

Opioid Receptor Ligands Derived from Food Proteins

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Abstract: During the last two decades a variety of food protein fragments has been demonstrated to elicit biological effects in various in vitro or in vivo test systems. A considerable part of these bioactive peptides are opioid receptor ligands, which may be regarded as exogenous supplements to the endogenous opioidergic systems of the human organism. Most of these food-derived opioid receptor ligands are fragments of the milk proteins alpha-, beta- or kappa-casein, alpha-lactalbumin, beta-lactoglobulin or lactotransferrin; however, also wheat gluten, rice albumin, bovine serum albumin or hemoglobin, i.e. possible constituents of meat, and even a protein from spinach could be demonstrated to contain fragments behaving like opioid receptor ligands. Practically all of these compounds display opioid agonist activity; only very few of them behave like opioid antagonists. Bioactive food protein derivatives have been termed "food hormones", which implies that these compounds display their bioactivities when released from food constituents, i.e. from their precursor molecules due to the action of gastrointestinal enzymes. The critical point in case of food protein-derived opioid receptor ligands is that only a minority of their bioactive effects demonstrated as yet has been observed upon oral or intragastric administration of these peptides or their precursor proteins and that most of these studies have been performed in animals. Thus, in terms of "evidence-based dietary supplementation" more studies are needed to prove effects of food protein-derived opioid receptor ligands or their precursors after oral administration in humans and, moreover, to prove a benefit for the consumer's organism.

Key Words: Opioid receptor ligands; Opioids; Opioid peptides; Beta-casomorphins; Gluten exorphins; Hemorphins; Bioactive substances; Functional food.

INTRODUCTION

For a long time basic foodstuffs such as milk or bread have just been regarded as "brick" or energy providers required by the food consumer's organism. During the last two decades, however, quite a few nutrients have been shown to contain or to release under gastrointestinal conditions "bioactive" substances apparently able to elicit effects in the recipient's organism [1]. Since milk is a foodstuff representing a "bridge" connecting the neonate's and its mother's organism after their separation for quite a long time, the detection of a variety of hormones and other essential agents in mother's milk was, in principle, not amazing [2, 3, 4, 5, 6]. However, during the last decade it became clear that milk contains bioactive substances which may be expected to elicit effects in the adult milk consumer's organism as well - at least in the gastrointestinal tract. The topic is drawing impressively increasing interest as may be concluded from the reviews written on the field during the last 5 years [7, 8, 9, 10, 11, 12, 13, 14]. Obviously, biologically active compounds derived from all kinds of food have been searched for [15] - mostly in view of their influence on intestinal functions [16], but also in view of effects on the central nervous system [17]. Adverse

effects of certain food constituents had to be considered also [18]. Recently even a database of biologically active peptide sequences has been offered [19]. Whereas terms like "bioactives" [13], "food peptides" or "food hormones" [20] or their short-cuts, e.g. "formones" [11] still refer to the effects of food-derived bioactive substances on the food consumer's organism, more recently coined terms such as "functional foods" or "nutraceuticals" [21, 22, 23] indicate a considerable interest in "formone"-containing "functional foods" - not only signalled by their consumers, but also by their producers.

From the very beginning of the development outlined above there was a considerable interest in screening basic foodstuffs for opioids potentially contained therein. In view of the peptide character of endogenous opioids found in the mammalian organism, food protein cleavage products appeared to be candidates. The search for food-derived opioid peptides was, in fact, successful [24].

In this review, the chase for food protein-derived opioid agonists or antagonists ending up with their demonstration and preliminary pharmacological characterization will be described under a historical point of view - with emphasis on the significance of food-derived opioid peptides as exogenous opioid receptor ligands in relation to the endogenous opioidergic systems of the human organism. Proven and potential physiological as well as pharmacological, i.e. nutraceutical significance of food protein-derived opioid receptor ligands will be discussed.

