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Altered gut microbiota and endocannabinoid system tone in vitamin D deficiency-mediated chronic pain

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ABSTRACT

Recent evidence points to the gut microbiota as a regulator of brain and behavior, although it remains to be determined if gut bacteria play a role in chronic pain. The endocannabinoid system is implicated in inflammation and chronic pain processing at both the gut and central nervous system (CNS) levels. In the present study, we used low Vitamin D dietary intake in mice and evaluated possible changes in gut microbiota, pain processing and endocannabinoid system signaling.

Vitamin D deficiency induced a lower microbial diversity characterized by an increase in Firmicutes and a decrease in Verrucomicrobia and Bacteroidetes. Concurrently, vitamin D deficient mice showed tactile allodynia associated with neuronal hyperexcitability and alterations of endocannabinoid system members (endogenous mediators and their receptors) at the spinal cord level. Changes in endocannabinoid (anandamide and 2-arachidonoylglycerol) levels were also observed in the duodenum and colon.

Remarkably, the anti-inflammatory anandamide congener, palmitoylethanolamide, counteracted both the pain behaviour and spinal biochemical changes in vitamin D deficient mice, whilst increasing the levels of Akkermansia, Eubacterium and Enterobacteriaceae, as compared with vehicle-treated mice. Finally, induction of spared nerve injury in normal or vitamin D deficient mice was not accompanied by changes in gut microbiota composition.

Our data suggest the existence of a link between Vitamin D deficiency – with related changes in gut bacterial composition – and altered nociception, possibly via molecular mechanisms involving the endocannabinoid and related mediator signaling systems.

1. Introduction

In addition to the typical classification of the central nervous system (CNS) as an immune privileged area, increasing evidence points to the reciprocal communication between the periphery and brain in neurophysiological features, including mood and behavior (Bercik et al., 2011; Johnston et al., 2014). In particular, the gut microbiota is increasingly considered one of the most important actors in the bidirectional interactions between the intestine and brain, in both physiological and pathological conditions (Scriven et al., 2018). Several findings indicate that gut microbiota perturbations may produce a local, and subsequently, systemic inflammation, which is believed to trigger neuroinflammation and alterations in brain functioning and behavior (Bercik et al., 2010; Guida et al., 2018; Valles-Colomer et al., 2019). These findings open the possibility of microbiota-modulating approaches for the treatment of psychiatric diseases and other CNS

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disorders (Kelly et al., 2017; Misra and Mohanty, 2017). Studies in germ-free mice and antibiotic-treated animals suggested that the gut microbiota may play a role in pain perception (Luczynski et al., 2017; O'Mahony et al., 2014; Verdu et al., 2006). However, no studies have explored the role of gut microbiota in chronic pain states, i.e. neuropathic pain. Neuropathic pain is a debilitating pathological condition characterized by abnormal pain perception that is manifested by painful responses to non-painful stimuli (allodynia), which can persist after the leading injury is repaired. This complex process involves plasticity phenomena exerted through modifications in cytokine, chemokine and lipid messenger release, mainly by non-neuronal cells – i.e. microglia and astrocytes - able to induce neuronal sensitization at the spinal cord level. In this context, endocannabinoids and biochemically related mediators, by being important modulators of glia-neuron crosstalk, are strongly implicated in inflammation-based pathological states, including neuropathic pain (Luongo et al., 2010). Specifically, the observed dysregulation of endocannabinoid signaling at CB1 and CB2 receptors (CB1R and CB2R), at both the gut (Borrelli and Izzo, 2009; Capasso et al., 2014) and CNS (Giordano et al., 2011; Petrosino et al., 2007) level, have suggested that compounds able to restore a physiological endocannabinoid tone, such as *N*-palmitoylethanolamine (PEA), may be used for the treatment of inflammation and pain (Guida et al., 2017; Guida et al., 2015b; Luongo et al., 2013), as well for the correction of some of the central effects associated with microbiota perturbation (Cristiano et al., 2018). Indeed, pathophysiological mechanisms linking the gut microbiota to the endocannabinoid system in dysbiosis-induced systemic inflammation have been recently proposed (Cani et al., 2016, Guida et al., 2018). Among them, increases in the abundance of *A. muciniphila* associated with an improved metabolic profile in obese mice have been correlated also with changes in the gut levels of endocannabinoids and endocannabinoid-like mediators (Everard et al., 2013; Everard et al., 2019).

In the present study, we used a model of Vitamin D deficiency to induce microbiota perturbation (Jin et al., 2015; Jin et al., 2005) in mice. Indeed, Vitamin D plays a significant role in modulating the immune system in both periphery and CNS. In fact, vitamin D is an important calcium-regulating factor essential for bone homeostasis, metabolism (Plum and DeLuca, 2010) and gut functioning (Sun, 2018). Altered Vitamin D status have been linked with changes in epithelial barrier functions and microbiome setting in clinical disorders, including irritable bowel syndrome (Jin et al., 2015; Lim et al., 2005; Shang and Sun, 2017; Su et al., 2016). In addition, Vitamin D, as a neuroactive steroid, exerts neuroimmune-modulating effects resulting in anti-inflammatory and neuroprotective actions potentially useful against neurodegenerative and autoimmune diseases (De Abreu et al., 2009). Indeed, an inverse correlation between Vitamin D levels and painful manifestations has been recently demonstrated to occur in disorders characherised by an over-activated immune system (Akyuz et al., 2017; Herly et al., 2018; Zadro et al., 2017).

On these bases, we tested the hypothesis that Vitamin D deficiency contributes to pain development, and evaluated whether it does so through the modulation of the endocannabinoid system and the gut microbiome. Therefore, we modified Vitamin D intake in healthy and neuropathic animals. Mice were submitted to behavioral, biochemical and electrophysiological tests to assess whether Vitamin D status may affect behavior and gut microbiota composition, together with the concentrations of central and peripheral endocannabinoids and related *N*-acylethanolamines. Moreover, since one of such *N*-acylethanolamines, PEA, is a potent modulator of inflammation and pain, this compound, administered as a micronized formulation previously suggested to exhibit elevated bioavailability (Impellizzeri et al., 2014), was used as pharmacological tool to investigate the role of endocannabinoid-like mediators in the consequences of Vitamin D deficiency in both the gut and spinal cord.

2. Material and methods

2.1. Animals and treatments

Male C57/bl6 mice (3 weeks) obtained from Envigo Laboratory were housed controlled illumination and environmental conditions for 1 week before the commencement of experiments.

The experimental procedures were approved by the Animal Ethics Committee of University of Campania of Naples. Animal care was in compliance with the IASP and European Community (E.C. L358/1 18/12/86) guidelines on the use and protection of animals in experimental research. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Mice were fed a diet with low vitamin D concentration (< 5 IU/kg cholecalciferol, purified vitamin D3 deficient diet, Art No E15312-24) or with normal vitamin D concentration (1500 IU/kg cholecalciferol, purified control diet, Art No E15000-04). Both diets were identical in terms of all other components including calcium and phosphorus and were obtained from Ssniff, Soest, Germany (Latimer et al., 2014). After 6 weeks, [25(OH)D] concentrations in serum were measured and mice were submitted to the behavioral testing. Afterwards, controls and Vitamin D deficient mice were treated intraperitoneally (i.p.) with palmitoylethanolamide (PEA) (10 mg/kg), or CB1 receptor agonist arachidonyl-2-chloroethylamide (ACEA) (10 mg/kg), or CB2 receptor agonist (JWH133) (1 mg/kg) or vehicle (5% Pluronic acid) (see Fig. 1 A). Ultramicronized PEA (um-PEA) has been provided by Epitech group Spa (Italy). The CB1 receptor agonist arachidonyl-2-chloroethylamide (ACEA), and CB2 receptor agonist (JWH133) were purchased were purchased from Tocris Bioscience, Bristol, UK.

After behavioral testing, animals were subjected to biochemical evaluations and microbiota analysis. A separate group of animals was used for *in vivo* electrophysiological recording.

2.2. Serum measurements

Serum samples were centrifuged and kept frozen at −20 °C until analysis. The levels of [25(OH)D] were measured by quantitative chemiluminescent microparticle immunoassay (CMIA) Architect 25-OH Vitamin D (Abbott Laboratories).

2.3. Behavioral testing

2.3.1. Pain

Mechanical allodynia was assessed by using a Simplified Up-Down method (SUDO), consisting in a modified version of the up-down method introduced by (Chaplan et al., 1994). Mice were placed on a wire mesh platform and, 1 h after habituation period, calibrated von Frey filaments (North Coast Medical, Gilroy, CA, USA; ranging from 0.002 to 2.0 g bending force) were applied to the hind paw for 3 to 4 s. Five von Frey filament presentations per test were executed and a lack of response to a filament dictates that the next higher filament is used in the following stimulation, while a positive response dictates the use of the next lower filament. The PWT is assumed to exist in the vicinity of a stimulus level where the animal first changes its response pattern: a negative response followed by a positive response or vice versa. The PWT estimation is recorded as the response to the fifth stimulus.

Mice were tested at day 0 (baseline), at 6 weeks (diet timing) before SNI and, at day 7 post SNI when the allodynia was established and after various pharmacological interventions every 10 min for 60 min. The mean of paw withdrawal thresholds (g) was reported for each experimental group.

