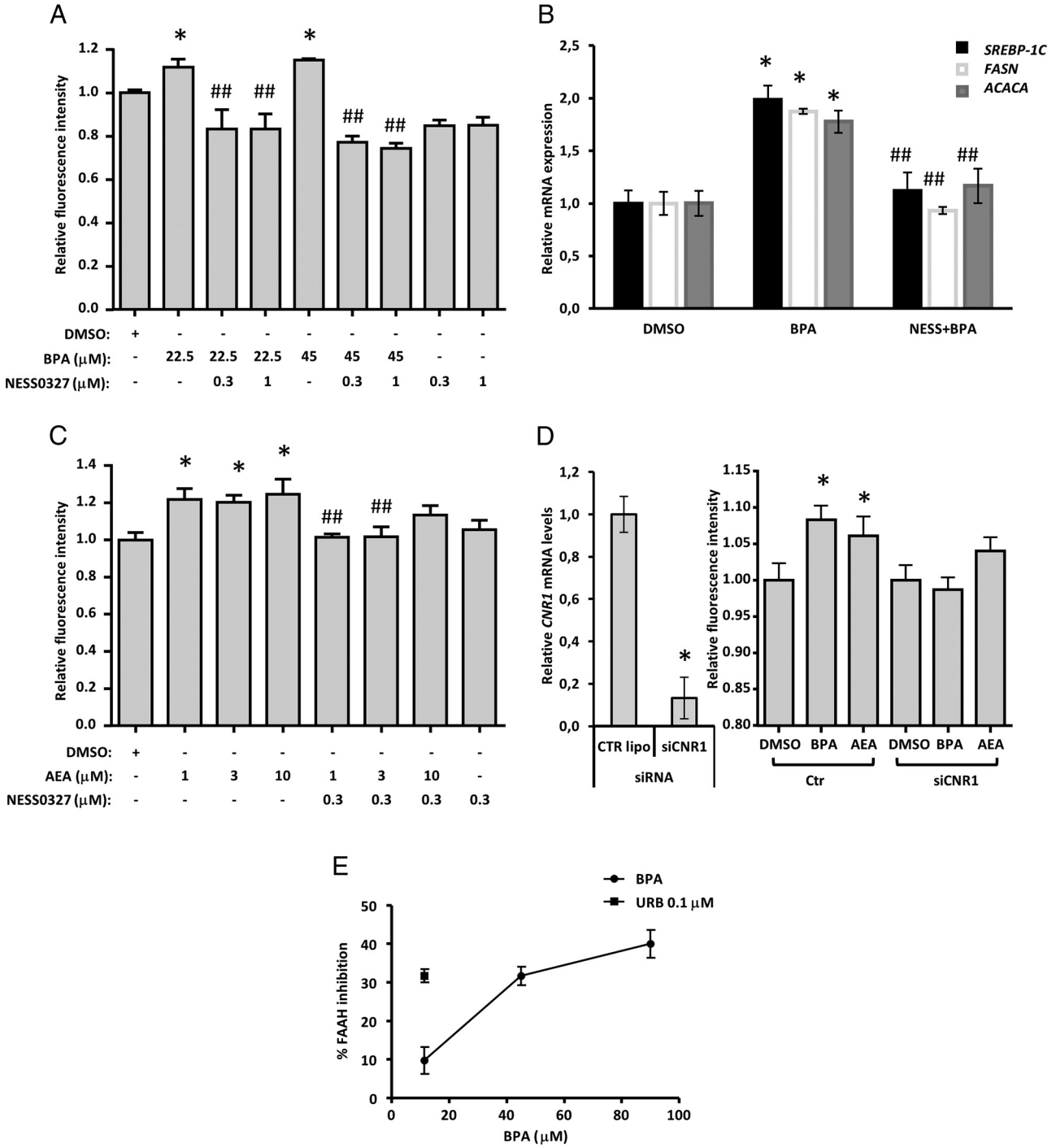

Figure 5. BPA triggers an AEA positive feed-back loop in HHL-5 cells by inhibiting FAAH enzymatic activity, involving CB1 activation and its downstream gene mediators. Adipored fluorescence signal on HHL-5 cells treated for 24 hrs with BPA and two doses of NESS0327 at 1 and 0.3 μM (**A**); the lowest dose of BPA (22.5 μM) and highest of NESS0327 (1 μM) were followed also for the qPCR analysis on *srebp-1c, fasn* and *acaca* the main *cnr1* downstream genes involved in de novo fatty acid synthesis (**B**); combinatory experiment with three doses of AEA at 1, 3, 10 μM and NESS0327 at 0.3 μM on HHL-5 exposed for 24 hrs (**C**). siRNA experiment was performed in triplicate with same experimental condition for the control and *cnr1* siRNA, after 24 hrs silencing the cells were treated with BPA at 45 μM and AEA at 3 μM for additional 24 hrs and then neutral lipid content was evaluated by Adipored fluorochrome (**D**). Total RNA was collected at the beginning and after the treatment with the aforementioned compounds at 24 hrs to confirm the *cnr1* knockdown (**D**). FAAH enzymatic activity was measured on HHL-5 membrane fraction after 30 minutes exposure with BPA at 11.25, 45, 90 μM (**E**), data are reported as percentage of inhibition of FAAH activity respect to the control group, setting as maximal inhibition URB597 at 0.1 μM. Adipored assay was measured as individual well fluorescence from nine separate points in a 3 $\times$ 3 grid by Genios pro plate reader, data is expressed as the average and standard error of the mean of signal from 96 wells, qPCR data is reported as relative expression $\pm$ SEM by BioRad CFX Manager Software. (* $P < .05$ vs DMSO control).