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OPIOIDERGIC SYSTEMS OF THE HUMAN ORGANISM

Endogenous Opioidergic Systems

Endogenous opioidergic systems of the human organism consist of opioid receptors and their ligands, endogenous opioids with alkaloid or with peptide structure. For extensive review up to the year 1992 see *Handbook of Experimental Pharmacology*, Volumes Opioids I and II [25]; short-cut and updated information about the field as far as relevant in context with this review has been presented as well [26].

Opioid Receptors

Opioid receptors have been found in the central and in the peripheral nervous system, in the immune system and in the endocrine system of mammals [27, 28, 29]. There are three types of opioid receptors, μ -, δ - and κ -opioid receptors; μ_1/μ_2 , δ_1/δ_2 and κ_1/κ_2 subtypes have been reported as well [30, 31, 32]. The chromosomal location of the receptor genes, OPRM (μ), OPRD (δ) and OPRK (κ), are 6q24-25, 1p34.3-36.1 or 8q11-12, respectively [32, 33]. A fourth receptor sharing a high degree of structural homology with those conventional opioid receptors, displaying, however, quite distinct pharmacological properties is the Orphanin FQ / nociceptin receptor, N/OFQ [34]. Further names for the receptors, MOP, MOR, OP₃ (for μ), DOP, DOR, OP₁ (for δ), KOP, KOR, OP₂ (for κ) or NOP, NOR, ORL1 (for N/OFQ) have not been widely accepted as yet [33, 35]. Opioid receptors belong to the family of G protein-coupled receptors and their activation results in adenylate cyclase inhibition (G_i), K⁺ channel activation (G_{i/o}) or Ca⁺⁺ channel inactivation (G_o) [31, 32, 36, 37].

Endogenous Opioid Receptor Ligands

Endogenous opioid receptor ligands may have alkaloid or peptide structure. There is only limited information about endogenous "opioid alkaloids" [38, 39]. By far most of the endogenous opioid receptor ligands are peptides; they almost exclusively behave like agonists at opioid receptors. These "opioid peptides" may be divided in two groups, "typical" and "atypical" opioid peptides.

Typical opioid peptides are liberated from three precursor proteins, from proenkephalin (PENK), from proopiomelanocortin (POMC) and from prodynorphin (PDYN). From PENK the enkephalin pentapeptides (and longer fragments containing the enkephalin sequences), from POMC the endorphins (after their cleavage from the precursor they may be N-terminally acetylated) and from PDYN the dynorphins (and further fragments such as neoendorphins) are released. Like the opioid receptors, PENK, POMC and PDYN are mainly expressed in the central and in the peripheral nervous system, in the immune and in the endocrine system [40, 41]. As a structural characteristic, all typical opioid peptides have the same N-terminal amino acid sequence, Tyr-Gly-Gly-Phe, which is their fragment able to interact with opioid receptors.

Atypical opioid peptides [42] differ from typical opioid peptides in view of structure and precursor proteins. All atypical opioid peptides have a N-terminal Tyr residue, but

the rest of the N-terminal amino acid sequence is not identical with that of the typical opioid peptides and shows considerable variation. Their precursor proteins, e.g. hemoglobin, appear to be quite different in terms of distribution or function in comparison with PENK, POMC or PDYN [42].

Functional Significance of Opioidergic Systems

Receptors and ligands of the endogenous opioidergic systems are biosynthesized within cells, tissues or compartments of the immune system, the endocrine system, the central and the peripheral nervous system. As compatible with this distribution, the ligands, as far as administered like drugs, elicit effects on their receptors located within these systems, most of which are well-known classical opioid effects such as antinociception, respiratory depression, sedation, gastrointestinal motility reduction, etc. In particular on cells of the immune system, however, additional effects are elicited - apparently due to the opioid peptides' additional interaction with target molecules not identical with opioid receptors [43, 44]. However, for most of these endogenous ligands it is not clear as yet, under which conditions they interact with opioid receptors. Although opioidergic systems appear to be involved in a wide variety of functions such as nociception, cardiovascular regulation, respiration, neuroendocrine, neuroimmune or various behavioural activities, etc. [25, 45], inactivation of PENK, POMC, PDYN, MOR, DOR and KOR genes in mice by homologous recombination (gene knockout mice) revealed very interesting, but still limited information about the physiological significance of opioidergic systems in mammals [46]. Information about consequences of gene deficiencies in humans is still meagre. POMC gene mutations just led to early onset severe obesity, red hair pigmentation and adrenal insufficiency [47], and allelic variation in the PDYN gene promoter as studied did not prove to be correlated with physiological or pathophysiological phenomena such as schizophrenia or heroin addiction [48, 49]. Variations in the human MOR gene appeared to correlate with heroin addiction [50] or with idiopathic absence epilepsy [51], whereas human DOR gene variations as studied did not correlate with heroin or alcohol dependence [52].