Tail flick test. Mice were gentle placed on the tail flick unit (Ugo Basile, Italy) and the thermal stimulus was elicited by a radiant heat source focused on the mice tail approximately 1–3 cm from the tip. Tail-flicks were elicited before and after injecting drugs every 10 min for 60 min. The mean (sec) of paw withdrawal thresholds was reported for

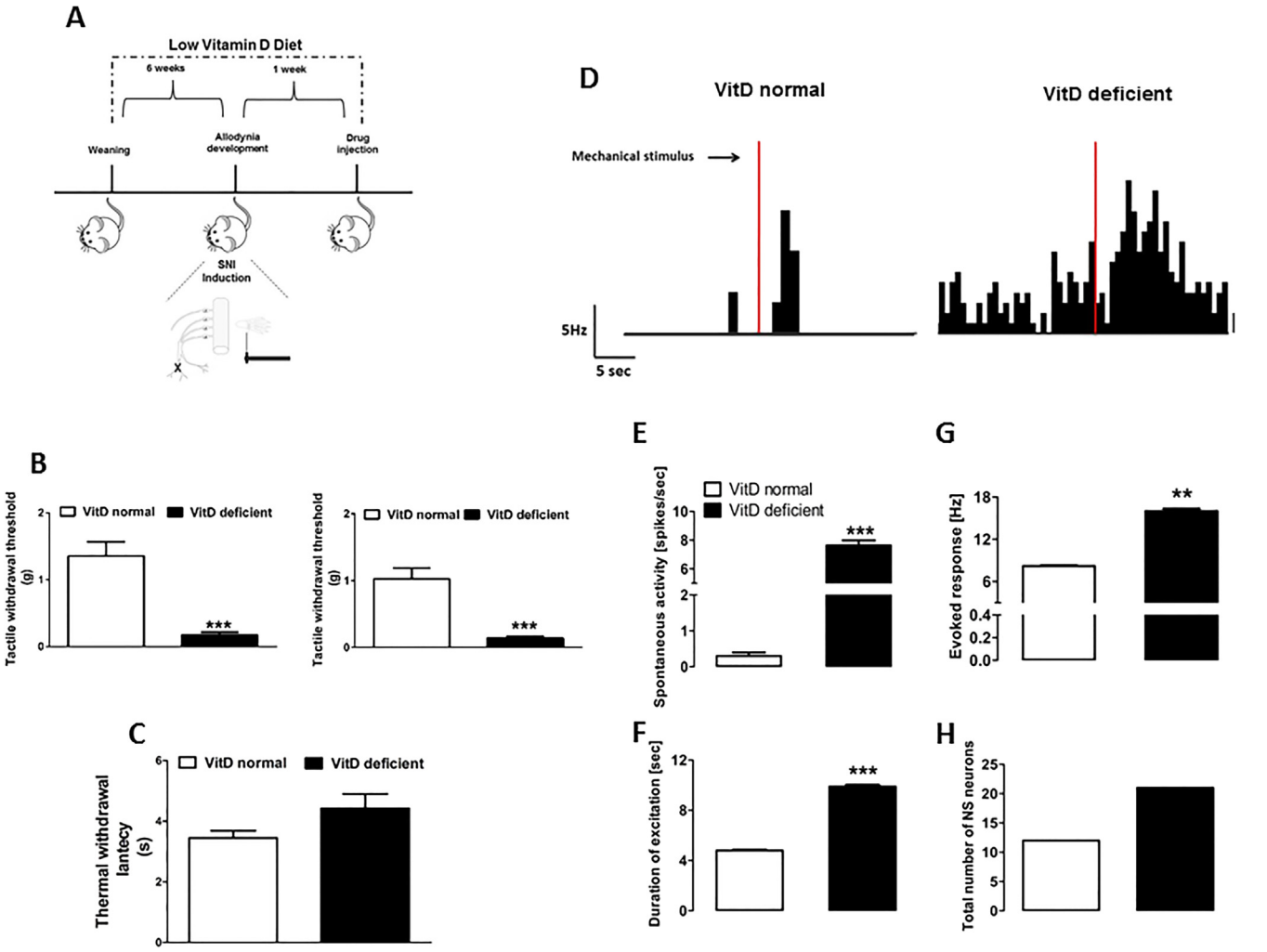

Fig. 1. Effects of Vitamin D deficiency on pain behavior and spinal neuronal sensitization. A) Experimental design of diet administration, induction of neuropathic pain (SNI), drug injection and pain measurements. B) Tactile withdrawal threshold, right and left paw. C) thermal nociceptive responses in normal (VitD normal) and vitamin D deficient (VitD deficient) mice. Data are expressed as mean ± SEM, (***) $P < 0.001$ versus VitD normal mice, (n = 14). D) Peristimulus time histograms (PSTHs) of responses to the paw painful pressure in nociceptive specific neurons (NS) of the VitD normal (left) and VitD deficient (right) mice. H) Number of total NS neurons in both VitD normal and deficient mice. E), G) and F) Spontaneous activity (spikes/s), the frequency (Hz) and the duration (sec) of NS neurons responses evoked by noxious mechanical pressure applied to the paw for 2 s, in VitD normal and in VitD deficient mice, respectively. Data are expressed as mean ± SEM, (**) indicates $P < 0.01$, and (***) $P < 0.001$ versus VitD normal mice, (n = 5). One way ANOVA, post-hoc Tukey's. H) Total number of NS neurons recorded in VitD normal and in VitD deficient mice.

each experimental group.

2.4. Neuropathy induction

The spared nerve injury. Mononeuropathy was induced according to the method of Decosterd and Woolf (Boccella et al., 2018; Decosterd and Woolf, 2000). Mice were anesthetized by intraperitoneal injection of ketamine xylazine (60 mg/kg + 10 mg/kg). The sciatic nerve was exposed at the level of its trifurcation into sural, tibial, and common peroneal nerves. The tibial and common peroneal nerves were ligated tightly with 7.0 silk threads and then transected just distal to the ligation, leaving the sural nerve intact. Sham mice were anesthetized, and the sciatic nerve was exposed at the same level, but it was not transected.

2.5. Western blotting analysis

Each animal, previously anesthetized, was decapitated and spinal cord was removed, washed twice in cold PBS (without Ca^{2+} and Mg^{2+}, pH 7.4) and homogenized in protein lysis buffer [HEPES 25 mM; EDTA 5 mM; SDS 1%; Triton X-100 1%; PMSF 1 mM; MgCl2 5 mM; Protease Inhibitor Cocktail (Roche, Mannheim, Germany); Phospahatase Inhibitor Cocktail (Roche, Mannheim, Germany)] as previously described (20). Lysates were then centrifuged for 15 min at 13,000 g at 4 °C, and the supernatants transferred into clear tubes and quantified by DC Protein Assay boiled for 5 min in Laemmli SDS loading buffer and loaded on 10–15% SDS-polyacrylamide gel electrophoresis and then transferred to a PVDF membrane. Filters were incubated overnight at 4 C with the following antibodies: (a) polyclonal anti-VDR antibody (1:500, Elabscience: E-AB-33210); (b) polyclonal anti-CB2 (1:500, Abcam: Ab3561); (c) mouse anti-CB1 antibody (1:1000, Calbiochem: 209550); (d) Anti-PPARα antibody (1:500, Abcam: ab2779). The monoclonal GAPDH (1:5000, Santa Cruz Biotechnology, Dallas, Texas) antibody was used to check for equal protein loading. Reactive bands were detected by chemiluminescence (ECL or ECL-plus; Perkin-Elmer). Images were analyzed on a C-DiGit™ Chemiluminescent Western Blot Scanner (LI-COR Biosciences, 4647 Superior Street Lincoln, NE 68504-5000).

2.6. Extracellular recordings of nociceptive specific neurons (NS)

For *in vivo* single unit extracellular recording, mice were initially anesthetized with Avertin (1.25%). After tracheal cannulation, a catheter was placed into the right external jugular vein to allow continuous infusion of propofol (5–10 mg/kg/h, i.v.). Spinal cord segments L4–L6 were exposed medially by laminectomy, near the dorsal root entry zone, up to a depth of 1 mm (McGaraughty et al., 2010). An elliptical rubber ring (about 3 × 5 mm) was tightly sealed with silicone gel onto the surface of the cord. This ring formed a trough with about 50 µL capacity over the spinal segments used for topical spinal drug application. It also provided access to the spinal neurons that receive input from the ipsilateral paw, where the mechanical stimulation was applied. Animals were then secured in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) supported by clamps attached to the vertebral processes on either side of the exposure site. The exposed area of the spinal cord was initially framed by agar and then filled with mineral oil. Body temperature was maintained at 37 °C with a temperature-controlled heating pad. A glass-insulated tungsten filament electrode (3–5 MΩ; FHC Frederick Haer & Co., ME, USA) was used to record single unit extracellular activity of dorsal horn NS neurons. NS neurons were defined as those neurons that respond only to high-intensity (noxious) stimulation (Telleria-Diaz et al., 2010). To confirm NS response patterns, each neuron was characterized while applying a mechanical stimulation to the ipsilateral hind paw using a von Frey filament with 97.8 mN bending force (noxious stimulation) for 2 s until it buckled slightly (Boccella et al., 2015; Simone et al., 2008). Only neurons that responded specifically to the noxious hind paw stimulation, without responding to stimulation of the surrounding tissue, were included. The recorded signals were amplified and displayed on a digital storage oscilloscope to ensure that the unit under study was unambiguously discriminated throughout the experiment. Signals were also fed into a window discriminator, whose output was processed by an interface CED 1401 (Cambridge Electronic Design Ltd., UK) connected to a Pentium III PC. Spike2 software (CED, version 4) was used to create peristimulus rate histograms online and to store and analyze digital records of single unit activity offline. Configuration, shape, and height of the recorded action potentials were monitored and recorded continuously using a window discriminator and Spike2 software for online and offline analysis. This study only included neurons whose spike configuration remained constant and could be clearly discriminated from activity in the background throughout the experiment, indicating that the activity from one neuron only and from the same neuron was measured. The neuronal activity was expressed as spikes/sec (Hz). The total number of NS neurons recorded per electrode descent (track) was also calculated, since the number of neurons recorded is a valid, indirect index of the percentage of neurons that are active during *in vivo* electrophysiological recordings (Bambico et al., 2007). At the end of the experiment, each animal was killed with a lethal dose of urethane.

2.7. Endocannabinoids measurement

Spinal cord, jejunum, ileum, colon and duodenum were homogenized with pestle in 2:1:1 choloroform/methanol/TRIS-HCl (50 mM, pH 7.4) and sonicated containing deuterated standards (5 pmol of d8-AEA, 50 pmol of d5-2AG, d4-PEA, d2-OEA, 10 pmol of d4-DHEA and d4-EPEA). The homogenates were extracted with chloroform and the organic phase collected and evaporated under nitrogen stream. The lipid-containing organic phase was dried down, weighed and pre-purified by open bed chromatography on silica gel. Fractions were obtained by eluting the column with 99:1, 90:10 and 50:50 (v/v) chloroform/methanol. The 90:10 fraction was used for AEA, 2-AG, PEA, OEA, DHEA and EPEA quantification by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS), as previously described and using selected ion monitoring at M + 1 values for the four compounds and their deuterated homologues.