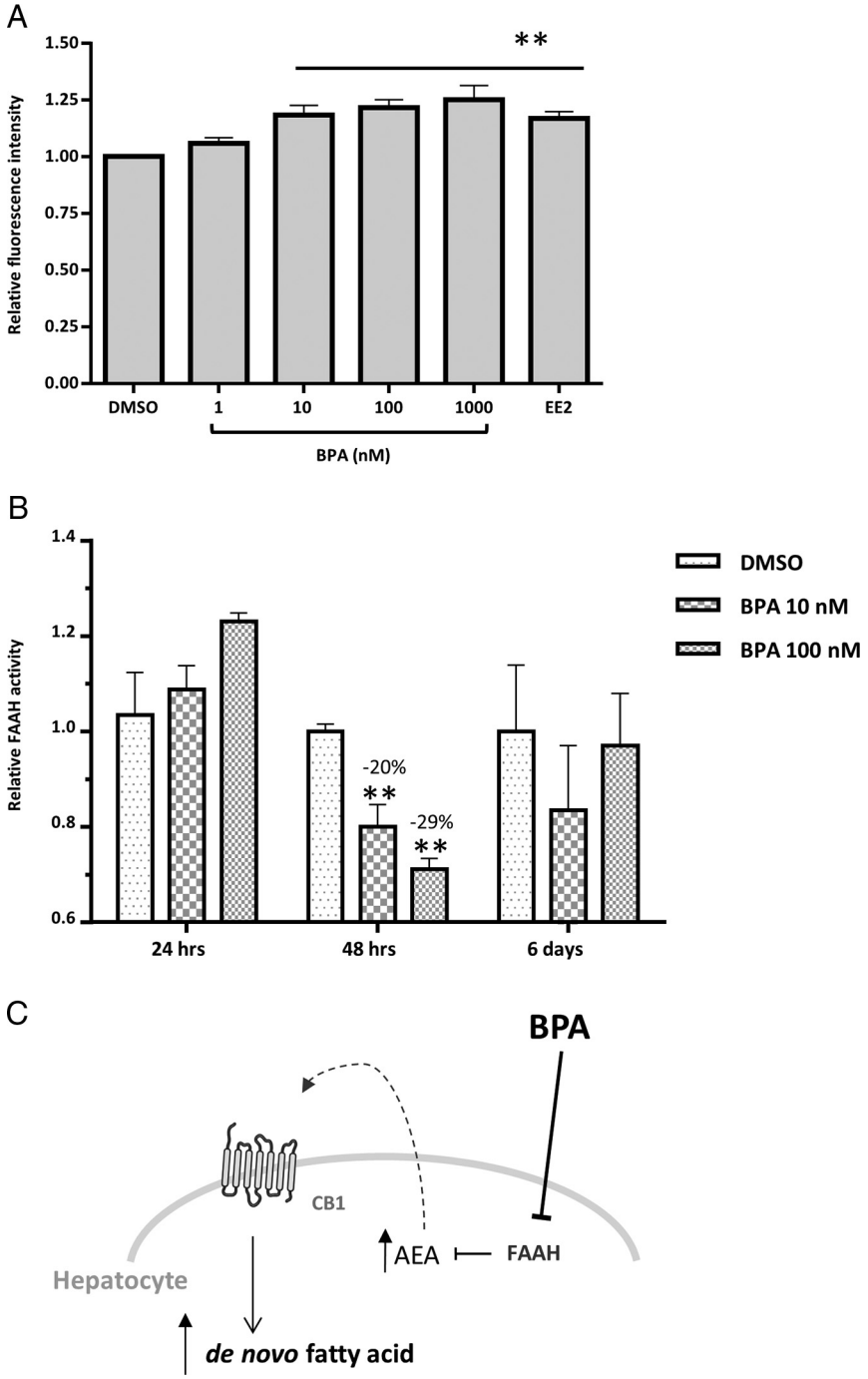

Figure 6. Adipored assay and FAAH activity in HHL-5 cells chronically treated with nanomolar doses of BPA. HHL-5 cells were chronically treated with BPA using DMSO as vehicle at the indicated concentrations and EE2 (2 μM) as an estrogen control for six days (n = 11) (**A**). Data are shown as relative values with respect to the corresponding DMSO control. Individual well fluorescence was measured from twelve separate points with an Envision plate reader. HHL-5 cells were exposed for 24, 48 hrs or 6 days to 10, 100 nM of BPA or DMSO vehicle, and then harvested and FAAH activity assayed (**B**). FAAH activity values were measured by pmol of hydrolyzed [^{14}C]AEA after 30 minutes incubation with 7 μg of membranes. Data are expressed as relative value respect to the DMSO control and standard error of the mean. (* $P < .05$ vs DMSO control, ** $P < .01$). A model of BPA-mediated NAFLD via CB1 activation (**C**). BPA exerts its steatogenic effects by inhibiting FAAH activity, resulting in increased AEA levels and subsequent CB1 activation. This initiates a positive feedback loop which up-regulates CB1 gene expression and its downstream targets involved in de novo fatty acid synthesis.

Discussion

The estrogenic activity of bisphenol A (BPA) has been known since the 1930s, yet it is still used as an emollient in polycarbonate plastics and epoxy resins resulting in sustained environmental contamination and daily exposure. BPA has a wide range of biological targets, exerting metabolic interference by antagonizing thyroid receptors (29), modulating insulin and glucagon responses in pancreatic cells (13), and influencing body weight and adipokine release (12, 30). Here, we evaluated the effects of BPA environmental contamination on liver energy metabolism using zebrafish as an in vivo model. Furthermore, given the role of ECS dysregulation in metabolic disorders, and its conserved function in zebrafish (31), we assessed the effects of BPA on this system in this vertebrate model and in human hepatocytes. We propose the ECS as a target through which BPA acts to induce fatty acid accumulation in the liver and possibly disrupt appetite regulation.

