Neuroscience 277 (2014) 885–901

DISTURBANCE OF THE GUT MICROBIOTA IN EARLY-LIFE SELECTIVELY AFFECTS VISCERAL PAIN IN ADULTHOOD WITHOUT IMPACTING COGNITIVE OR ANXIETY-RELATED BEHAVIORS IN MALE RATS

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Abstract—Disruption of bacterial colonization during the early postnatal period is increasingly being linked to adverse health outcomes. Indeed, there is a growing appreciation that the gut microbiota plays a role in neurodevelopment. However, there is a paucity of information on the consequences of early-life manipulations of the gut microbiota on behavior. To this end we administered an antibiotic (vancomycin) from postnatal days 4–13 to male rat pups and assessed behavioral and physiological measures across all aspects of the brain–gut axis. In addition, we sought to confirm and expand the effects of early-life antibiotic treatment using a different antibiotic strategy (a cocktail of pimaricin, bacitracin, neomycin; orally) during the same time period in both female and male rat pups. Vancomycin significantly altered the microbiota, which was restored to control levels by 8 weeks of age. Notably, vancomycin-treated animals displayed visceral hypersensitivity in adulthood without any significant effect on anxiety responses as assessed in the elevated plus maze or open field tests. Moreover, cognitive performance in the Morris water maze was not affected by early-life dysbiosis. Immune and stress-related physiological responses were equally unaffected. The early-life antibiotic-induced visceral hypersensitivity was also observed in male rats given the antibiotic cocktail. Both treatments did not alter visceral pain perception in female rats. Changes in visceral pain perception in males were paralleled by distinct decreases in the transient receptor potential cation channel subfamily V member 1, the α-2A adrenergic receptor and cholecystokinin B receptor. In conclusion, a temporary disruption of the gut microbiota in early-life results in very specific and long-lasting changes in visceral sensitivity in male rats, a hallmark of stress-related functional disorders of the brain–gut axis such as irritable bowel disorder.

Key words: visceral pain, brain–gut axis, microbiota, antibiotic, neonatal, behavior.

INTRODUCTION

Increasing evidence points to a crucial role for the gut microbiota in health and disease (Matamoros et al., 2013; Petschow et al., 2013; Quigley, 2013; Cenit et al., 2014; Moloney et al., 2014; Nylund et al., 2014). Alterations in the microbiota have been shown in a variety of disorders ranging from obesity, diabetes to inflammatory bowel disorder and irritable bowel syndrome (IBS) (Kostic et al., 2014; Mayer et al., 2014; Moreno-Indias et al., 2014; Walsh et al., 2014). IBS is now recognized as a disorder of the brain–gut microbiome axis, characterized by visceral hypersensitivity, enhanced stress and anxiety (Mayer et al., 2001, 2014; Longstreth et al., 2006; Mayer and Tillisch, 2011; Fashner and Gitu, 2013). The early postnatal period is the most dynamic stage of intestinal microbiota development. During the first three years of life the abundance, composition and diversity of the gut microbiome goes through rapid and large modification (Matamoros et al., 2013; Nylund et al., 2014). Disruption of these developmental patterns prior to the attainment of a more stable adult-like gastrointestinal (GI) tract microbiota is increasingly being linked to adverse health outcomes (Dominguez-Bello et al., 2011; Versalovic, 2013; Clarke et al., 2014; Moloney et al., 2014). Gut microbes play essential roles in the

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Abbreviations: α2AAR, α-2A adrenergic receptor; CCK2R, cholecystokinin B/2 receptor; CRD, colorectal distension; GI, gastrointestinal; IBS, irritable bowel syndrome; IL, interleukin; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time PCR polymerase chain reaction.

http://dx.doi.org/10.1016/j.neuroscience.2014.07.054
0306-4522/

development of pain pathways, immune system development, and the production of short chain fatty acids (Amaral et al., 2008; Cryan and O'Mahony, 2011; Collins et al., 2012). These interactions between microbiota and the host during the early-life period can significantly influence the central nervous system (Sudo et al., 2004; Bravo et al., 2011; Diamond et al., 2011; Diaz Heijtz et al., 2011; Collins et al., 2012; Clarke et al., 2013; Foster and McVey Neufeld, 2013; Farmer et al., 2014). This has led to the concept of the brain–gut–microbiota axis (Rhee et al., 2009; Collins et al., 2012; Cryan and Dinan, 2012). It can, therefore, be readily appreciated that factors perturbing the microbiota during the early-life could result in long-lasting effects on the gut and even brain function that persist into adulthood (Hsiao et al., 2013; Borre et al., 2014). The parallel development of the central nervous system (CNS) and the microbiota in early-life and the bi-directional communication between these organs puts the bacterial components in a position where they may exert substantial influence over the developing brain (Borre et al., 2014; Stilling et al., 2014).

