Milk protein-derived peptides induces 5-HT$_{2C}$-mediated satiety in vivo

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Abstract

This study investigates the ability of milk protein-derived peptides to specifically activate the serotonin 2C (5-HT$_{2C}$) receptor, a key receptor in central regulation of food intake. A dose dependent 5-HT$_{2C}$ receptor activation by the 1 kDa ultrafiltration permeates of a sodium caseinate (NaCNH-1 kDa permeate) and a whey protein hydrolysate (WPH-1 kDa permeate) was demonstrated using an intracellular calcium mobilization assay in human embryonic kidney (Hek) cells expressing the 5-HT$_{2C}$ receptor. Both samples activated the 5-HT$_{2C}$ but not the 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors. NaCNH-1 kDa permeate significantly (p < 0.01) reduced cumulative food intake when administered to male mice (C57Bl/6) by intraperitoneal injection at 500 mg kg$^{-1}$ body weight. In contrast, no effect of WPH-1 kDa permeate could be seen on food intake in vivo. These results demonstrate the promising appetite-suppressing potential of NaCN-derived peptides, targeting the 5-HT$_{2C}$ receptor.
1. Introduction

As the incidence of obesity and its co-morbidities are increasing worldwide, so too does the need for novel anti-obesity intervention strategies (Bloom et al., 2008; Nguyen et al., 2012). Current anti-obesity treatments consist of dietary and lifestyle interventions as well as pharmaceutical drug treatments and surgery (Aronne, Powell, & Apovian, 2011; Cecchini et al., 2010; Derosa & Maffioli, 2012; Halford, Boyland, Blundell, Kirkham, & Harrold, 2010; Kang & Park, 2012; Powell, Apovian, & Aronne, 2011; Powell & Khera, 2010; Schellekens, Dinan, & Cryan, 2009). However, most pharmaceutical drug candidates have so far failed to reach the market, due to their low potency and efficacy or side effects and safety concerns (Aronne et al., 2011; Chakrabarti, 2009; Derosa & Maffioli, 2012; Kang & Park, 2012; Kennett & Clifton, 2010). Moreover, diets are generally associated with a low success rate due to poor adherence and compliance. Bioactive peptides from natural sources may have potential as weight management ingredients and are becoming of considerable interest in the food industry to counteract obesity when incorporated into the diet (Gonzalez-Castejon & Rodriguez-Casado, 2011; Gooda Sahib et al., 2012).

Weight loss can be achieved following the modulation of appetite and satiety signalling pathways in the central nervous system (CNS) (Obici, 2009; Valentino, Lin, & Waldman, 2010). Modulation of the central serotonergic system, in particular activation of the centrally expressed serotonin (5-hydroxytryptamine, 5-HT) 2C receptor (5-HT$_{2C}$), stimulates satiety via excitatory neurotransmission. Therefore, activation of the 5-HT$_{2C}$ receptor is a target for anti-obesity strategies (Dutton & Barnes, 2006; Garfield & Heisler, 2009; Lam et al., 2008; Miller, 2005b; Schellekens, Clarke, Jeffery, Dinan, & Cryan, 2012a; Somerville, Horwood, Lee, Kennett, & Clifton, 2007; Tecott et al., 1995; Tecott, 2007; Vickers, Clifton, Dourish, & Tecott, 1999). Several drugs targeting the central serotonergic system, such as sibutramine,
and fenfluramine, have been specifically developed to induce satiety, or as for m-
chlorophenylpiperazine (mCPP), found to reduce food intake as a secondary effect (Dalton,
Lee, Kennett, Dourish, & Clifton, 2004, 2006; Halford, Harrold, Boyland, Lawton, &
Blundell, 2007; Vickers et al., 1999). However, these drugs have been associated with poor
efficacy and safety concerns due to heart and pulmonary vasculature side-effects and non-
specific effects (Miller, 2005b). Recently, the specific 5-HT$_{2C}$ receptor agonist, Lorcaserin,
was approved for the treatment of obesity (Jandacek, 2005; Martin et al., 2011; O’Neil et al.,
2012; Redman & Ravussin, 2010). This highlights the benefits of targeting the 5-HT$_{2C}$
receptor in weight management strategies. Natural compounds, including milk protein-
derived bioactive peptides, are presumed to be safe because they are derived from food
sources. In addition, they have not been associated with the undesirable side effects, which
are often encountered with pharmaceutical drugs.

Milk proteins represent a unique source of biologically active peptides which have been
associated with various health benefits (FitzGerald, Murray, & Walsh, 2004; Korhonen,
2009; Kreider et al., 2011; Lonnerdal, 2003; Meisel & FitzGerald, 2003; Nagpal et al., 2011;
Phelan & Kerins, 2011). These include anti-hypertensive, mineral binding, antimicrobial,
immuno-regulatory, antidiabetic, antioxidant and opioid peptides (Booij, Merens, Markus, &
Van der Does, 2006; Froetschel, Azain, Edwards, Barb, & Amos, 2001; Korhonen, Marnila,
& Gill, 2000; Kumar et al., 2012; Nongonierma & FitzGerald, 2012b; Nongonierma &
FitzGerald, 2013; Orosco et al., 2004; Power, Jakeman, & FitzGerald, 2013; Virtanen,
Pihlanto, Akkanen, & Korhonen, 2007). In particular, the satiating effects of milk proteins
and milk protein-derived peptides may play a role in appetite regulation, weight loss and/or
prevention of weight gain (Anderson, Tecimer, Shah, & Zafar, 2004; Burton-Freeman, 2008;
Dougkas, Reynolds, Givens, Elwood, & Minihane, 2011; Luhovyy, Akhavan, & Anderson,
2007; Pilvi, Korpela, Huttunen, Vapaatalo, & Mervaala, 2007; Pilvi et al., 2008; Ricci-
Cabello, Herrera, & Artacho, 2012; Staljanssens et al., 2011; van Meijl, Vrolix, & Mensink, 2008; Veldhorst et al., 2009; Zemel, 2005).