Food Protein-derived Opioid Receptor Ligands: Exogenous Supplements to Endogenous Opioidergic Systems

Opioid receptor ligands not biosynthesized by the human organism were used thousands of years before the detection of their endogenous counterparts; as constituents of opium they were used as analgesics or antidiarrhoics and their major representative, morphine, was isolated from opium around 1800 by a pharmacist, *F. W. Sertürner*. Morphine and some other opioid alkaloids are still the only analgesics effective for treatment of patients with extremely severe pain. After the demonstration of opioid receptors in 1973 as well as, during the following years, of a series of peptide ligands cleaved from precursor proteins in the human organism the interest in exogenous opioid receptor ligands shifted from opioids of alkaloid structure to opioids of peptide structure. In fact, a series of such exogenous opioid peptides has been demonstrated, which just can be

regarded as exogenous supplements to the endogenous opioidergic systems (Fig. 1); these exogenous opioid receptor ligands were food protein cleavage products exclusively [24] - most of them derived from milk proteins [26, 53, 54, 55, 56]. They proved to be atypical opioid peptides exclusively [42, 57]. It appears more than likely that among these exogenous opioid receptor ligands at least the milk protein-derived opioid peptides are essential supplements of the opioidergic systems of the recipient's organism (Fig. 1).

SEARCH FOR FOOD PROTEIN-DERIVED OPIOID RECEPTOR LIGANDS

The chase for exogenous ligands of opioid receptors accompanied the chase for their endogenous counterparts from the very beginning of the research on opioidergic systems. Screening of basic foodstuffs for materials with opioid agonist or antagonist activity soon succeeded in the demonstration and subsequently in the pharmacological characterization of food protein-derived opioid receptor ligands.

Screening Basic Foodstuffs for Food Protein-derived Materials with Opioid Agonist or Antagonist Activity

Most basic foodstuffs, i.s. milk, cereals, maize, rice, soy, eggs or blood as a constituent of meat were screened for

protein fragments with opioid agonist or antagonist activity (Table 1). From a historical point of view credit should be given for pioneer work to the group of *Werner Klee* and *Christine Zioudrou* [58], who conducted the first systematic study on this field or to Seymour Ehrenpreis' group [59] for raising the first finding on a milk protein-derived opioid-like acting material. The first food constituent identified as an opioid receptor ligand was a bovine beta-casein fragment [60] later followed by the identification of further opioid agonists or antagonists derived from milk proteins [61].

Two strategies were used for identification of the opioids. Frequently, preparations of food, mixtures of food proteins or proteins isolated from food were subjected to peptic, tryptic, or chymotryptic hydrolysis or to digestion by various gastrointestinal enzymes. The hydrolysates were screened for materials with opioid activity using either an adenylate cyclase test [58] or one of two bioassays employed very frequently for testing materials for opioid activity, i.s. the guinea pig ileum longitudinal muscle myenteric plexus preparation ("strip") or the mouse vas deferens preparation; a material was assumed to contain a peptide with opioid agonist activity, when its inhibitory effects on these organ bath preparations could be antagonized by the opioid antagonist naloxone. Further, radioligand competition assays with opioid receptor preparations were also used for assessment of opioid properties. Opioid-like behaving materials were subjected to chromatographical purification and isolation procedures and