We show for the first time that BPA produces hepatosteatosis in zebrafish, similar to the reported effects on mouse liver (15). These effects were associated with decreased OEA, a potent anorexic signal involved in the peripheral regulation of food intake (24), in the CNS. Alternatively 2-AG, also a regulator of feeding behavior (32), showed a trend toward an increase in response to BPA. In contrast, AEA brain levels did not change in response to BPA, although they were reduced by EE2. A better understanding of central endocannabinoid tone comes from the gene expression profile of receptors and regulatory enzymes. A consistent increase of *cnr1* transcript levels in BPA-treated animals suggests a possible increase of food intake mediated by 2-AG, as no significant

changes were found for *dagla* and *mgll* genes. Alternatively, it is possible that the elevation of brain 2-AG levels was due to other factors, such as the availability of biosynthetic precursors and substrates for DAGLα, or changes in the protein and enzymatic activity levels of this enzyme and MAGL. Regarding AEA production, we reported a compensation between the two synthesizing enzymes *adbh4* and *nape-pld* in treated animals, which might explain why no net difference in AEA levels were found in the brains of treated zebrafish. *Faah2a* expression, however, did exhibit a significant down-regulation following both BPA and EE2 exposure, underlying a possible estrogen influence on the levels of this gene. Indeed, the presence of an estrogen response element (ERE) within the *faah* promoter has been reported, which may lead to a direct (33) or indirect (34) regulation of zebrafish brain *faah2a*. Among the hypothalamic signals that control food intake, *cart4* gene exhibited a decreased expression correlated with *cnr1* up-regulation, in agreement with a relationship already described in zebrafish (35) and mammals (21). Taken together these findings reveal a general reduction of brain anorexigenic signals linked to the endocannabinoid system following BPA treatment. However, the neuropeptide Y/agouti-related protein system did not change in correlation with the ECS profile, in line with previous studies, which suggested independent stimulation of food intake by CB1 receptors and neuropeptide Y (20). At any rate, since we did not see any significant changes in food intake following zebrafish exposure to BPA (Supplemental Figure 1E), we cannot speculate on the functional consequences of the alterations of the transcripts and of endocannabinoid levels, other than concluding that BPA-induced hepatosteatosis in zebrafish does not appear to be a consequence of increased energy intake.

Despite the role of the ECS in the CNS, peripheral perturbations driven by this xenoestrogen compound appear to be more important. Given the observed hepatosteatosis, elevated hepatic 2-AG levels in BPA and EE2 groups suggest a possible production of this endocannabinoid from stellate cells working through a CB1-mediated pathway to enhance lipogenic gene expression, similar to alcohol-induced steatosis in mice (36). Increased 2-AG levels were not correlated with changes in the expression of its metabolic enzymes, strengthening the hypothesis that 2-AG is mostly synthesized in stellate cells (which account only for 5%–8% of the total liver cells (37)), and suggests that *mgll*, the mRNA levels of which were increased, is not involved in 2-AG level control as much as general lipid metabolism (38). Concerning hepatic AEA levels, we reported a concrete increase of these too, suggesting a potential synergistic action with 2-AG to act as local enhancers of triglyceride levels in hepatocytes through CB1 stimulation (39). In fact, this overproduction of AEA correlated with increased expression of *cnr1* transcripts in treated zebrafish livers, similar to results obtained in mouse organotypic liver slices (40), as well as with the up-regulation of the mRNAs of AEA biosynthetic enzymes. We also observed up-regulation of *srebf2* transcripts in the liver of both treated groups, consistent with data linking *cnr1* to *srebp-1c* expression and elevated de novo fatty acid synthesis in zebrafish livers (41). Additional evidence of intense lipogenesis comes from the elevated expression of leptin, adiponectin (*acrp30*) and adipsin (*cfdl*), which are expressed only in fatty livers (28). BPA-mediated NAFLD progression, may result not only from enhanced lipogenesis but also thorough reduced lipolysis. Endocannabinoid-like molecules, such as PEA, present noncannabimetic activities, such as stimulation of PPARα, which mediates peripheral lipolytic activities (25). A significant hepatic reduction of this acylethanolamide was observed in the livers of both treated groups, which correlates with the observed down-regulation of *ppara* gene expression within the liver, suggesting a possible reduction of lipolysis.