It has been demonstrated that diets rich in \(\alpha\)-lactalbumin could enhance 5-HT release in rats (Orosco et al., 2004) and in humans (Booij et al., 2006; Nieuwenhuizen et al., 2009). This was attributed to a higher bioavailability of tryptophan, the precursor of 5-HT. In addition, the milk protein-derived bioactive peptide, \(\beta\)-casomorphin-7 (Tyr-Pro-Phe-Val-Glu-Pro-Ile), has been shown to bind and antagonize 5-HT\(_2\) receptors (Sokolov et al., 2005).

Recently, it was demonstrated that milk protein hydrolysates generated from different starting substrates, including sodium caseinate (NaCN), acid casein, skim milk powder and glycomacropeptide, behave as 5-HT\(_{2C}\) receptor agonists \textit{in vitro} (Nongonierma, Schellekens, Dinan, Cryan, & FitzGerald, 2013). The aim of this study was to assess the role of NaCN-derived and whey protein (WP)-derived bioactive peptides on 5-HT\(_{2C}\) receptor agonism. In addition, the specificity of NaCN and WP-derived bioactive peptides for the 5-HT\(_{2C}\) receptor over the closely related 5-HT\(_{2A}\) and 5-HT\(_{2B}\) receptors was studied. Finally, the effect of NaCN and WP hydrolysates on cumulative food intake in mice was assessed as a measure of the satiety inducing potential of these milk protein-derived peptides \textit{in vivo}. Milk protein-derived bioactive peptides with 5-HT\(_{2C}\) receptor agonist properties may have considerable potential in the development of non-pharmacological weight management ingredients and may be exploited in the development of anti-obesity functional foods.
2. Materials and methods

2.1. Generation of the milk protein hydrolysates and their associated ultrafiltration fractions

The experimental design of this study is summarized in Fig. 1. Sodium caseinate (NaCN, 90.4% (w/w) protein) was provided by Kerry Ingredients (Listowel, Co. Kerry, Ireland) and whey proteins (WP, 88.3% (w/w) protein) were from Carbery Milk Products (Ballineen, Co. Cork, Ireland). The 240 min NaCN hydrolysate (NaCNH) was produced by enzymatic hydrolysis as per Nongonierma et al. (2013). The whey protein hydrolysates (WPH) were generated using similar enzymatic hydrolysis conditions as for the NaCNH. Briefly, an aqueous solution of WP (10% (w/v) on a protein basis) was rehydrated for 1 h at 50°C with gentle mixing. The pH was adjusted to 3.0 with 1.0 N HCl before addition of pepsin. The pH of the reaction mixture was maintained constant throughout hydrolysis using a pH stat titrator (Titrando 843, Tiamo 1.4 Metrohm, Dublin, Ireland) for 4 h. In addition, aliquots of the WPH were withdrawn at 10, 30, 60, 120 min throughout the hydrolysis reaction. WPH was generated in two independent duplicates (n=2). The enzyme was heat inactivated at 90°C for 20 min. The 4h WPH was further fractionated by ultrafiltration (UF) using a cross-flow ultrafiltration unit (Sartoflow Alpha filtration System, Sartorius, Göttingen, Germany). WPH was sequentially UF fractionated through a 10, 5 and then a 1 kDa molecular weight cut-off (MWCO) stabilized cellulose UF cassette (Sartorius). Ultrafiltration was carried out at 30°C. Six fractions of WPH were collected (10, 5 and 1 kDa permeate and retentate). The samples were freeze-dried (FreeZone 18L, Labconco, Kansas City, U.S.A.) and stored at -20°C until utilization.
2.2. Reverse phase-ultraperformance liquid chromatography (RP-UPLC) and gel permeation high performance liquid chromatography (GP-HPLC) analysis of WPH and its associated UF fractions

Unhydrolysed whey proteins (WP), WPH and its UF fractions were analysed with an Acquity UPLC (Waters, Dublin, Ireland) as described by Nongonierma and FitzGerald (2012a). Samples were resuspended at 0.5 % (w/v) in 0.1 % (v/v) trifluoro acetic acid (TFA, Sigma-Aldrich, Dublin, Ireland) in HPLC grade water (VWR, Dublin, Ireland). Separation of peptides and individual milk proteins was carried out at 30°C using a 2.1 x 50 mm, 1.7 µm Acquity UPLC C18 BEH column mounted with a 0.2 µm inline filter (Waters). The absorbance of the eluant was monitored at 214 nm.

The molecular mass profiles of the proteins and peptides present in the samples was determined by GP-HPLC as described by Spellman, O’Cuinn, and FitzGerald (2009). Samples were resuspended at 0.25 % (w/v) in 0.1% TFA and 30 % HPLC grade acetonitrile (ACN, WVR) in HPLC grade water. A 600 x 7.5 mm I.D. TSK G2000 SW column mounted with a 75 x 7.5 mm I.D. TSKGEL SW guard column (Tosoh Bioscience, Stuttgart, Germany) was used for the separation. The absorbance was monitored at 214 nm.