2.8. DNA extraction and 16S rRNA amplicon sequencing

Total DNA from fecal samples was used for DNA extraction, by using the DNeasy PowerSoil kit (Qiagen). V3-V4 region of the 16S rRNA gene was amplified by using primer S-D-Bact-0341 and S-D-Bact-0785 as previously described (Berni Canani et al., 2017). PCR products were purified with the Agencourt AMPure XP beads and quantified using a Plate Reader AF2200. Amplicon multiplexing, pooling and sequencing was carried out following the Illumina 16S Metagenomic Sequencing Library Preparation protocol, on a MiSeq platform and using the MiSeq Reagent kit v3, leading to 2 × 300 bp, paired-end reads.

3. Statistical analysis

Data were represented as mean ± S.E.M. The behavioural data were analysed using one-way ANOVA, followed by One-way ANOVA, post-hoc Tukey's. The unpaired *T*-test with Mann Whitney test for intra-groups analysis (N = 10–12). Electrophysiological data were analysed using one-way ANOVA, followed by Holm-Sidack post hoc comparisons or *t*-test (N = 6–8). Biomolecular data were expressed as mean ± SD and were analysed by one-way ANOVA, post-hoc Tukey's (N = 5–6).

3.1. Microbiota data analysis

Demultiplexed, forward and reverse reads were joined by using FLASH (Magoc and Salzberg, 2011). Joined reads were quality trimmed (Phred score < 20) and short reads (< 250 bp) were discarded by using Prinseq (Schmieder et al., 2012). High quality reads were then imported in QIIME 1.9 (Caporaso et al., 2010). Operational taxonomic units (OTUs) were picked through a *de novo* approach and uclust method and taxonomic assignment was obtained by using the Ribosomal Database Project (RDP) classifier and the Greengenes (McDonald et al., 2012) database, following a pipeline previously reported (Berni Canani et al., 2017). In order to avoid biases due to the different sequencing depth, OTU tables were rarefied to the lowest number of sequences per sample. Statistical analyses and visualization were carried out in R environment (https://www.r-project.org). Alpha-diversity analysis was carried out in QIIME on rarefied OTU tables. Kruskal-Wallis and pairwise Wilcoxon tests were used in order to determine significant differences in alpha diversity parameters or in specific taxa abundance. Correction of p-values for multiple testing was performed when necessary.

The 16S rRNA gene sequences produced in this study are available at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information under accession number SRP170185.

4. Results

4.1. Vitamin D deficiency induces allodynia associated with spinal neuronal sensitization

After weaning, mice were fed a diet with normal or low vitamin D concentrations for 6 weeks. No differences in food intake or corresponding body weights were observed between groups throughout the experimentation (not shown). The influence of Vitamin D on the rate of pain perception was evaluated by measuring the mechanical sensitivity of the hind paw plantar surface. When compared with controls, Vitamin D deficient mice showed reduced mechanical threshold (right: 0.17 ± 0.04 g vs 1.35 ± 0.21 g, left: 0.14 ± 0.32 g vs 1.029 ± 0.16; $t[26] = 5.47$, $p < 0.0001$) (Fig. 1 B) (Supplementary Fig. 1), while no changes were observed in the thermal nociceptive threshold, as revealed by the tail flick test (3.6 ± 0.24 g vs 4.43 ± 0.48 g, $t[6] = 1.83$, $p = 0.12$) (Fig. 1C).

In vivo electrophysiological experiments were performed in order to

investigate possible changes in spinal NS neuron activity induced by dietary manipulation. The results are based on NS neurons (one cell recorded from each animal) at a depth of 0.7–1 mm from the surface of the spinal cord. This cell population was characterized by a mean rate of spontaneous firing of 0.3 ± 0.08 spikes/sec and only cells showing this pattern of basal firing were chosen for the experiment. Compared with Vitamin D normal mice (0.27 ± 0.02 spikes/sec), we found a considerable increase in the spontaneous activity (7.95 ± 0.6 spikes/sec, $t[3] = 42$, $p = 0.0006$), as well as, in the frequency (16 ± 0.5 spikes/sec, $t[3] = 11.79$, $p = 0.0071$) and in the duration (9.90 ± 0.15 sec, $t[3] = 77.50$, $p = 0.0002$) of the evoked activity of NS neurons in Vitamin D deficient mice, as revealed by unpaired *t*-test (Fig. 1 D, E, F and G, respectively). Moreover, Vitamin D deficiency also affected the total number of NS neurons. Indeed, we found a total number of 21 NS in Vitamin D deficient mice as compared to 12 NS in Vitamin D normal mice (Fig. 1 H).

4.2. Spared nerve injury increases spinal VDR expression without affecting serum vitamin D levels

To evaluate the correlation between a vitamin D status and neuropathic pain, we applied the spared nerve injury model in mice in the presence or absence of Vitamin D deficiency. Seven days of SNI reduced the tactile threshold as compared with sham animals (sham: 0.81 g ± 0.12 vs SNI: 0.03 g ± 0.004, $F5,66 = 26.39$, $p < 0.0001$) in normal condition, while it did not significantly change the nociceptive threshold (0.17 g ± 0.04 sham vs 0.03 g ± 0.006, $F5,66 = 26.39$, $p < 0.0001$) in Vitamin D deficient animals with respect to sham mice (Fig. 2 A). Blood levels of [25(OH)D] were measured in the same animals. Circulating 25OHD levels were 60.9 ng/ml ± 0.9 in control mice, while they were significantly ($P < 0.0001$) reduced to 12.23 ng/ml ± 0.2 in low Vitamin D group (Fig. 2 B). Interestingly, SNI did not affect the circulating 25OHD levels (Fig. 2 B), however it significantly increased spinal VDR expression, in both Vitamin D normal and deficient mice (5.58 ± 0.15 vs 6.16 ± 0.055, $F3,12 = 28.39$, $p < 0.0001$) (Fig. 2 C). Notably, spinal VDR expression levels resulted significantly augmented also in sham animals exposed to the low Vitamin D diet, as compared with sham animals fed with control diet (1 ± 0.028 vs 1.91 ± 0.032, $F3,12 = 28.39$, $p < 0.0001$) (Fig. 2 C).

4.3. CB1R and CB2R involvement in Vitamin D deficiency-induced allodynia

Compared with controls, Vitamin D deficient animals showed reduced spinal CB1R expression levels, as revealed by western blotting analysis (0.43 ± 0.042 vs 1 ± 0.062, $F3,12 = 41.76$, $p < 0.0001$) (Fig. 3 A). The intraperitoneal administration of synthetic CB1R agonist ACEA (10 mg/kg i.p.) did not change the mechanical threshold in all experimental groups (Fig. 3 B) (BL:0.68 ± 0.19 vs post-ACEA:0.84 ± 0.16, $t[8] = 0.65p = 0.53$; (BL:0.08 ± 0.03 vs post-ACEA:0.11 ± 0.03, $t[8] = 0.80p = 0.45$). However, ACEA, starting from 15 min post injection, transiently increased the thermal nociceptive threshold in controls (3.450 ± 0.51 s vs 5.50 ± 0.60 s, $t[6] = 2.61$, $p = 0.04$), while it was not effective in Vitamin D deficiency (4.15 ± 0.62 s vs 4.60 ± 0.47 s, $t[5] = 0.54$, $p = 0.61$) (Fig. 3 C), in agreement with the reduced levels of CB1R in Vitamin D deficient mice.

As already reported for other neuropathic pain states (Guida et al., 2012; Wang et al., 2007), SNI mice showed increased spinal CB1R expression levels, as compared with sham animals (0.088 ± 0.013 vs 1.52 ± 0.18, $F3,12 = 41.76$, $p < 0.0001$). Interestingly, the SNI surgery further reduced the CB1R expression when applied under vitamin D deficiency (0.088 ± 0.01 vs 0.43 ± 0.04, $F3,12 = 41.76$, $p < 0.0001$) (Fig. 3 A). As a result, Vitamin D deficient SNI mice exhibited a significantly lower expression of CB1R compared to Vitamin D normal SNI mice. Consistently, ACEA injection (10 mg/kg i.p.) was able to increase the mechanical threshold in SNI controls (0.05 ± 0.006 s vs 0.37 ± 0.09 s, $t[8] = 3.84$, $p = 0.005$), while it was ineffective in SNI Vitamin D deficient animals 0.05 ± 0.005 s vs 0.12 ± 0.07 s, $t[9] = 1.6$, $p = 0.24$), (Fig. 3 D).

Interestingly, Vitamin D deficient animals showed increased CB2R expression levels as compared with control animals (3.99 ± 0.02 vs 1.00 ± 0.02, $F3,4 = 7962$, $p < 0.0001$) (Fig. 3 E). However, the single intraperitoneal administration of the synthetic CB2R agonist JWH133 (1 mg/kg i.p.) did not change the mechanical and thermal thresholds in all experimental groups (0.13 ± 0.01 g vs 0.14 ± 0.05 g, $t[19] = 0.37$, $p = 0.71$, 0.59 ± 0.15 g vs 0.63 ± 0.10 g, $t[11] = 0.23$, $p = 0.82$; 3.6 ± 0.36 s vs 4.04 ± 0.16 s, $t[8] = 1.11$, $p = 0.30$, 4.2 ± 0.40 s vs 4.2 ± 0.44 s, $t[2] = 0p = 1$, respectively) (Fig. 3 F and G). As compared with sham animals, 7 days of SNI induced significant CB2R over-expression in both the normal and Vitamin D deficient diet (25.25 ± 0.24 vs 1.00 ± 0.02 and 65.39 ± 0.62 vs 3.99 ± 0.02, respectively, $F3,4 = 7962$, $p < 0.0001$) (Fig. 3 E). Nevertheless, JWH133 reversed pain behavior in SNI controls, while it was ineffective in SNI Vitamin D deficient animals (0.32 ± 0.10 g vs 0.09 ± 0.02 g, $t[10] = 2.27$, $p = 0.05$, 0.083 ± 0.03 g vs 0.03 ± 0.04 g, $t[10] = 1.57p = 0.15$) (Fig. 3 J). These data suggest that CB2R might become dysfunctional (i.e. uncoupled to its signaling pathway) during vitamin D deficiency in SNI mice.