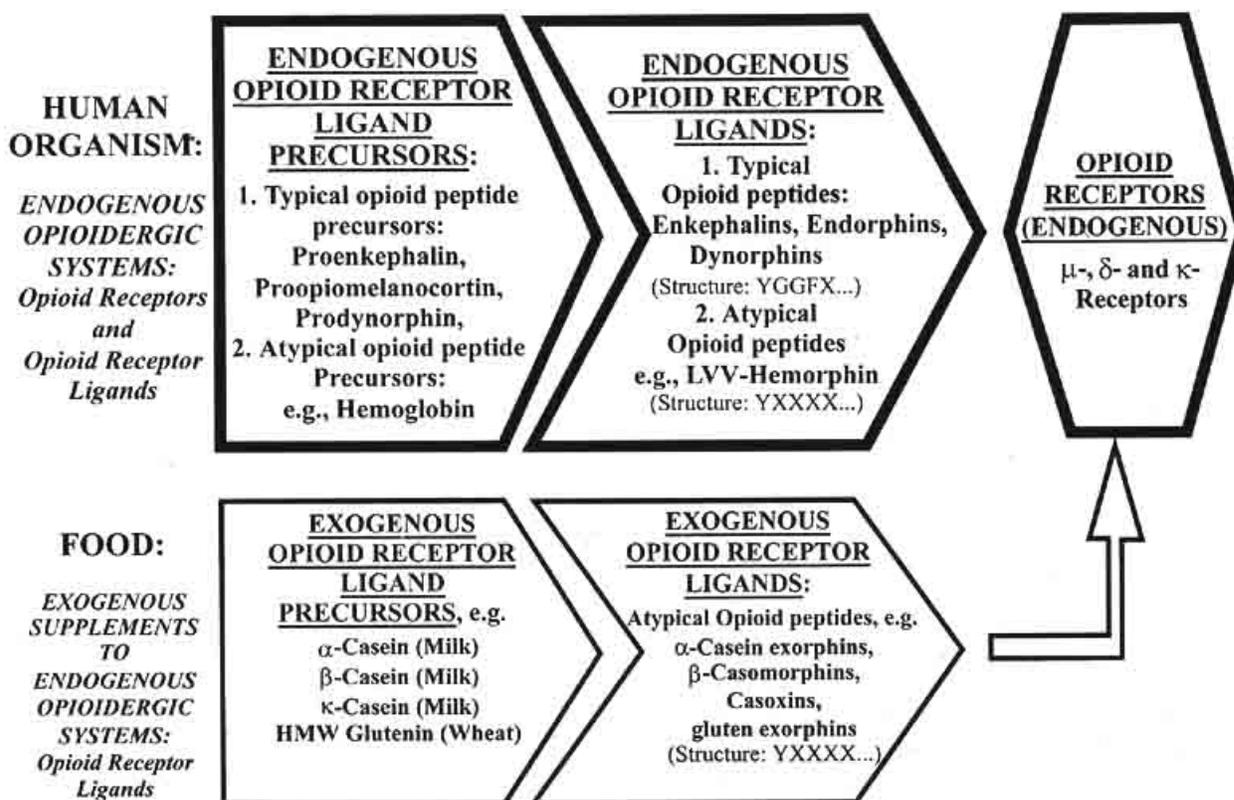


Fig. (1). Opioidergic systems.

Table 1. Screening Hydrolysates of Food Proteins for Materials with Opioid Agonist or Antagonist Activity

FOODSTUFF:	PROTEIN:	MATERIAL WITH OPIOID AGONIST OR ANTAGONIST ACTIVITY			[REF.]
		Looked for:	Found:	Identified:	
Milk	-Casein	+	+	+	[58,62,63,64]
	-Casein	+	+	+	[60,65,66,67,68,69,70,71]
	-Casein	+	+	+	[72,73]
	-Lactalbumin	+	+	+	[58, 70, 74]
	-Lactoglobulin	+	+	+	[70]
	Lactotransferrin	+	+	+	[73]
Wheat	Gluten	+	+	+	[58,77,78]
	Gliadin	+	+	+	[58, 79,80]
Barley	Hordein	+	+	-	[58]
Oats	Avenin	+	-	-	[58]
Rye	Secalin	+	-	-	[58]
Maize	Zein	+	+	-	[58]
Rice	Albumin	+	+	+	[76]
Soy	-Protein	+	-	-	[58]
Eggs	Ovalbumin	+	-	-	[58]
Blood (const. of meat)	Albumin	+	+	+	[58, 75]
	Hemoglobin	+	+	+	[81, 82,83,]
	-Globulin	+	-	-	[58]
Spinach	RUBISCO (D-Ribulose-1, 5-biphosphate carboxylase / oxygenase)	+	+	+	[84]