Indeed, in preclinical studies, germ-free mice have an exaggerated stress response and altered anxiety (Sudo et al., 2004; Clarke et al., 2013). Moreover, anxiety-related behavior can be transmissible from one strain of mouse to another via the microbiota (Bercik et al., 2011). Germ-free mice also show memory deficits (Gareau et al., 2011) and social deficits of relevance to autism (Desbonnet et al., 2014) and decreased pain responses (Amaral et al., 2008). On the other hand probiotic bacteria have been shown to reduce anxiety, improve cognition and visceral pain (Eutamene et al., 2007; Rousseaux et al., 2007; McKernan et al., 2010; Bravo et al., 2011; Johnson et al., 2011; Stilling et al., 2014). Moreover transplantation of microbiota from IBS patients induces visceral pain in germ-free rats (Bercik et al., 2012). Together these results highlight the range of complex behaviors and signaling pathways which may be modulated by the maturing GI microbiome during critical neurodevelopmental windows.

Early-life stress in the form of maternal separation induces marked perturbations in fecal microbiota diversity (Bailey and Coe, 1999; O'Mahony et al., 2009). However, it is unclear if there is a direct link between such microbiota disturbances and the other early-life stress-induced alterations such as increased visceral hypersensitivity, increased anxiety, cognitive changes, enhanced pro-inflammatory cytokine production, changes in mast cell phenotype and elevations in corticosterone (O'Mahony et al., 2009).

Thus in this study we use broad-spectrum non-absorbable antibiotics as tools to disrupt the colonizing microbiota in early life and assess behavioral and physiological parameters in adulthood. Antibiotic administration, while essential to modern medicine, disrupts the developing bacterial ecosystem (Penders et al., 2006). Early-life antibiotic exposure is associated with altered microbiota (Fouhy et al., 2012), allergic disease (Marra et al., 2009) and inflammatory bowel disease (Shaw et al., 2010). Administration of broad-spectrum antibiotics, frequently used in pediatric practices, has been shown to reduce the biodiversity of fecal microbiota and delay the colonization by probiotic strains in infants (Bennet et al., 2002). However, the functional consequence of such modifications on health outcomes and disease susceptibility is unclear, especially at the level of the brain–gut axis.

We hypothesized that disruption of the microbial milieu during this critical period may result in long-lasting disturbances in brain–gut axis function relevant to stress-related psychiatric disorders and IBS.

EXPERIMENTAL PROCEDURES

Animals

Pregnant Sprague–Dawley female rats (Harlan, UK) and their offspring were used. They were kept under controlled conditions (21 ± 1 °C) on a 12-h light/dark cycle (lights on 7:00 am) and fed *ad libitum*. The dam and litter and later the offspring were maintained in animal cages (15 × 22 × 9 cm). All experiments were conducted in accordance with the European Directive 86/609/EEC, the Recommendation 2007/526/65/EC and approved by the Ethics Committee of University College Cork and the Health Department, Dublin.

Compounds

While the doses of vancomycin we used are higher than those seen in the clinic and vancomycin is usually prescribed along with a gram-negative antibiotic such as gentamicin and given intravenously we intended this study more as a proof of concept than one to replicate the effect of vancomycin in the clinic. It is non-absorbable from the GI tract and we were confident that any effects seen were due to disrupted microbiota and not systemic effects of the antibiotic (Yap et al., 2008). We chose to repeat the 100-mg/kg dosing of vancomycin only in study two as it appeared to have the strongest effect (both threshold and total pain behaviors) on visceral sensitivity in study one. The antibiotic cocktail (pimaricin 5/2.5-mg/kg, bacitracin 100/50-mg/kg, neomycin 100/50-mg/kg) and regime (reducing to half dose after day 5) has been previously shown to induce visceral hypersensitivity when administered to adult mice (Verdu et al., 2006).

Study 1: The impact of early-life vancomycin on behavioral and physiological measures across the brain–gut-microbiota axis

Male (n = 15) and female (n = 15) Sprague–Dawley rats were purchased from Harlan, UK. These were allowed to habituate to the animal facility for 1 week. The rats were mated after this and all 15 females became pregnant. The day of birth was designated as postnatal day 0. On postnatal day 4 the female pups were culled and males were randomly assigned to treatment groups. Treatment groups included: 10, 30 or 100-mg/kg of vancomycin (0.1 ml volume) or sterile water (n = 14–16) from postnatal day 4 for 10 days via oral gavage. The pups were monitored daily for an adverse reaction to the treatments. Fecal pellets were collected at four (first