4.4. PEA reduces the allodynia and neuronal sensitization in Vitamin D deficiency

Having established that both CB1R and CB2R agonists are ineffective at correcting the allodynia induced by vitamin D deficiency, we next evaluated the effect of exogenous PEA, and endocannabinoid-like molecule which does not directly activate the two cannabinoid receptors and acts instead via several different molecular targets (Petrosino and Di Marzo, 2017). The dose of PEA was chosen according to our previously published studies (Guida et al., 2017; Guida et al., 2015a; Luongo et al., 2013).

Whereas a single injection of PEA (10 mg/kg, i.p.) did not exert any change in mechanical threshold in normal diet group, it significantly improved the pain behavior associated with the absence of Vitamin D (0.43 ± 0.10 g vs 0.59 ± 0.13 g, $t[16] = 0.99$, $p = 0.34$, 0.26 ± 0.06 g vs 0.06 ± 0.01 s, $t[7] = 2.85$, $p = 0.02$) (Fig. 4 A). PEA injection did not change the thermal nociceptive response in the tail flick test in both diet regimens (not shown). Electrophysiological data showed that PEA administration (10 mg/kg) reduced the spontaneous activity (58 ± 9%, $F3,16 = 27.58$, $p < 0.0001$), the frequency (48.6 ± 7%, $F3,16 = 73.70$, $p < 0.0001$) and the duration (21.95 ± 5%, $F3,16 = 145.8$, $p < 0.0001$) of evoked activity of NS neurons in Vitamin D deficient mice starting from 25 min post-injection, as revealed by two-way ANOVA followed by Bonferroni post-hoc test (Fig. 4 C–E). No differences in NS activity in Vitamin D normal mice were observed after PEA injection, as compared to vehicle-treated animals ($p > 0.05$) (Fig. 4 B, C–E). Remarkably, Vitamin D deficient mice showed increased (~20-fold) expression levels of PPAR-α at the spinal cord level, compared with normal vitamin D animals (23.36 ± 0.18 vs 1 ± 0.11, $F3,12 = 3167$, $p < 0.0001$) (Fig. 4 F). Moreover, we found increased expression levels of PPAR-α in both normal and Vitamin D deficient SNI mice (72 ± 0.86 and 72 ± 0.9, $F3,12 = 3167$, $p < 0.0001$). This upregulation might underlie PEA normalization of SNI-induced allodynia in both diet regimens (0.30 ± 0.07 g vs 0.03 ± 0.04 g, $t[10] = 3.44$, $p = 0.006$, 0.28 ± 0.07 g vs 0.027 ± 0.004 g, $t[10] = 3.68$, $p = 0.04$) (Fig. 4 G), although we did not perform any experiments with the compound in the presence of a PPAR-α antagonist to confirm or discard this hypothesis.

4.5. Changes in endocannabinoid and endocannabinoid-like mediator levels in the spinal cord

Next, we wanted to assess if the alterations in tactile threshold

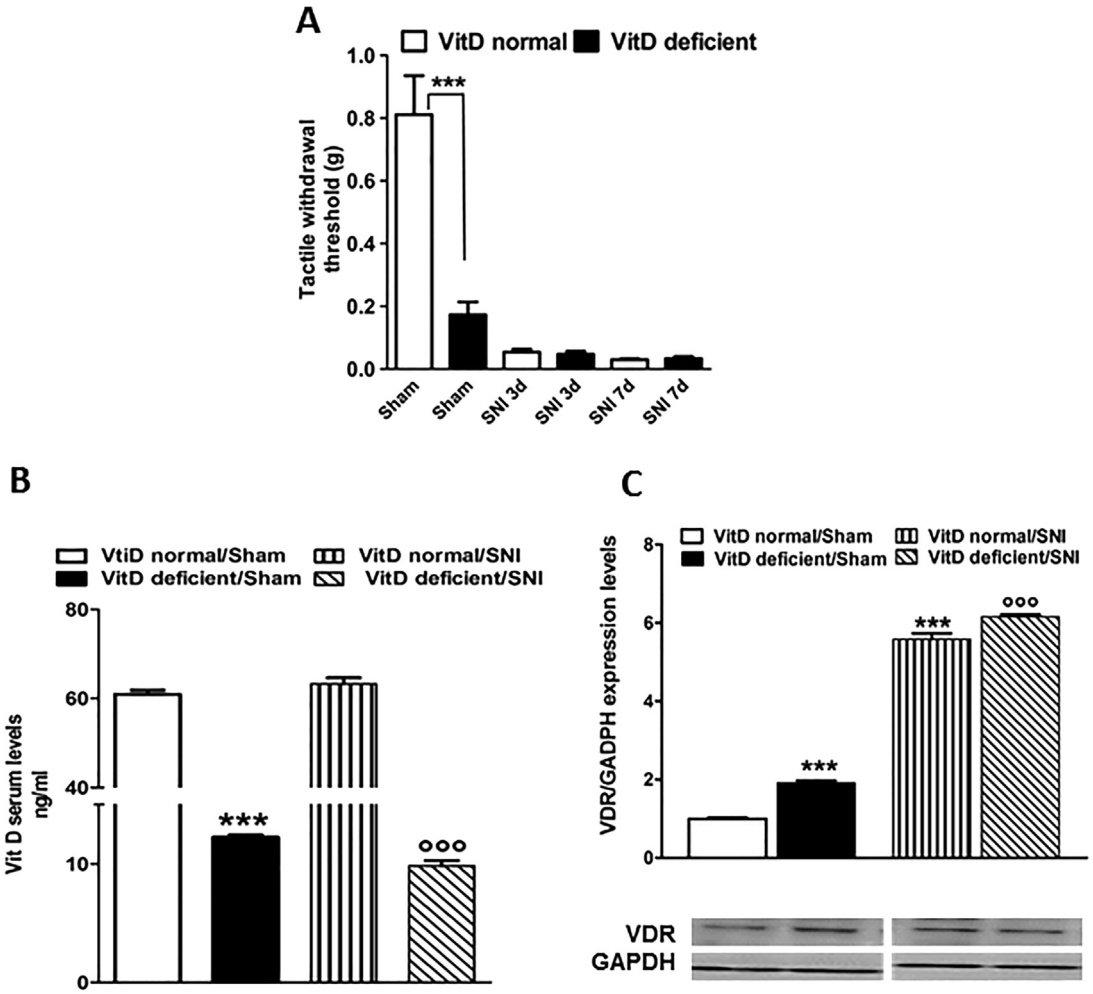

Fig. 2. Effects of spared nerve injury on vitamin D signaling. A) Mechanical withdrawal threshold in sham and SNI VitD normal and VitD deficient, 3- or 7-days post-surgery. Data are expressed as mean ± SEM. (***) P < 0.001 versus VitD normal/sham mice, (n = 12). B) Vitamin D serum level (ng/ml) in sham and SNI VitD/ normal and VitD/deficient 7 days post-surgery. (***) P < 0.001 versus VitD normal/sham mice, (ooo) P < 0.001 versus VitD normal/SNI mice, (n = 5–6). C) Representative western blot image and related quantification showing the expression of VDR, using GAPDH as a loading control. Data were shown as mean ± SEM, (n = 4). (***) P < 0.001 versus VitD normal/sham mice, (ooo) P < 0.001 versus VitD deficient/Sham mice. One-way ANOVA, post-hoc Tukey's.

observed in Vitamin D deficiency, as well as following SNI performed in presence or absence of Vitamin D, were accompanied by changes in endocannabinoid and endocannabinoid-like mediators (including PEA) in the spinal cord and gut, as such changes have been suggested to contribute to inflammatory and chronic pain. In the spinal cord, significant increases of AEA and DHEA levels were detected in Vitamin D deficient mice (Fig. 5 A and E). On the other hand, spinal AEA and DHEA levels were decreased in SNI animals, as compared with the sham group (Fig. 5 A and E). Interestingly, the employment of the SNI procedure in Vitamin deficient mice did not result in statistically significant effects on any of the mediators measured (Fig. 5 A–F).

The injection of PEA determined a significant increase in PEA spinal cord levels in SNI vs. sham mice, indicating that during neuropathic conditions the blood spinal cord barrier might become more permeable to exogenous PEA (Fig. 5 C). Interestingly, this effect was observed only in normal Vitamin D conditions, while no significant changes were induced by PEA treatment in vitamin D deficient animals. Remarkably, together with the restoration of tactile thresholds, PEA treatment normalized the increased AEA and DEHA levels in Vitamin D deficient mice. PEA also decreased 2-AG and DHEA levels in SNI vitamin D deficient mice, and 2-AG levels in non-SNI vitamin D normal mice (Fig. 5 A, B and E). These latter data can be the consequence of possible adaptive mechanisms aimed at reducing the endogenous effects of analgesic mediators in response to the administration of a *per se* analgesic compound.

4.6. Changes in endocannabinoid and endocannabinoid-like mediator levels in the gut

The levels of AEA, 2-AG, PEA, OEA, DHEA and EPEA in the mouse jejunum, ileum, colon and duodenum are shown (Fig. 6 A and B; supp. Fig. 3). In all four tissues, PEA treatment was found to lead to the expected strong and significant increase in PEA levels only in vitamin D normal mice, and much less so in vitamin D deficient mice, indicating that the absence of vitamin D in the diet modulates the absorption or metabolism of this compound in both the small and large intestine in a manner to reduce its intestinal levels. Interestingly, the co-occurrence of neuropathy reduced the bioavailability of PEA in the ileum and duodenum, but not in the colon and jejunum (Fig. 6 A and B; supp. Fig. 3).