the isolated peptides, in most cases, were identified by Edman degradation techniques and amino acid analysis. Synthesis of the identified peptide was an additional step necessary for confirmation of the peptide's sequence. A second method proved to be faster: Any food protein fragment expected to interact with opioid receptors due to a Tyr residue in N-terminal position was synthesized and tested for opioid activity using the aforementioned techniques. In case of a positive result, evidence for its release under gastrointestinal conditions had to be provided subsequently.

In fact, a variety of enzymatic digests of milk proteins, -casein [58, 62, 63, 64], -casein [60, 65, 66, 67, 68, 69, 70, 71], -casein [72, 73], -lactalbumin [58, 70, 74], -lactoglobulin [70], and lactotransferrin [58, 73] were shown to contain materials with opioid agonist or antagonist activity, which subsequently were all identified (Table 1). The opioid material contained in the peptic hydrolysate of -casein [58] appeared to be very pure from the very beginning [58] as it apparently also was the case with the opioid demonstrated in a peptic bovine serum albumin

digest [58]; however, their identification took considerable time - more than a decade [62, 63, 75].

Also peptic hydrolysates of proteins from wheat, barley, oats, rye, maize or soy were screened for opioid agonist or antagonist activity very early [58] and indeed gluten, gliadin, hordein and zein hydrolysates proved to contain materials with opioid agonist activity as shown in the adenylate cyclase test [58]. The peptides from gliadin and gluten were identified later [77, 78, 79, 80]; this, however, took never place with the materials from hordein and zein. Also a tryptic digest of rice seeds was successfully screened for materials with opioid receptor ligand properties; the material was identified and appeared to show opioid agonist as well as antagonist properties [76].

Peptic ovalbumin and -globulin hydrolysates tested as well did not contain opioid agonist or antagonist activity [58], but enzymatic digests of bovine serum albumin [58, 75] and of whole bovine blood did contain opioid-like acting materials, which were shown to be fragments of albumin [75] or hemoglobin [81], respectively. Later further

hemoglobin derivatives, which behaved like opioid agonists as well, were shown to be released from bovine hemoglobin upon peptic digestion of the protein [82, 83].

Very recently, D-ribulose-1,5-biphosphate carboxylase/oxygenase (RUBISCO), a spinach constituent, was shown to contain a fragment, which displayed opioid activity as well [84].

Identification of Food Protein-derived Materials with Opioid Agonist or Antagonist Activity

In most cases the demonstration of a material with opioid agonist or antagonist activity in hydrolysates of food preparations, protein mixtures or single food proteins (Table 1) was followed by its isolation and the identification of its amino acid sequence and, in case of hydrolysates of more than one protein, its precursor was identified as well (Table 2). Tests for assessment of its opioid activities were then performed with the synthetic peptide. In a second type of approach, skipping hydrolysis, isolation and identification procedures, fragments of food proteins with a Tyr residue in N-terminal position, which indicated the possibility of an interaction with opioid receptors (see preceding sections) were synthesized and tested for opioid agonist or antagonist activity (Table 2). In case of a positive result usually the peptide's release from its precursor under gastrointestinal conditions was studied.

For all categories of food protein derivatives (single derivatives or groups of derivatives) with opioid agonist or antagonist activity as far as identified as yet, in Table 2 origin, i.e. foodstuff, precursor protein and species (whenever appropriate), way of identification, modification of sequence during isolation procedures or arbitrary modification by design of a synthetic opioid receptor ligand as well as the names of the food protein derivative or of the derivative group are listed. Opioid receptor ligands derived from milk proteins of the human species were included in view of a potential need to substitute those mother's milk constituents by nutrients.