2.3. Cell culture, in vitro transfection and lentiviral transduction

Human embryonic kidney cells (Hek293A) were cultured and maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich) supplemented with 10 % (v/v) heat inactivated foetal bovine serum (FBS, Sigma-Aldrich) and 1 % (v/v) Gibco® MEM non-essential amino acids (NEAA, Life technologies, Biosciences, Dublin) in an atmosphere of 95 % air and 5 % CO₂ at 37°C. Stably transfected Hek293A cells were maintained in complete media supplemented with 300 ng μL⁻¹ geneticin (G418, Merck, Darmstadt, Germany) as maintenance antibiotic. Hek293A cells stably expressing the
unedited 5-HT<sub>2C</sub>-INI or the partly edited 5-HT<sub>2C</sub>-VSV isoform of the 5-HT<sub>2C</sub> receptor were generated as per Schellekens et al. (Schellekens, Dinan, & Cryan, 2013). Briefly, Hek293A cells were transfected with plasmid construct expressing the unedited INI (H3309; Accession code, NM_000868, Genecopeia, MD, U.S.A.) or the partly edited VSV isoform of the 5-HT<sub>2C</sub> receptor (Genecopeia, T0336, Accession code: AF208053.1) with a C-terminal-EGFP tag, from a CMV promoter using lipofectamine® LTX plus reagent from Invitrogen™ (Life technologies, Biosciences, Dublin ), according to the manufacturer's instructions. The 5-HT<sub>2A</sub> receptor was amplified from the commercial construct 3xHA-tagged (N-terminus) 5-HT<sub>2A</sub> (HTR02ATN00, Missouri S&T cDNA Resource Center; MO, U.S.A.) and cloned into the pEGFP-N1 expression vector (6085-1, Clontech, Saint-Germain-en-Laye, France). Cells stably expressing the insert gene-EGFP fusion proteins were selected using flow assisted cell sorting (FACS, Becton Dickinson (BD) FACSVantage SE, Oxford, UK) and G418 as a selection antibiotic. Hek293A cells expressing the 5-HT<sub>2B</sub> receptor were generated using lentiviral transductions, using a third generation packaging, gene delivery and viral vector production system developed by Naldini and colleagues (Follenzi & Naldini, 2002a, 2002b; Naldini et al., 1996; Naldini, 1998; Vigna & Naldini, 2000). The 5-HT<sub>2B</sub> receptor (Missouri S&T; HTR02BTN00) construct was cloned into the lentiviral expression vector, pHR-SIN-BX-EGFP, modified from the lentiviral expression plasmid pHR-SIN-BX-ires-EmGFP vector (gift of Adrian Thrasher, Institute of Child Health, London, United Kingdom). The generated HIV-based lentiviral vector (lv) particles, pseudotyped with the vesicular stomatitis virus G (VSV-G), expressing the G-protein coupled receptor (GPCR) constructs from a spleen focus-forming virus (SFFV) promoter were produced using 293T-17 cells, following transient cotransfection of the cloned expression construct, pHR-5-HT<sub>2B</sub>-EGFP, the packaging construct, pCMVΔR8.91 and the envelope construct, pMD.G–VSVG. Cells were transduced with lv expressing the lv5-HT<sub>2B</sub>-EGFP receptor diluted in transduction media,
consisting of DMEM with 2 % (v/v) heat-inactivated FBS, 1 % (v/v) NEAA and an additional 8 μg mL⁻¹ polybrene® (H9268, Sigma-Aldrich). Fluorescence was monitored using flow cytometry (BD FACS Calibur, BD Biosciences) as an indicator of receptor expression.

2.4. Calcium (Ca²⁺) mobilization assay

Receptor mediated changes in intracellular Ca²⁺ were monitored on a Flex station II multiplate fluorometer (Molecular Devices Corporation, Sunnyvale, CA, U.S.A.). Stably transfected cells were seeded in sterile black-wall and clear flat bottom 96-well microtiter plates (3904, Costar, Fisher Scientific, Dublin, Ireland) at a density of 2.5 x 10⁵ cells mL⁻¹ (i.e., 2.5 x 10⁴ cells/well) and maintained for ~ 24 h at 37°C in a humidified atmosphere containing 5 % CO₂. After removal of the growth medium, cells were incubated with 25 μL of assay buffer (1x Hank’s buffered salt solution (HBSS) supplemented with 20 mM HEPES buffer) and 25 μl of Ca²⁺ 4 dye (R8141, Molecular Devices Corporation) according to the manufacturer’s protocol. FBS (Sigma-Aldrich) and 5-HT (H9523; Sigma-Aldrich) diluted in assay buffer were used as controls. Milk protein hydrolysates were resuspended in assay buffer (1x HBSS supplemented with 20 mM HEPES buffer). Suspensions were centrifuged at 2000 rpm (Jouan C3i multifunction centrifuge, Thermo Scientific, Dublin, Ireland) for 5 min at 4°C and filtered through 0.45 μm low protein binding filters (Sartorius, Dublin, Ireland). Hydrolysates were tested at the concentration of 1 mg mL⁻¹ in duplicate or triplicate. Test and control samples (25 μL/well) were directly added to cells using a Flexstation II and fluorescent readings were taken for 80 s in flex mode with excitation and emission wavelengths of 485 and 525 nm, respectively. The change in intracellular Ca²⁺ was monitored as an increase in relative fluorescence units (RFU), and the difference between maximum and minimum RFU (Vmax-Vmin) was compared and depicted as percentage of
maximum Ca\(^{2+}\) influx as elicited by the controls (5-HT or FBS, as indicated). For dose response curves, data analysis was performed using PRISM 4.0 (GraphPAD Software Inc., CA, U.S.A.).