The levels of other *N*-acylethanolamines and 2-AG in the four intestinal tissues did not significantly change with most of the treatments. Notable exceptions to this rule were: 1) the colon, where vitamin D deficiency per se reduced 2-AG levels (Fig. 6 A), and PEA elevated AEA, OEA and DHEA levels only in vitamin D normal SNI mice (Fig. 6 A); and 2) the duodenum, where PEA elevated DHEA levels in both vitamin D normal and deficient non-SNI mice (Fig. 6 B). These findings may suggest, respectively, that: 1) vitamin D deficiency may also be

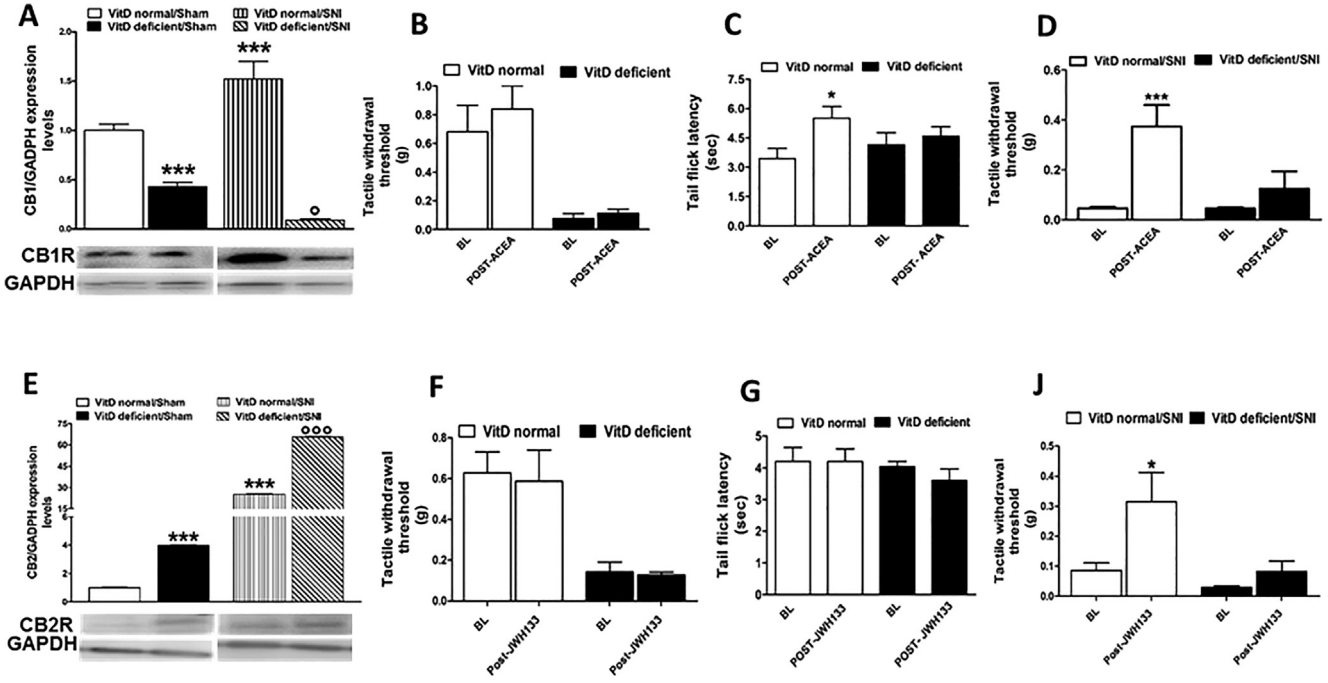

Fig. 3. Involvement of CB1 and CB2 receptors in vitamin D deficiency-induced allodynia. A, E) Representative western blot image and related quantification showing the expression of CB1 and CB2 using GAPDH as a loading control. Data were shown as mean ± SEM, (n = 4). (***) $P < 0.001$ versus VitD normal/sham mice, (∘) $P < 0.05$ versus VitD deficient/Sham mice. One-way ANOVA, post-hoc Tukey's. B, F) Mechanical withdrawal threshold in VitD normal and VitD deficient mice pre (BL) and post (20 min) treatment with ACEA (10 mg/kg, i.p.) or AM630 (1 mg/kg, i.p.). C, G) Thermal withdrawal threshold in VitD normal and VitD deficient mice pre (BL) and post (20 min) treatment with ACEA (10 mg/kg, i.p.) or JWH133 (1 mg/kg, i.p.). D, J) Mechanical withdrawal threshold in VitD normal/SNI and VitD deficient/SNI mice pre (BL) and post (20 min) treatment with ACEA (10 mg/kg, i.p.) or JWH133 (1 mg/kg, i.p.). Data are expressed as mean ± SEM. (***) $P < 0.001$, (*) $P < 0.05$ versus relative control (BL), unpaired T-test (n = 5–6).

accompanied by increased pain and inflammation due to reduced endocannabinoid (i.e. 2-AG) signaling at intestinal CB1R and CB2R, and its subsequent amelioration by PEA, and 2) PEA may produce anti-inflammatory effects in the small intestine in both vitamin D normal and deprived mice by elevating DHEA levels. This hypothesis will require ad hoc studies on the effects of vitamin D deficiency, with or without PEA administration, in models of visceral pain and intestinal inflammation.

In view of the previously reported effects of endocannabinoids and *N*-acylethanolamines on the gut microbiota (Cristiano et al., 2018), the findings in the small intestine overall may also suggest that vitamin D deficiency, on the one hand, and PEA administration, on the other hand, might be accompanied by changes in fecal microbiota composition, which reflects colonic and, to a smaller extent, small intestinal, microbiota composition.

4.7. Changes in gut microbiota

Gut microbiota composition and diversity was strongly affected by the decreased intake of vitamin D, while animals following a standard diet clustered together regardless of the neuropathy or PEA treatment (Fig. 7 A). VitD deficient mice showed a lower microbial diversity compared to control mice (Fig. 7 B, $p < 0.05$).

In healthy, untreated mice the vitD deficient diet was linked to an increase in Firmicutes and a decrease in Verrucomicrobia and Bacteroidetes ($p < 0.05$). Moreover, the treatment with PEA in animals following a diet deficient in vitamin D led to higher levels of *Akkermansia*, *Eubacterium* and *Enterobacteriaceae* (Fig. 8).

5. Discussion

The aim of this study was to explore the role of the microbiota-gut-CNS axis in vitamin D deficiency-associated pain processing. First, we showed that vitamin D deficiency causes allodynia, spinal neuronal sensitization, and modifications in endocannabinoid signaling at the spinal and gut levels. Moreover, we proved that the administration of the endocannabinoid-like mediator and *N*-acylethanolamine, PEA, currently marketed as medical food against neuropathic pain (Petrosino and Di Marzo, 2017), counteracts pain and modifies several of the biochemical and functional changes induced by vitamin D deficiency. Finally, and remarkably, we identified specific gut bacteria populations associated with Vitamin D deficiency as well as PEA pharmacological effects.

Low Vitamin D levels are suspiciously common in people suffering of chronic pain (Shipton and Shipton, 2015; Straube et al., 2009). However, the neurophysiological and biochemical mechanisms through which the Vitamin D status affects pain processes have been poorly investigated. In the present study, we demonstrated that increased nociception associated with central sensitization is part of the phenotype of Vitamin D deficiency, pointing out the role of Vitamin D/VDR signaling in the transmission of noxious signals from the periphery to the spinal cord. Indeed, vitamin D deficient mice showed a decreased tactile threshold to innocuous stimuli, typically associated to damage to the CNS (i.e. neuropathic pain), but not thermal threshold, which is more related to changes in pro-inflammatory factors or nociceptor activation. Differently to the typical mononeuropathies induced by peripheral nerve lesion, in our model the occurrence of neuropathic-like symptoms was bilateral, indicating an overall sensitization. Such abnormal pain perception was not linked to evident musculoskeletal impairments (Girgis et al., 2015; Mallya et al., 2016; Tague et al., 2011), since Vitamin D deficiency, at least under the experimental conditions used here, did not affect muscle strength or motor coordination and, concurrently, did not cause any macroscopic alterations in bone and muscle structures. Electrophysiology indicated an over-excitability of nociceptive-specific neurons in the spinal cord, suggesting a likely "maladaptive spinal plasticity" possibly due to changes in Vitamin D-related signaling. Spinal VDR expression was increased, perhaps as a