As far as milk protein-derived opioid receptor ligands are concerned, the relevant data have been completely included together with the respective references in Table 2 for synoptic reasons and they also have been accomplished by adding data about a few novel compounds; however, since the field of milk protein-derived opioid receptor ligands has been reviewed extensively [26, 53, 54, 55, 56, 61], in the text those data will not be further referred to.

In peptic hydrolysates of gluten and gliadin, i.s. two preparations from wheat, either of which has to be regarded as a mixture of proteins, already Zioudrou and coworkers [58] had found materials with opioid activity. Upon further digestion of a gluten hydrolysate, e.g. with trypsin, chymotrypsin or thermolysin [77, 78] or with pancreatic elastase or pancreatin [92] a couple of peptides with opioid activity was released, i.s. gluten exorphin A4, A5, B4 and B5 [77, 92] or gluten exorphin C [78]. Two α -gliadin fragments, α -gliadin (43-47) and (43-49), were synthesized and successfully tested for opioid agonist activity [80,

93]; they were named gliadorphins. Rice protein hydrolysates had not been tested during the very first screening period in the late seventies [58], but since rice is one of the most widely consumed nutrients in the world, this has been done more recently [76]; in fact, from a tryptic rice protein digest a peptide, named oryzatensin, was isolated, which displayed opioid activity [76]; recently, the C-terminal fragment of oryzatensin was synthesized and was shown to possess opioid activity as well [94].

Blood proteins may play a role as nutrients in so far as they represent constituents of meat or sausages and thus it may be worth including them into this review under this aspect. A peptic digest of bovine serum albumin had been shown to contain a material with opioid activity [58], which was in fact later isolated and identified [75]; it was named serorphin. Upon treatment of bovine blood with gastrointestinal enzymes a protein hydrolysate was obtained, from which a couple of opioid materials were isolated, two of which turned out to be fragments of bovine hemoglobin [81]; they were named hemorphins. This finding coined the name for a group of hemoglobin-derived opioid peptides, all of them containing the original hemorphin amino acid sequence, e.g. VV-hemorphin(1-7) and LVV-hemorphin(1-7) [82] or VV-hemorphin(1-5) and LVV-hemorphin(1-5) [83]. Subsequently a big series of hemoglobin fragments was synthesized or isolated from various non-nutrient sources and tested for opioid activities [95, 96, 97].

Finally, a synthetic fragment of spinach D-ribulose-1,5-biphosphate carboxylate/oxygenase (RUBISCO), named rubiscolin, was shown to behave like an opioid agonist [84]; rubisco is an abundant constituent of green leaves of all kinds of plants. The material was, however, not demonstrated to be released under gastrointestinal conditions, as yet.

Characterization of Food Protein Derivatives with Opioid Agonist or Antagonist Activity

In Table 3 some characteristics of food protein derivatives with opioid agonist or antagonist activity (representatives of derivative groups; in some cases the group just consists of one compound as listed in Table 2), i.s. name, amino acid sequence, position within the precursor sequence, agonistic or antagonistic properties and selectivity for μ -, δ - or κ -opioid receptors are given. As group representatives compounds with a somewhat outstanding history, e.g. compounds, which had been demonstrated among the first ones of the group or compounds, about which most data had been collected, were chosen, which necessarily was somewhat biased by arbitrary decisions. In general, the affinities to opioid receptors, i.e. the potencies of these natural exogenous opioid receptor ligands proved to be rather weak, although some synthetic derivatives thereof showed high affinities for opioid receptors and were used as standard substances in many laboratories [26, 42]. As already outlined above, the field of milk protein-derived opioid receptor ligands has been extensively reviewed [26, 53, 54, 55, 56, 61] and, thus, findings raised on these compounds as listed in Table 3 for synoptic reasons will not be discussed in this context.