2.5. Reverse-phase high performance liquid chromatography (RP-HPLC) to quantify L-tryptophan and serotonin

For quantification of L-tryptophan, the HPLC system consisted of a 510 pump, a 717 plus autosampler cooled at 4°C, a 486 absorbance detector, a bus SAT/IN module (Waters, Dublin), a croco-cil column oven and a 1046A Fluorescent Detector (Hewlett Packard, supplied by Agilent Technologies, Dublin). All samples were injected onto a RP Luna 3 μm C18 150 × 2 mm column (Phenomenex, London, UK), mounted with a Krudkatcher pre-column filter and security guard cartridges (Phenomenex). The analytical method was adapted from Herve, Beyne, Jamault, and Delacoux (1996). The mobile phase consisted of 50 mM acetic acid (Alkem/Reagecon, Cork, Ireland), 100 mM zinc acetate (Alkem/Reagecon) with 3 % (v/v) acetonitrile (Alkem/Reagecon) and was filtered through a 0.45 μm Millipore Durapore filter (AGB, Dublin) and vacuum degassed prior to use. Separations were achieved by isocratic elution at 0.3 mL min\(^{-1}\). The fluorescent detector was set to excitation and emission wavelengths of 254 and 404 nm, respectively. The UV detector was set at 330 nm. Samples were deproteinized by the addition of 20 μL of 4 M perchloric acid (Alkem/Reagecon) to 200 μL of sample spiked with 3-nitro-L-tyrosine (Sigma-Aldrich) as internal standard. A 20 μL aliquot of sample or standard (L-tryptophan, Sigma-Aldrich) was injected onto the HPLC column and results were processed using Empower® software (Waters). Analytes were identified based on their retention time and concentrations were determined using analyte:internal standard peak height ratios which were compared with standard injections. Results were expressed as ng L-tryptophan mg\(^{-1}\) of sample.
Serotonin concentration was determined using a different RP-HPLC system according to a modification of a previously described procedure (O’Mahony et al., 2008). The HPLC system consisted of a SCL 10-Avp system controller, LC-10AS pump, SIL-10A autoinjector (with sample cooler maintained at 4°C), CTO-10A oven, LECD 6A electrochemical detector (Shimadzu, Kyoto, Japan) and an online Gastorr degasser (ISS, Kent, UK). A RP column Kinetex 2.6 μm C18 100 × 4.6 mm (Phenomenex) maintained at 30°C was employed for analyte separation at a flow rate 0.9 mL.min⁻¹. The glassy carbon working electrode combined with an Ag/AgCl reference electrode (Shimadzu) was operated a + 0.8 V and the results were analysed using Class-VP 5 software (Shimadzu). The mobile phase, which contained 0.1 M citric acid, 0.1 M sodium dihydrogen phosphate, 0.01 mM EDTA (Alkem/Reagecon), 5.6 mM octan-1-sulphonic acid (Sigma-Aldrich) and 9 % (v/v) methanol (Alkem/Reagecon), was adjusted to pH 2.8 using 4 N sodium hydroxide (Alkem/Reagecon). Briefly, samples were diluted either 1/3 or 1/10 in chilled (4°C) mobile phase spiked with 0.1 ng μL⁻¹ of N-methyl 5-HT (Sigma-Aldrich) as internal standard. Diluted samples were then centrifuged at 14,000 rpm (Hettich MIKRO 22 R, DJB Labcare, UK) for 15 min at 4°C and 20 μL of the supernatant was injected onto the HPLC column. Serotonin was identified by its retention time as determined from injection of serotonin standards. Serotonin concentrations were determined using the analyte:internal standard (N-methyl 5-HT) peak height ratios, which were compared with that of serotonin standard injections. Results were expressed as nM serotonin mg⁻¹ of sample

2.6. Cumulative food intake experiment.

Animals, male C57Bl/6 mice were purchased from Harlan (Blackthorn, UK). Mice of 7 to 8 week-old were received at the facility. Animals were group-housed (4 to 5 mice per cage) in standard holding cages with controlled light-dark cycle (12-h light; lights on at 7:45 a.m.) and...
in a temperature- (21 ± 1°C) and humidity-controlled (55 ± 10 %) environment. Water and
standard lab chow (2018S Teklad Global 18 % Protein Rodent Diet, Harlan, UK) were
available *ad libitum*. All mice were weighed (precision 0.1 g) twice weekly and on the day of
the study between 9:00 and 10:00 a.m. All experiments were in full accordance with the
European Community Council directive (86/609/EEC) and approved by the Animal
Experimentation Ethics Committee of University College Cork. Cumulative food intake was
analysed during the light cycle following a 16 h fast during the dark phase as described
previously (Vickers, Dourish, & Kennett, 2001). The fasting period prior to the study is
warranted to induce a robust baseline feeding response, enabling studies with fewer animals.
On the day of the study, mice were placed into individual housing cages and left to
acclimatize for > 15 min. Animals were randomized into two groups (n = 10) and received an
intraperitoneal (IP) injection with either vehicle (HBSS and 20 mM HEPES) or milk protein
hydrolysate (WPH-1 kDa permeate or NaCNH-1 kDa permeate) resuspended in HBSS and
20 mM HEPES. The milk protein hydrolysates were administered at 200 or 500 mg kg⁻¹ body
weight and animals were returned to their individual holding cages. A pre-weighed amount
(5.00 ± 0.01 g) of standard lab chow was placed in the cages 20 min after IP injection of the
sample or vehicle. Food intake was determined at regular intervals by measuring the
cumulative amount of food consumed.

2.7. Statistical analysis

Statistical analyses were performed using SPSS software (IBM SPSS statistics 20, Chicago,
IL, U.S.A.). A mean multi comparison for the Ca²⁺ mobilization assay was performed using
one way ANOVA followed by Bonferroni post-hoc tests at a significance level of p < 0.05.
Food intake measurements between groups were analysed using a one way, repeated
measures ANOVA followed by estimation of the parameters. If the data was non-spherical a Huynh-Feldt correction was applied. Graphs were expressed as mean ± SEM.