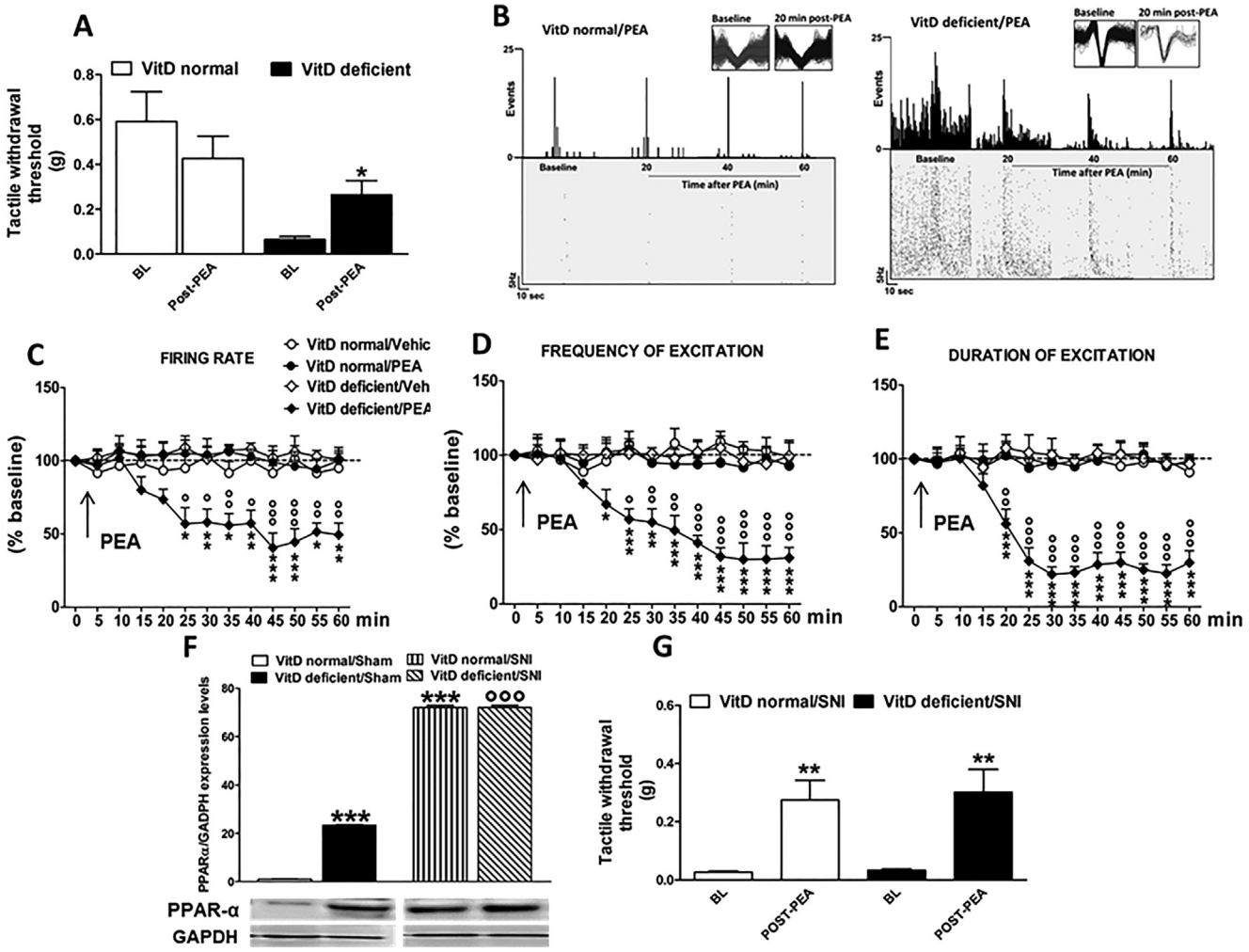

Fig. 4. Effects of PEA treatment on allodynia and neuronal sensitization induced by vitamin D deficiency. A) Mechanical withdrawal threshold in VitD normal and VitD deficient mice pre (BL) and post (20 min) treatment with PEA (10 mg/kg, i.p.). Data are expressed as mean ± SEM, (*) $P < 0.05$ versus relative control (BL). B) Representative ratemeters of NS neurons illustrating the effect of noxious pressure-evoked excitation before and after injection of PEA (10 mg/kg, i.p.) in VitD normal (left) and VitD deficient mice (right), respectively. C), D), and E) Firing rate, frequency, and duration of excitation of NS neuron evoked by paw noxious pressure in VitD normal and VitD deficient mice, respectively. Data are expressed as mean (% of baseline) ± SEM, (n = 5–6). A single neuron was recorded per group. (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ versus baseline (time 0) and (°) $P < 0.05$, (°°) $P < 0.01$, and (°°°) $P < 0.001$ versus Vehicle. F) Representative western blot image and related quantification showing the expression of PPAR-α using GAPDH as a loading control. Data were shown as mean ± SEM, (n = 4). (***) $P < 0.001$ versus VitD normal/sham mice, (ooo) $P < 0.001$ versus VitD deficient/Sham mice. One-way ANOVA, post-hoc Tukey's. G) Mechanical withdrawal threshold in VitD normal/SNI and Vit D deficient/SNI mice pre (BL) and post (20 min) treatment with PEA (10 mg/kg, i.p.). Data are expressed as mean ± SEM. (**) $P < 0.01$ versus relative control (BL), unpaired T-test (n = 5–6).

consequence of compensatory mechanism of reduced Vitamin D levels (Filipović et al., 2013; Tague et al., 2011). As a neuroactive steroid, Vitamin D may modulate a number of signal transduction systems participating in pain processes. The endocannabinoid system, i.e. one of the most important such systems, was, in fact, also found here to be altered. We found reduced spinal CB1R expression levels, which might result from the altered genomic VDR pathways or, alternately, from receptor over-stimulation through the increased synthesis of agonists (i.e. AEA, DEHA) detected in this study, and might underlie long-term functional changes leading to allodynia that would not be sensitive any longer to CB1R agonists. In fact, systemic administration of a CB1R agonist did not reverse the Vitamin D deficiency-mediated allodynia, although it did counteract the thermal pain sensitivity in vitamin D normal mice. On the contrary, spinal CB2R was strongly increased, possibly as an adaptive mechanism to counteract the potential spread of inflammation. Surprisingly, however, a CB2R agonist failed to ameliorate pain symptoms. As already suggested in physiological conditions (Rossi et al., 2013; Guida et al., 2017), it is possible that CB2R may control different immune-related responses i.e. cells proliferation and autophagy, also in Vitamin D deficiency (Wu and Sun, 2011), and that its increased expression is not accompanied by its coupling to anti-allodynic pathways.

Remarkably, the injection of a multi-target endocannabinoid-like *N*-acylethanolamine, i.e. PEA, reverted chronic pain and neuronal excitability, together with spinal endocannabinoid levels dysregulation (AEA and DHEA). The analgesic effects of PEA have been previously demonstrated in different animal models associated with chronic pain (Guida et al., 2017; Guida et al., 2015b; Impellizzeri et al., 2014; Luongo et al., 2013). However, our data suggest novel therapeutic properties of PEA, which may be helpful to compensate, or amplify the endogenous protection mechanisms deployed in vitamin D deficiency-associated dysfunctions. Although investigating PEA mechanism of action was not among the aims of the present study, we found that PPAR-α, known to be responsible for several pharmacological properties of PEA (Citraro et al., 2013; Di Cesare Mannelli et al., 2013; Scuderi et al., 2012; Serena et al., 2018), was increased in the absence of

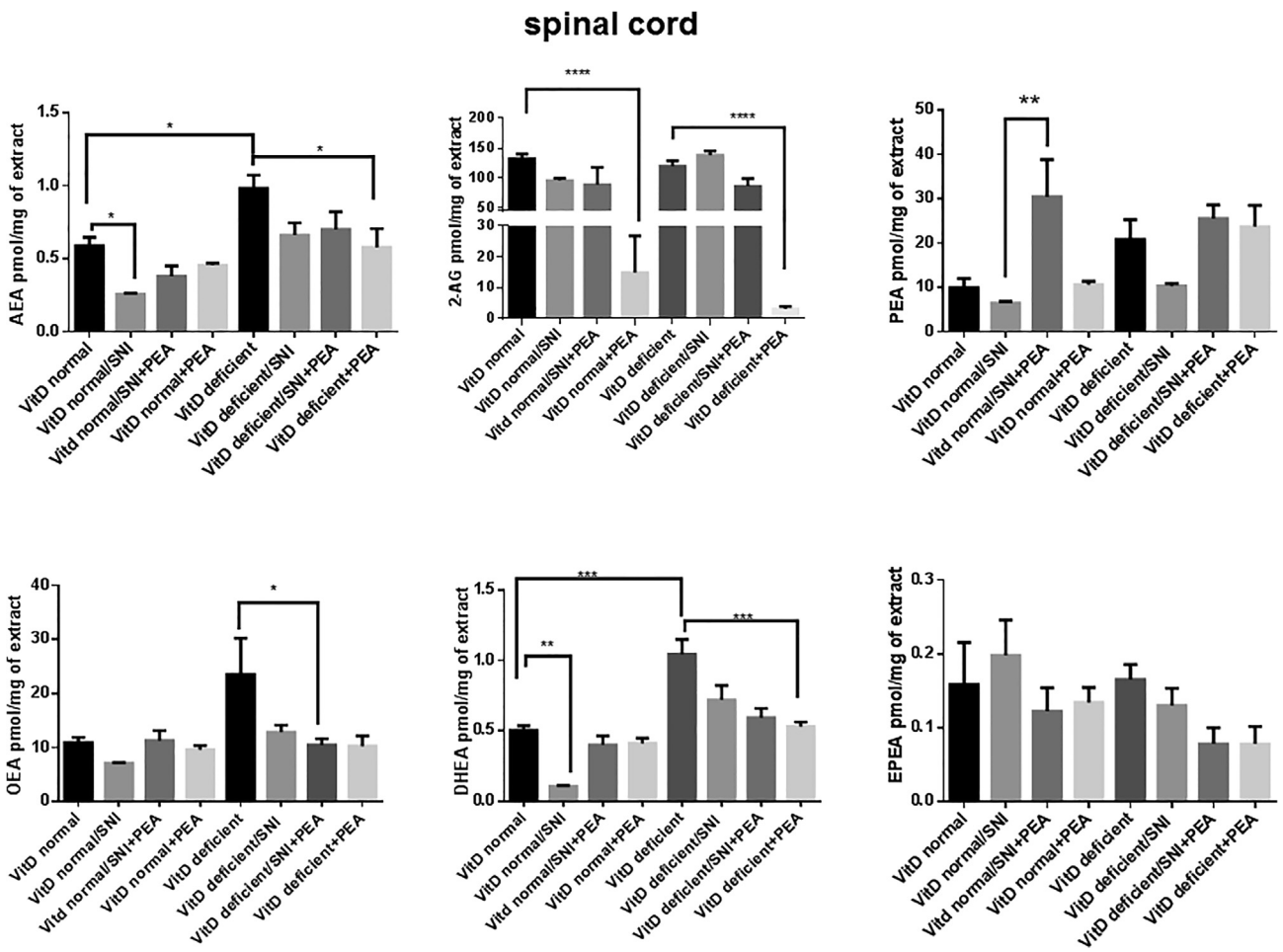

Fig. 5. Endocannabinoids and endocannabinoids-like levels in the spinal cord. Levels of A) AEA, B) 2-AG, C) PEA, D) OEA, E) DEHA, F) EPEA in the spinal cord of VitD normal/sham and VitD deficient/sham or VitD normal/SNI and VitD deficient/SNI in presence or not of PEA treatment (10 mg/ kg, i.p.). Data are expressed as mean ± SEM (n = 5). (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ versus relative control, One-way ANOVA, post-hoc Tukey's.

Vitamin D. Indeed, the interaction between VDR and PPAR-α, both requiring heterodimerization with retinoic × receptor (RXR) to exert their activity through response element binding, has been previously suggested (Sertznig and Reichrath, 2011; Zella et al., 2006). However, we cannot exclude that PPAR-α over-expression could be also due to possible metabolic alterations in lipid metabolism and glucose homeostasis commonly associated with Vitamin D deficiency, but normally observed in other tissues and organs (Nakamichi et al., 2017; Sharma and Staels, 2006; Takahashi et al., 2017).