BPA induced de novo fatty acid production also in the human HHL-5 hepatocyte cell line after 24 hrs, with magnitude similar to that of our estrogen control, consistent with the observations in mice (15), where BPA at low doses up-regulates lipogenic genes related to NAFLD. Since CB1 antagonist/inverse agonists have been reported to ameliorate hepatic insulin resistance and steatosis, by exerting important effects on lipid metabolism (16), we tried to block the steatogenic effect of BPA with a specific CB1 antagonist (AM251). Importantly, blocking CB1 did reverse the effect of the most ED of BPA. Consistent with the findings in zebrafish liver, following exposure to BPA, we observed a reduction of the two acylethanolamides, PEA and OEA, which are involved in lipolysis independent of CB1. These BPA-induced changes were not blocked by AM251, further suggesting CB1-independent effects on lipolytic processes within the liver. However, BPA also caused an increase of AEA levels in HHL-5 cells, which in line with the in vivo data, and AM251 reversed the BPA-induced increase. CB1 neutral antagonism (NESS0327) also blocked BPA upregulation of AEA, supporting an essential role for CB1 activation in BPA lipogenic activity not only by stimulating per se the expression of lipogenic enzymes, but also by further enhancing AEA levels and hence causing a potential vicious circle leading to more triglyceride accumulation in hepatocytes. qPCR analysis also confirmed the partial dependence of BPA steatogenic potential on CB1 activity; in fact, the increased expression of key genes involved in hepatic lipogenesis induced by

BPA was partially counteracted by NESS0327. Finally, knockdown experiments underlined the essential role of the CB1 receptor in this pathway (19, 42), since BPA and AEA were totally ineffective at triggering de novo fatty acid synthesis when *CNR1* was silenced. Once outlined the involvement of the ECS in acute exposure conditions, we confirmed a similar steatogenic potential of BPA also following chronic treatments, using low doses of this contaminant in line with the human exposure levels (43). After six days of treatment with nanomolar doses of BPA, human hepatocyte showed a significant triglyceride increase comparable to the steatotic insult previously described in the acute experiments. As a biomarker for the chronic BPA obesogenic effect, we monitored FAAH activity over time. Interesting, we noticed a transient inhibition of FAAH activity that culminated after 48 hrs exposure with 10 and 100 nM of BPA, with no differences being found at earlier or later time points. The reduction in FAAH activity (roughly 25% of inhibition) is comparable with the inhibition of this hydrolytic enzyme after 24 hrs of acute BPA exposure, and therefore we can speculate that this effect is translated to AEA accumulation.

Steatogenic agents increase CB1 activity by stimulating synthesis or reducing degradation of endocannabinoids (44); therefore we believe that, in BPA-treated hepatocytes, AEA is feeding a positive feedback loop together with the CB1 receptor, which is necessary for the progression of BPA-induced NAFLD (Figure 6C). Indeed, AEA was able to produce steatosis in HHL-5 cells and this effect was totally abolished by CB1 antagonism. Similarly, BPA was unable to exert its steatotic influence in *cnr1*-knockdown hepatocytes. Moreover, similar to a HFD condition, we showed that the pollutant was able to directly inhibit the hydrolytic activity of the human FAAH, thus potentially explaining its selective increase of AEA levels (19). Taken together these data show for the first time that BPA is able to produce NAFLD in adult zebrafish in a manner mediated, at least in part, by endocannabinoid action at CB1 receptors. Furthermore, we showed the steatogenic effect of BPA also in human hepatocytes and confirmed the presence of positive feedback loop between AEA and CB1 resulting from FAAH inhibition which is essential for the priming of de novo fatty acid production. These results clearly indicate that BPA induces metabolic alterations in humans as well as in *Danio rerio* hepatocytes. In addition, the present study highlights for the first time the effects of BPA on the ECS, suggesting the use of endocannabinoids and related mediators, as well as of their metabolic enzymes, as novel biomarkers for BPA monitoring.

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