3. Results and Discussion

3.1. Serotonergic activity of WPH and its associated UF fractions

In this study, WP-derived enzymatic hydrolysates were exposed to Hek cells expressing two isoforms of the 5-HT$_{2C}$ receptor, the unedited 5-HT$_{2C}$-INI receptor or the partially edited 5-HT$_{2C}$-VSV receptor isoform. The partly edited 5-HT$_{2C}$–VSV results from the distinctive ability of the 5-HT$_{2C}$ receptor to be modified by post-transcriptional RNA editing on 5 specific nucleotide positions, converting an adenosine to inosine residues, causing amino acid sequence changes (Burns et al., 1997). It is the most abundantly expressed 5-HT$_{2C}$ receptor isoform in human brain regions, in particularly in the hypothalamus (Niswender, Copeland, Herrick-Davis, Emeson, & Sanders-Bush, 1999; Werry, Loiacono, Sexton, & Christopoulos, 2008). In addition, increased editing of the 5-HT$_{2C}$ receptor has been associated with an altered feeding behaviour and fat mass, supporting the role for the 5-HT$_{2C}$ receptor in obesity (Kawahara et al., 2008; Olaghere da Silva et al.; Schellekens, Clarke, Jeffery, Dinan, & Cryan, 2012b). An in-house established medium throughput cellular based screening platform was utilized to screen whey protein-derived hydrolysates for their 5-HT$_{2C}$ receptor activating potential.

The WPHs sampled at different hydrolysis times were able to significantly induce intracellular Ca$^{2+}$ mobilization in Hek cells stably expressing both isoforms of the 5-HT$_{2C}$ receptor compared to wild type cells, albeit lower in cells expressing the partially edited 5-HT$_{2C}$–VSV isoform (Fig. 2A). This was expected, as increased 5-HT$_{2C}$ receptor editing has been associated with a decreased receptor signalling (Berg, Clarke, Cunningham, & Spampinato, 2008; Burns et al., 1997; Niswender et al., 1999; Olaghere da Silva et al., 2010;
Schellekens et al., 2012a). Interestingly, unhydrolysed WP was also able to increase intracellular Ca\(^{2+}\) to some extent in cells expressing the 5-HT\(_{2C}\) receptor isoforms. The relative Ca\(^{2+}\) influx in Hek cells expressing the 5-HT\(_{2C}\)–INI isoform was similar at the different hydrolysis times studied. However, with the 5-HT\(_{2C}\)–VSV isoform, an increase in relative Ca\(^{2+}\) influx was observed with increased hydrolysis time. In a previous study it was also demonstrated that with NaCNH, the 5-HT\(_{2C}\) receptor mediated Ca\(^{2+}\) influx was significantly (p < 0.05) enhanced with increasing hydrolysis time (Nongonierma et al., 2013). Indeed, NaCNH was shown to induce a Ca\(^{2+}\) influx > 60 % of 5-HT\(_{2C}\) receptor mediated Ca\(^{2+}\) signalling compared to control. In contrast, here we demonstrate that WPHs herein were only able to mediate 50 % of 5-HT\(_{2C}\) receptor mediated Ca\(^{2+}\) signalling compared to control (Fig. 2A). Nevertheless, the independent duplicate of WPH gave similar activation of the serotonin 5-HT\(_{2C}\) receptor (Table 1 and Fig. 2A), suggesting that the generation of the bioactive peptides was reproducible.

Next, UF fractionation of the WPH 240 min hydrolysate was carried out. The RP-UPLC profiles of WP, WPH and its associated UF fractions are illustrated in Fig. 3. The WPH (Fig. 3B) still contained unhydrolysed whey proteins (Fig. 3A), notably a high proportion of β-lactoglobulin was still present within the hydrolysate. This is in agreement with the relatively high resistance of β-lactoglobulin to peptic hydrolysis (Reddy, Kella, & Kinsella, 1988). In agreement with the MWCO of the membranes, the unhydrolysed proteins were retained in the 10 kDa retentate (Fig. 3C) and the 10, 5 and 1 kDa permeates did not contain any unhydrolysed WPs (Fig. 3D, 3E and 3F). The relative 5-HT\(_{2C}\) receptor-mediated increase in intracellular Ca\(^{2+}\) was significantly higher in the 1, 5 and 10 kDa permeates compared to their associated retentates (Table 1). A one-way Anova comparisons revealed an overall significant increase in 5-HT\(_{2C}\) receptor activation with UF fractionation (Table 1) of F(7) = 270.120; p < 0.001. The molecular mass distribution profile of WPH and its associated UF fractions is
illustrated in Fig. 4. An enrichment in low molecular mass peptides was seen during sequential UF fractionation, with the 1 kDa permeate containing ~ 92% peptides < 1 kDa.

The 5-HT2C receptor-mediated calcium increases were significantly higher in UF fractions enriched with low molecular mass peptides compared to unhydrolysed WP and WPH 240 min (p < 0.001) (Table 1). This indicates that the low molecular mass peptides within the UF permeates were responsible for activation of the serotonin 5-HT2C receptor. Similar results were previously found with the NaCNH, also showing an increase in the 5-HT2C receptor activation with peptides having a molecular mass < 1 kDa (Nongonierma et al., 2013).

Comparison of concentration response curves for NaCNH-1 kDa and WPH-1 kDa permeates demonstrated a similar efficacy (75-100 %) (Fig. 2B). However, the WPH-1 kDa permeate had a significantly higher (P < 0.05) potency when compared to NaCNH-1 kDa permeate (Fig. 2B), with absolute half maximal effective concentration (EC50) values of 0.022 and 0.111 mg mL⁻¹, respectively.