With regard to PEA-induced reduction of spinal AEA and DEHA levels observed after PEA injection in vitamin D deficient mice, it is reasonable to hypothesize that this effect was not responsible for pain amelioration, but instead represents an adaptive mechanism to PEA-induced analgesia, possibly exerted via one or more of the different targets of this mediator, such PPAR-α or the orphan G-protein-coupled receptor GPR55 (Raso et al., 2014).

In the gut, vitamin D deficiency produced significant decreases of 2-AG levels in the colon, confirming the dual role of the endocannabinoid system in gut pathophysiology (Cani et al., 2016). Endogenous PEA levels were not affected by diet, but they resulted increased, as expected, after injection of exogenous PEA. This effect was more evident in control (colon, jejunum and duodenum) than Vitamin D deficient mice, suggesting possible changes in drug bioavailability/distribution or anatomical changes induced by the pathological situation. Interestingly, PEA strongly increased DHEA levels in both vitamin D normal and deprived mice in the duodenum, suggesting a possible link between PEA protective effects in the small intestine and elevated DHEA levels. This hypothesis should be investigated in future studies, and is supported by the previous finding that DHEA and its metabolites exhibit anti-inflammatory and organ-protective properties (Yang et al., 2011).

The correlation between vitamin D status and neuropathic pain was investigated by applying the SNI to Vitamin D deficient mice. This is a well-established model of neuropathic pain associated with neuronal firing changes at the spinal and supraspinal levels, as well as with neuropsychiatric disorders (Guida et al., 2015a; Sagheddu et al., 2015). Although circulating vitamin D levels were not changed, SNI increased spinal VDR expression, suggesting that VDR signaling plays a role in neuropathic pain states. Moreover, we cannot exclude that SNI may affect, and possibly reduce, Vitamin D levels in the spinal cord. Interestingly, the down- and up-regulation of spinal CB1R and CB2R expression, respectively, induced by Vitamin D deficiency was reproducible under neuropathic pain condition, and both CBR1 and CBR2 agonists did not decrease pain behaviour in SNI-vitamin D deficient mice. On the other hand, consistently with PPAR-α expression profiles, PEA treatment was effective at reducing neuropathic pain regardless of Vitamin D intake. Interestingly, SNI mice showed reduced spinal AEA and DHEA levels. These results are in agreement with previous findings (Bishay et al., 2013; Petrosino et al., 2007) and, based on the neuroprotective, antinociceptive and anti-inflammatory properties of these bioactive lipids (Yang et al., 2011), may suggest that this dysregulation participates in SNI-induced allodynia, inflammation and neuronal damage. As expected, spinal PEA levels resulted increased after the injection of exogenous PEA, although such an increase was strong and statistically significant only in SNI mice, suggesting an increased

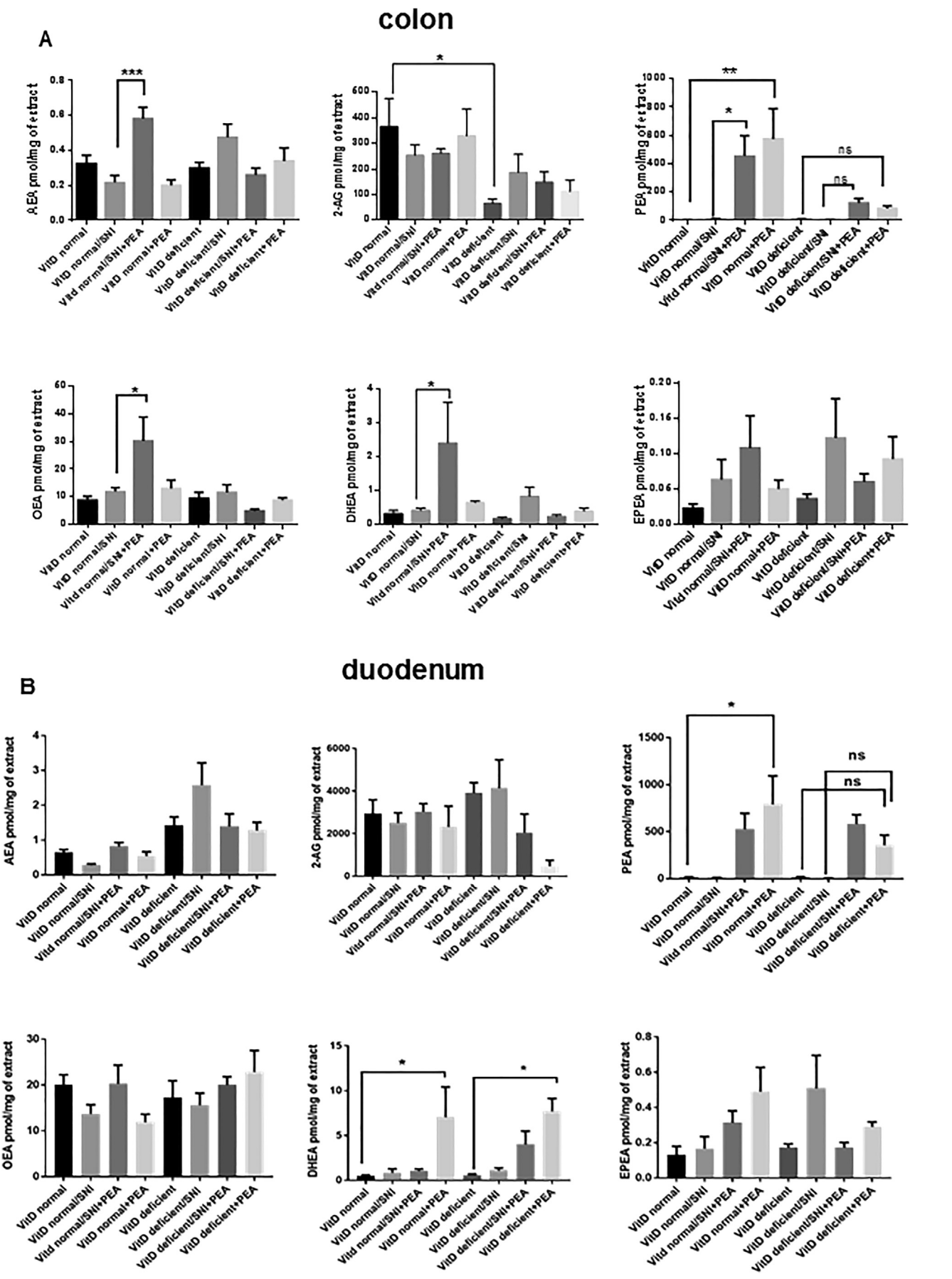

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Fig. 6. Endocannabinoids and endocannabinoids-like levels in the gut. Levels of endocannabinoids and endocannabinoids-like in the gut of VitD normal/sham and VitD deficient/sham or VitD normal/SNI and VitD deficient/SNI in presence or not of PEA treatment (10 mg/kg, i.p.). A) AEA, 2-AG, PEA, OEA, DEHA, EPEA in the colon and B) AEA, 2-AG, PEA, OEA, DEHA, EPEA in the duodenum. Data are expressed as mean ± SEM (n = 5). (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ versus relative control, One-way ANOVA, post-hoc Tukey's.

permeability of the blood spinal cord barrier following this neuropathic condition (Guida et al., 2015b). Interestingly, the effect of PEA on spinal AEA, 2-AG and DHEA levels described above for vitamin D deficient mice was not observed in SNI mice, with or without vitamin D deficiency. This finding is counterintuitive given: 1) the well-known "entourage effect" of PEA on AEA and 2-AG levels (Re et al., 2007), 2) the analgesic actions of AEA and 2-AG and the anti-inflammatory action of DHEA (Yang et al., 2011), and 3) the fact that PEA ameliorated allodynia. If, as mentioned above, the negative effects of PEA on the spinal cord levels of these mediators may be explained as a possible adaptive mechanism aimed at reducing the effects of endogenous analgesic mediators in response to an exogenous and *per se* analgesic compound, the fact that these negative effects are missing in the corresponding SNI mice may suggest, instead, that the analgesic effect of PEA in these neuropathic mice are partly due also to the lack of inhibition on endogenous analgesic/anti-inflammatory mediator signaling. All together, these data suggest the SNI-induced changes in spinal cord endocannabinoid and *N*-acylethanolamine signaling are not affected by Vitamin D deficiency.

At the gut level, we found that the co-occurrence of neuropathy reduced the bioavailability/distribution of PEA, specifically in the ileum and duodenum, but not in the colon and jejunum. Since PEA was equally effective at reducing allodynia in both SNI mice and vitamin D deficient mice, it can be surmised that the levels of this compound in the small and large intestine do not determine its analgesic efficacy. Moreover, in the colon PEA treatment elevated AEA, OEA and DHEA levels only in vitamin D normal SNI mice, suggesting the contribution of these bioactive lipids in the protective effects of PEA in the gut (Capasso et al., 2014; Esposito et al., 2014).

In the gut, altered vitamin D status may influence commensal bacterial composition and viceversa (Luthold et al., 2017). In our model the absence of vitamin D generated a marked dysbiosis, with a loss of bacterial diversity. We also demonstrated that SNI does not impact on microbiota composition. However, PEA treatment was seemingly associated with higher levels of some specific gut bacterial species, restrictively in the low Vitamin D condition (with or without SNI), including *A. muciniphila*. This latter species, together with other bacteria, resulted instead decreased by low vitamin D diet. Associations between *Eubacterium* and *Enteronbacteriaceae* with PEA or other neuroactive compounds have not been documented. Beneficial endocannabinoidome mediators such as PEA may contribute to correcting the effects of dysbiosis (Cristiano et al., 2018). *A. muciniphila* administration to high fat diet-fed mice induces an increase in intestinal levels of endocannabinoid-related mediators, with protective effects on gut-barrier integrity and inflammation (Cani, 2014). Conversely, deletion of a N-acylethanolamine biosynthetic enzyme from the adipose tissue was accompanied by reduced *A. muciniphila* levels and systemic inflammation only when resulting in the decrease of the endogenous levels of these non-endocannabinoid N-acylethanolamines, including PEA, and the increase in AEA levels (Geurts et al., 2015; Cani et al., 2016). Thus, the link between *A. muciniphila* abundance and the analgesic effects mediated by PEA may be related to of a connection between commensal bacteria and endocannabinoids related mediators in inflammation and host metabolism (Everard et al., 2013; Muccioli et al., 2010), at least in cases of in vitamin D deficiency. Given that PEA concentrations did not increase in the spinal cord following administration of exogenous PEA, whereas they did increase in the small intestine, it is tempting to speculate that at least part of the analgesic actions of PEA might be due to its effects on this peripheral tissue, including the observed trends towards the decrease in AEA levels and the increase of *A. muciniphila* abundance, with subsequent reduction of intestinal permeability (Alhamoruni et al., 2012) and systemic inflammation (Cani et al., 2016).