3.2. Specificity of the NaCNH and WPH 1 kDa permeate samples across the serotonin 2 receptor family

As already outlined, the 5-HT2 receptor family constitutes three different receptor types, the 5-HT2A, 5-HT2B and the 5-HT2C receptors (Hoyer et al., 1994; Hoyer, Hannon, & Martin, 2002). Activation of these receptors has been implicated in several physiological functions of the CNS and in pathologies including anxiety, depression, migraine, satiety and schizophrenia (Baxter, Kennett, Blaney, & Blackburn, 1995). Previous studies have identified that activation of the 5-HT2B receptor by serotonergic drugs, including Fenfluramine, plays an important role in the occurrence of negative side-effects, including pulmonary hypertension, impaired regulation of plasma serotonin level and valvulopathy (Miller, 2005b; Rothman et
al., 2000). The first specific 5-HT$_{2C}$ receptor agonist, Lorcaserin, was recently approved for the treatment of obesity (Jandacek, 2005; Martin et al., 2011; O'Neil et al., 2012; Redman & Ravussin, 2010), reinforcing the importance of the 5-HT$_{2C}$ receptor as a target for weight management. Nevertheless, serotonergic drugs, including Lorcaserin, are associated with safety concerns due to non-specificity, in particular caused by the non-selective activation of the 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors at the therapeutic dose. This has been suggested to be causal to heart and pulmonary vasculature side-effects (Miller, 2005a). The development of natural satiety inducing bioactives, such as milk protein–derived bioactive peptides specifically targeting the 5-HT$_{2C}$ receptor, may help to avoid the side-effects generally associated with the utilisation of pharmaceutical drugs. We therefore set out to investigate the 5-HT$_{2C}$ receptor specificity for the most active compounds, i.e., the NaCNH-1 kDa and WPH-1 kDa permeates (Fig. 5). Both 1 kDa permeates induced significant Ca$^{2+}$ mobilization in cells expressing the 5-HT$_{2C}$ receptor but not in cells expressing either the 5-HT$_{2A}$ or 5-HT$_{2B}$ receptor, while 5-HT was shown to equally activate the three 5-HT$_2$ receptors when tested at 1 mg mL$^{-1}$ (Fig. 5). This shows that the milk-derived 1-kDa permeates are more specific for the 5-HT$_{2C}$ receptor compared to the 5-HT$_{2A}$ and 5-HT$_{2B}$ receptor, which indicates an advantageous safety profile.

3.3. Serotonin and L-tryptophan concentrations in the NaCNH and WPH 1 kDa permeates

It is well known that 5-HT and the 5-HT precursor L-tryptophan have appetite-suppressing activities. Therefore, 5-HT and free L-tryptophan concentrations within WP, WPH 240 min and the WPH-1 kDa permeate were determined. In addition, 5-HT and free L-tryptophan concentrations were determined in NaCN, NaCNH 240 min and the NaCNH 1 kDa permeate. None of the samples contained 5-HT. Moreover, unhydrolysed WP and NaCN were also void of free L-tryptophan. The NaCNH 240 min and WPH 240 min samples contained very low
concentrations of free L-tryptophan, i.e. 6 and 11 ng mg\(^{-1}\), respectively. The WPH and NaCNH-1 kDa permeates contained high to moderate concentrations of L-tryptophan, i.e., 114 and 25 ng mg\(^{-1}\), respectively. Although L-tryptophan cannot directly bind the 5-HT\(_{2C}\) receptor, it may contribute to potential satiety effects overall \textit{in vivo}. However, this cannot explain the potent direct activation of the 5-HT\(_{2C}\) receptor \textit{in vitro} by the WPH-1 kDa permeate. This suggests that milk protein-derived peptides within NaCNH-1 kDa permeate were responsible for activation of the 5-HT\(_{2C}\) receptor.

3.4. Effect of NaCNH and WPH 1 kDa permeates on cumulative food intake in mice

Milk protein-derived bioactives have been shown to stimulate the release of various gut hormones associated with the regulation of food intake, including cholecystokinin (CCK) (Bowen, Noakes, Trenerry, & Clifton, 2006b; Figlewicz et al., 1992; Hall, Millward, Long, & Morgan, 2003), peptide YY (PYY) (Calbet & Holst, 2004; le Roux & Bloom, 2005), glucagon-like peptide 1 (GLP-1) (Aziz & Anderson, 2003; Hall et al., 2003) and ghrelin (Bowen, Noakes, & Clifton, 2006a; Bowen et al., 2006b; Williams & Cummings, 2005). Lactoferrin has recently been reported to reduce abdominal obesity via modulation of lipopolysaccharide levels and composition (Ono, Morishita, & Murakoshi, 2013). In humans, chronic intake of lactoferrin has also been associated with a decrease in visceral fat (Ono et al., 2010). Similarly, intake of \(\alpha\)-lactalbumin has been reported to reduce hunger, increase energy expenditure, and to regulate protein and fat balance (Hursel, van der Zee, & Westerterp-Plantenga, 2010). The satiety effects of \(\alpha\)-lactalbumin have been linked with the higher bioavailability of tryptophan, the 5-HT precursor. Thus, ingestion of \(\alpha\)-lactalbumin increases 5-HT release in rats (Orosco et al., 2004) and in humans (Booij et al., 2006; Nieuwenhuizen et al., 2009), as a consequence. The effect of the NaCNH and WPH 1 kDa permeates on cumulative food intake in male mice (C57Bl/6) was studied. No effect on food
intake was seen after administration of 200 mg kg\(^{-1}\) body weight (Fig. 6A and 6B). Nevertheless, following 500 mg kg\(^{-1}\) body weight administration of the NaCNH-1 kDa permeate, a significant reduction of cumulative food intake was observed (Fig. 6C and 6D). Repeated measures ANOVA showed a significant main effect of the treatment NaCNH-1 kDa permeate vs. vehicle (\(F(1,18)=8.552; p < 0.01\)) and a significant main effect of time (\(F(4.447,80.049)=834.962; p < 0.001\)). However, no significant interaction of time × treatment (\(F(4.447,80.049)=1.429; p = 0.228\)) was seen. Unhydrolyzed WP and NaCN did not demonstrate an effect on food intake, as no significant main effect of WP or NaCN compared to vehicle was observed, confirming the specificity of the anorexigenic effect of NaCNH-1 kDa permeate (Fig. 6G and 6H). Surprisingly, no significant effect on food intake was observed following IP administration of the WPH-1 kDa permeate when tested at 500 mg kg\(^{-1}\) body weight (Fig. 6E and 6F).

The lack of bioactivity seen with WPH-1 kDa permeate may be explained by the fact that the bioactive peptides within this sample were not bioavailable possibly due to their degradation by peptidases in the circulation or to a poor uptake in the target tissues. Future studies are needed to assess the orexigenic effect of these dairy-derived peptides following oral ingestion as well as the resulting plasma concentrations and kinetics of these orally administered bioactives. Bioactive peptides within the milk protein hydrolysates investigated in this study need to be able to cross the gut barrier and resist degradation by epithelial and serum peptidases. Previously, it has been shown that only minor fractions of dietary peptides reach the blood circulation due to proteolytic instability and poor absorption. However, intestinal transport of intact peptides has been reported in vivo (Gardner, 1983). The bioavailability of peptides is thought to be governed by their stability to gastrointestinal digestion, their permeability through the intestinal mucosa and stability to brush border and serum peptidases.
Casein-derived bioactive peptides could be detected in the jejunum of human subjects intubated with a nasogastric tube (Boutrou et al., 2013). Transepithelial transport of oligopeptides through a Caco-2 cell layer has been reported to be mainly governed by the hydrolytic activity of brush border peptidases (Shimizu, Tsunogai, & Arai, 1997). Degradation of the angiotensin converting enzyme (ACE) inhibitory peptide, Leu-His-Leu-Pro-Leu-Pro, by brush border peptidases of Caco-2 cells has been reported to occur as early as 5 min (Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008). However, the peptide released (His-Leu-Pro-Leu-Pro) by brush border peptidases was still bioactive and could cross the Caco-2 cell layer. Degradation of Val-Pro-Pro has also been shown, but intact material could be found in the basolateral compartment, showing that a certain proportion of Val-Pro-Pro could cross the Caco-2 cell monolayer without being degraded (Satake et al., 2002).

A few studies have demonstrated that specific peptide sequences could reach the circulation without being degraded. Following ingestion of a lacto-tripeptide enriched yogurt beverage, the ACE inhibitory peptide, Ile-Pro-Pro was found in its intact format in the blood circulation (Foltz et al., 2007). Other relatively hydrophobic dipeptides (Leu-Trp, Phe-Tyr and Ile-Tyr) were identified in the plasma following ingestion of the lacto-tripeptide enriched yoghurt beverage (Foltz et al., 2007). Moreover, the bioactive peptides found within hydrolysed NaCN are relatively small and hydrophobic in nature, which may suggest they have the potential to survive gastrointestinal digestion and allow for intestinal permeation (Morifuji et al., 2010; Nongonierma et al., 2013; Shimizu et al., 1997). Larger peptides may also cross the intestinal barrier and it has been reported that of peptides ≥ 5 amino acid length could permeate through a layer of Caco-2 cells (Quirós et al., 2008; Vermeirssen et al., 2002).
4. Conclusion

In this study the 5-HT$_{2C}$ receptor activating potential of enzymatic hydrolysates of WP and NaCN was demonstrated *in vitro*. It was shown that the serotonergic activation observed with the WHP-1 kDa permeate and the NaCNH-1 kDa permeate was specific for the 5-HT$_{2C}$ receptor, with no activation seen for the 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors. This suggests that the bioactive peptides may induce less side effects generally associated with non-specific activation of the 5-HT$_{2B}$ receptor, which may lead to valvulopathy. The *in vitro* 5-HT$_{2C}$ activating potential of NaCNH-1 kDa permeate translated to a reduced cumulative food intake in mice. However, these effects were only observed at a high dose of sample following IP administration. In addition, no *in vivo* effect was seen upon administration of the WHP-1 kDa permeate, despite the high potency on 5-HT$_{2C}$ receptor activation *in vitro*. The reason for the absence of an *in vivo* effect of the WPH derived hydrolysate permeate could be multifaceted and may be related to peptide stability, bioavailability or mal-absorption, and should be investigated further. The results described herein demonstrate the potential of milk-protein derived bioactive peptides with 5-HT$_{2C}$ receptor agonist properties as satiating ingredients. These may be further exploited in the development of functional foods with anti-obesity properties.

Acknowledgements

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References


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breakfasts containing alpha-lactalbumin, or gelatin with or without added tryptophan, on hunger, 'satiety' hormones and amino acid profiles. *Br J Nutr*, 101, 1859-1866.


Table 1. Increased whey protein hydrolysate (WPH)-mediated intracellular calcium ($\text{Ca}^{2+}$) in Hek-5-HT$_{2C}$-INI cells upon enrichment of low molecular mass peptides in the ultrafiltration (UF) samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Relative increase in intracellular $\text{Ca}^{2+}$ (% RFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WP</td>
<td>-11.8 ± 0.9</td>
</tr>
<tr>
<td>WPH</td>
<td>40.7 ± 2.2$^a$</td>
</tr>
<tr>
<td>WPH-10 kDa Ret</td>
<td>-0.5 ± 2.6$^b$</td>
</tr>
<tr>
<td>WPH-10 kDa Perm</td>
<td>67.9 ± 2.7$^{a,b}$</td>
</tr>
<tr>
<td>WPH-5 kDa Ret</td>
<td>42.8 ± 3.2$^{a,b}$</td>
</tr>
<tr>
<td>WPH-5 kDa Perm</td>
<td>86.7 ± 3.5$^{a,b}$</td>
</tr>
<tr>
<td>WPH-1 kDa Ret</td>
<td>76.7 ± 1.3$^{a,b}$</td>
</tr>
<tr>
<td>WPH-1 kDa Perm</td>
<td>97.1 ± 1.8$^{a,b}$</td>
</tr>
</tbody>
</table>