Further analysis will be necessary to identify possible biomolecules

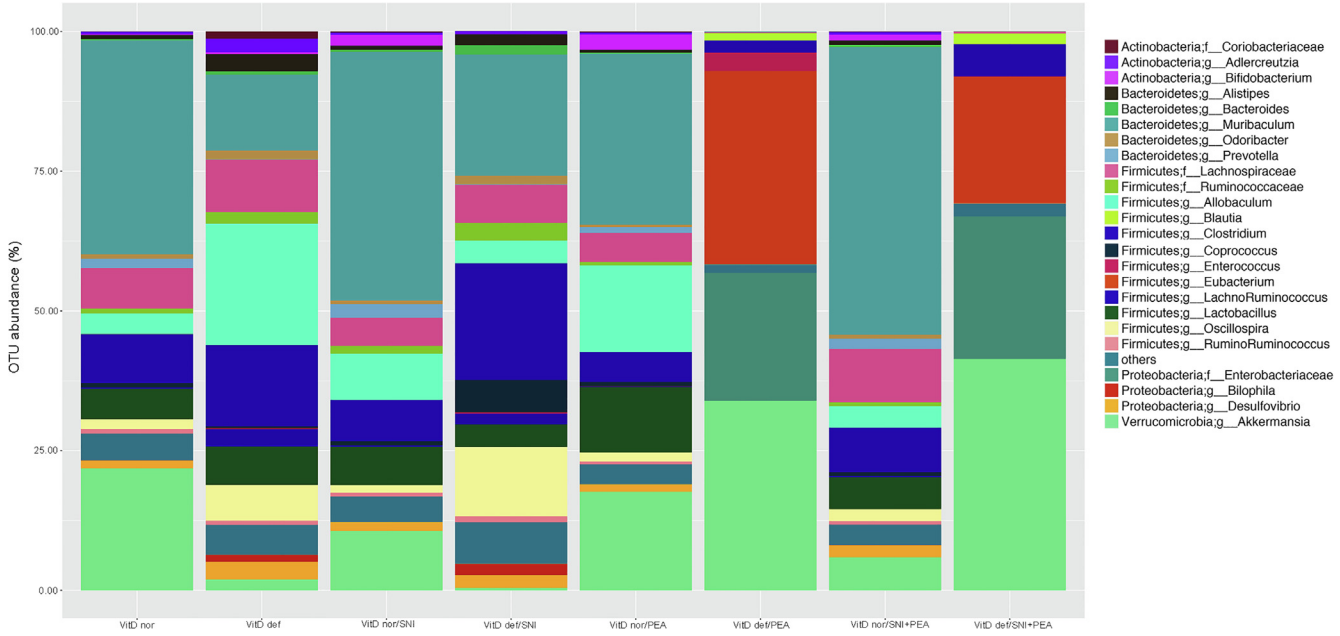

Fig. 7. Effect of diet and treatment on the gut microbiota. Principal Components Analysis (PCA, panel A) based on the bacterial community composition at genus level. Only genera present in at least 10% of samples were included. B) Box plots showing number of observed OTUs in the samples analyzed in this study. Boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside represents the median (2nd quartile). Whiskers denote the lowest and the highest values within 1.5 IQR from the first and third quartiles, respectively.

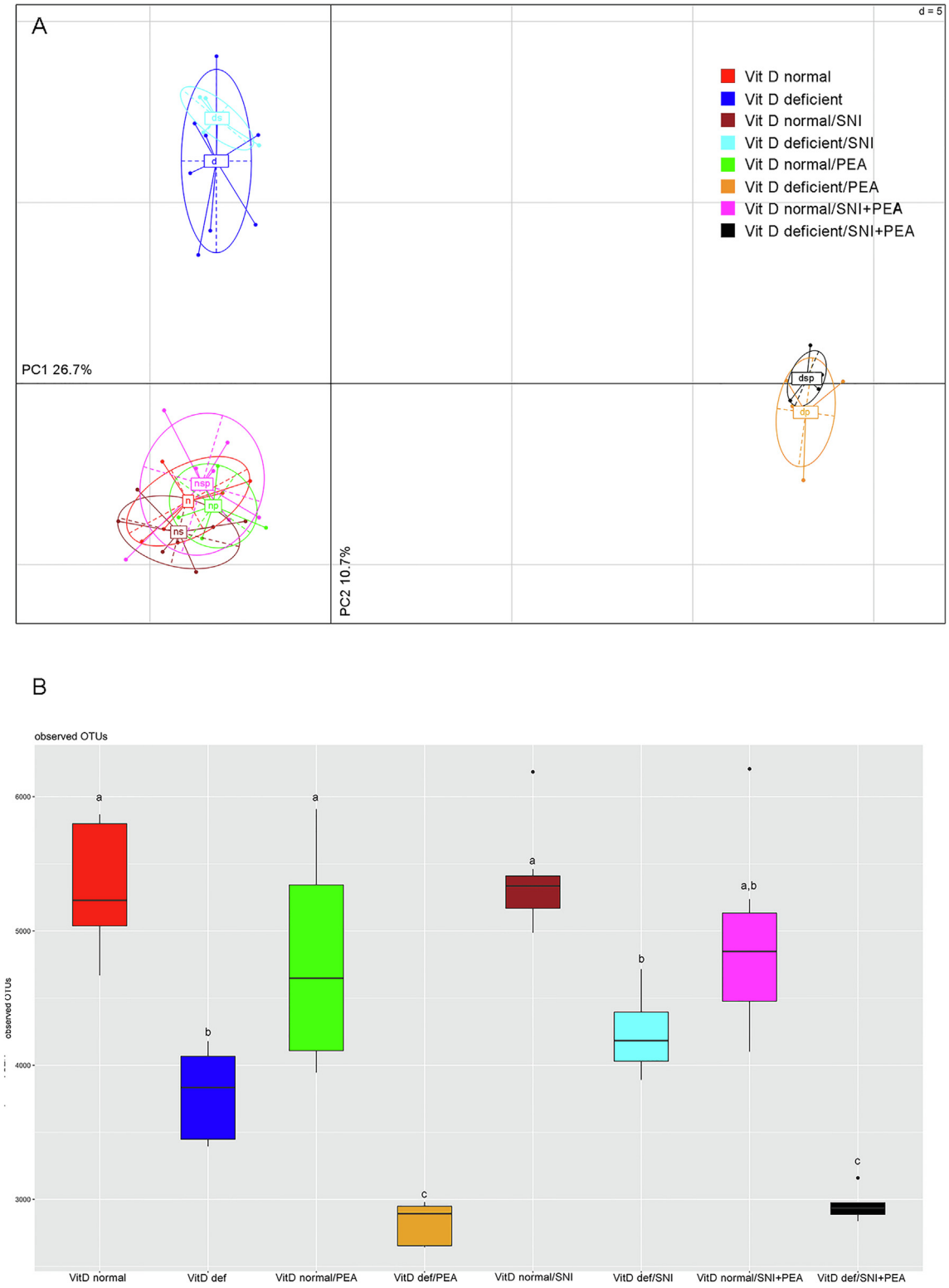

Fig. 8. Stacked bar showing the bacterial populations in the analyzed samples. The average value of the replicates is reported for each group. Only genera with abundance > 0.5% in at least one group are shown.

or bacterial products released from the gut into the systemic circulation, which may exert a role in the pathophysiology of pain. Moreover, given the presence of high levels of VDR in the brain (hippocampus, hypothalamus, thalamus, cortex, and substantia nigra) (Moretti et al., 2018), we cannot exclude that vitamin D deficiency may directly affect neuronal well-functioning in specific brain regions involved in pain processing.

6. Conclusions

In conclusion, our results suggest that Vitamin D deficiency, and associated changes in spinal cord sensory neuron activity, possibly together with altered gut bacterial composition, may cause pain behavior via molecular mechanisms involving at least in part the endocannabinoid and N-acylethanolamine signaling systems. We also

demonstrate that the neuropathic pain state induced in the present study, instead, does not affect microbiota composition. This latter finding seems to reinforce the hypothesis that, of the vitamin D deficiency-induced effects observed here, it is the dysbiosis that may concur to increase pain rather than the contrary. The fact that exogenous PEA: 1) modifies gut microbiota composition, and 2) produces certain changes in the spinal levels of endocannabinoids and related molecules, only during vitamin D deficiency-induced, and not in SNI-induced, pain also suggests a specific role of these two complex systems in the consequences of this defective dietary condition. Indeed, we have pointed out the potential relationship between particular gut microbial relative abundances and endocannabinoid and endocannabinoid-like molecules in the course of vitamin D deficiency-induced pain, as well as in its pharmacological treatment with one of such molecules.

We suggest novel therapeutic properties for PEA, which may be advantageous to counteract vitamin D deficiency-associated dysfunctions, possibly also via its effects on the gut microbiome and endocannabinoidome.

Conflict of interest

The authors declare no conflict of interest

Author contributions

Conceived and designed the experiments: FG, SM.

Performed the experiments: SB, CB, MI, FP, FDF, SP, FR, IM.

Analyzed the data. Contributed materials/analysis tools: FG, FDF, DS, LL, DE, VDM.

Wrote the paper: FG, SM, VDM.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2019.04.006.

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