1 WP: whey protein, WPH: 240 min whey protein hydrolysate, Perm: ultrafiltration (UF) permeate, Ret: UF retentate.

2 Relative increased in intracellular $\text{Ca}^{2+}$ expressed as percentage of relative fluorescence units (RFU) with respect to serotonin (5-hydroxytryptamine, 5-HT) (10 nM). Samples were tested at 0.5 mg mL$^{-1}$. Average values ± SEM of three replicates. Statistical different values compared to WP (a; p<0.001) and compared to WPH (b; p<0.001) are indicated.
Figure captions

Fig. 1 Schematic representation of the experimental design including the enzymatic hydrolysis, ultrafiltration fractionation and bioassay testing of the 1 kDa permeates of the 240 min sodium caseinate (NaCNH-1 kDa permeate) and whey protein hydrolysates (WPH-1 kDa permeate).

Fig. 2 Whey protein hydrolysate (WPH)-mediated intracellular calcium (Ca\(^{2+}\)) in human embryonic kidney (Hek) wild type (wt) (□) cells, Hek-5-HT\(_{2C}\)-INI (■) and Hek-5-HT\(_{2C}\)-VSV (■) cells. Intracellular Ca\(^{2+}\) increase was depicted as a percentage of maximal Ca\(^{2+}\) increase as elicited by the control (3.3 % FBS). Graph represents the mean ± SEM of samples analysed in triplicate. Statistical different values are indicated for Ca\(^{2+}\) increases in Hek-5-HT\(_{2C}\)-INI cells compared to WP in Hek wt (a, p<0.001); for Hek-5-HT\(_{2C}\)-VSV compared to WP in Hek wt (b, p<0.01); for Hek-5-HT\(_{2C}\)-INI compared to WP in Hek-5-HT\(_{2C}\)-INI (c, p<0.01 and d, p<0.001) and for Hek-5-HT\(_{2C}\)-VSV compared to WP in Hek-5-HT\(_{2C}\)-VSV (e, p<0.001) (A). Both the WPH-1 kDa permeate (□) and the NaCNH-1 kDa permeate (●) induce Ca\(^{2+}\) mobilization in cells expressing the 5-HT\(_{2C}\) receptor as analysed in a concentration response curve. Intracellular Ca\(^{2+}\) increase was depicted as a percentage of maximal Ca\(^{2+}\) increase as elicited by the endogenous ligand, 5-HT (1uM). Graph represents the mean ± SEM of samples analysed in duplicate (B).

Fig. 3 Reverse-phase ultraperformance liquid chromatographic (RP-UPLC) profile of (A) the unhydrolysed whey protein (WP), (B) 240 min whey protein hydrolysate (WPH), (C) 10 kDa retentate of WPH, (D) 10 kDa permeate of WPH (E) 5 kDa permeate of WPH and (F) the 1
kDa permeate of WPH. 1: caseinomacropetide; 2: α-lactalbumin; 3: β-lactoglobulin. The analysis was conducted three times for the 240 min hydrolysate (n = 3), showing identical peptide profiles in the 3 instances.

**Fig. 4** Molecular mass distribution of the 240 min whey protein hydrolysate (WPH) and its associated ultrafiltration (UF) fractions. Molecular mass distribution: ■: > 5 kDa; □: 5-1 kDa, ■: < 1 kDa.

**Fig. 5** Specificity of the NaCNH-1 kDa permeate and WPH-1 kDa permeate samples compared to 5-HT2 receptor activation mediated by the endogenous ligand, 5-HT (1uM). Intracellular calcium (Ca^{2+}) increase was depicted as a percentage of maximal Ca^{2+} increase as elicited by control (1 µM 5HT) in Hek-5-HT2A (□) cells, Hek-5-HT2B (■) and Hek-5-HT2C (■) cells. Graph represents the mean ± SEM of samples analysed in duplicate. Statistical different values of 5-HT2C compared to 5-HT2A (a; p<0.001 and c; p<0.01) and compared to 5-HT2B (b; p<0.001) are indicated.

**Fig. 6** Cumulative food intake following administration of milk protein-derived hydrolysates. Food (regular chow) intake in C57Bl/6 male mice was determined following intraperitoneal (IP) injection with 200 mg kg^{-1} body weight (A, B) or 500 mg kg^{-1} body weight (C, D) 1 kDa permeate of the 240 min sodium caseinate hydrolysate (NaCNH-1 kDa permeate). The 1 kDa permeate of the 240 min whey protein hydrolysate (WPH-1 kDa permeate) was IP injected at 500 mg kg^{-1} body weight (E, F). Unhydrolyzed WP and NaCN were also IP injected at 500 mg kg^{-1} body weight (G, H). Cumulative food intake was determined at regular intervals after IP injection (A, C, E and G). Food intake per time bin was calculated (B, D, F and H). Statistical significance was determined using repeated measures ANOVA and estimation of
parameters; statistical significance depicted is notated as ** p < 0.01 and * p < 0.05; n = 10 mice per treatment group.
NaCN: sodium caseinate; NaCNH: 240 min sodium caseinate hydrolysate; WP: whey proteins; WPH: 240 min whey protein hydrolysate; RP-UPLC: reverse phase ultraperformance liquid chromatography; GP-HPLC gel permeation-high performance liquid chromatography; Hek: human embryonic cells; 5HT$_{2C}$: serotonin (5-hydroxytryptamine, 5-HT) 2C receptor.

**Fig. 1**
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6