Table 2. Identification of Food Protein-derived Materials with Opioid Agonist or Antagonist Activity

Foodstuff	Protein	Species	Way of Identification					Sequence modified	Food Protein Derivatives (Compounds, Groups)	[Ref.]
			A-1	A-2	A-3	A-4	B			
Milk	-Casein	bovine	P	+	+	+			_B -Casein exorphins	[58, 62]
	-Casein	bovine					+		_B -Casein exorphins	[58, 62]
	_{S1} -Casein	human	P/CT	+	+	+			Casoxin D	[64]
	_{S1} -Casein	human					+		_{S1(H)} -Casomorphin	[63]
	_{S1} -Casein	human					+	-NH ₂	_{S1(H)} -Casomorphin-amide	[63]
	-Casein	bovine	P etc, GIT	+	+	+			_B -Casomorphins	[60, 65,71,85, 86]
	-Casein	bovine	GIT	+	+	+	+	-NH ₂	Morphiceptin	[69,87, 88]
	-Casein	bovine	GIT	+	+	+			Neocasomorphin	[71]
	-Casein	w. buffalo	GIT	+	+	+			_{WB} -Casomorphins	[66, 89]
	-Casein	human					+		_H -Casomorphins	[67, 68, 70]
	-Casein	human					+	-NH ₂	_H -Casorphin	[61,70]
	-Casein	human					+	-NH ₂	Valmuceptin	[61, 68]
	-Casein	human					+		N.N. -Casein fragment	[61, 70]
	-Casein	bovine					+		Casoxins A, B	[72]
	-Casein	bovine	T	+	+	+			Casoxin C	[72]
	-Casein	bovine					+	-OCH ₃	Casoxin-4, -5	[72, 73]
	-Casein	bovine	T	+	+	+		-OCH ₃	Casoxin-6	[72, 73, 90]
	-Lactalbumin	bovine					+	-NH ₂	_B -Lactorphin	[70]
	-Lactalbumin	bovine	P,T,CT	+	+	+			Des-NH ₂ - _B -Lactorphin	[91]
	-Lactalbumin	human					+	-NH ₂	_H -Lactorphin	[70]
	-Lactalbumin	human	P,T,CT	+	+	+			Des-NH ₂ - _H -Lactorphin	[91]
	-Lactoglobulin	bovine					+	-NH ₂	_B -Lactorphin	[70]
	-Lactoglobulin	bovine	P,T,CT	+	+	+			Des-NH ₂ - _B -Lactorphin	[91]
	Lactoferrin	human	P	+	+	+		-OCH ₃	Lactoferoxins A, B, C	[73]
Wheat	Gluten		P,T,CT, GIT	+	+	+			Gluten exorphins A, B, C	[58, 77,78, 92]
	-Gliadin						+		Gliadorphins	[58, 80, 93]
Rice	Albumin		T	+	+	+			Oryzatensins	[76, 94]
Blood (const. of meat)	Albumin	bovine	P	+	+	+			Serorphin	[58, 75]
	Hemoglobin	bovine	P, GIT,	+	+	+			Hemorphins	[81, 82, 83, 95, 96, 97]
Spinach	RUBISCO (D-Ribulose-1, 5-biphosphate carboxylase / oxygenase)		P, LAP		+		+		Rubicolins	[84]

A-1: Demonstration of a material with opioid agonist or antagonist activity in food protein hydrolysates obtained upon peptic (P), tryptic (T) or chymotryptic (CT) digestion or upon digestion with gastrointestinal enzymes (GIT), sometimes not exactly defined in case of a commercially available digest used for screening studies; A-2: Isolation of the material from protein hydrolysate, A-3: Identification of the amino acid sequence of the isolated material; A-4: Demonstration of identity of the amino acid sequence with a fragment of the respective food protein and demonstration of opioid agonist or antagonist activity of the synthetic food protein fragment. B: Screening the amino acid sequences of food proteins and - skipping hydrolysis, isolation and identification procedures - direct synthesis of fragments with potential opioid receptor ligand properties; subsequent demonstration of opioid agonist or antagonist activity displayed by the synthetic food protein fragment.

Table 3. Characterization of Food Protein Derivatives with Opioid Agonist or Antagonist Activity

Food Protein Derivatives (Compounds / Group Representatives)	Protein Fragment	Sequence	Agonist / Antagonist	Opioid Receptor Selectivity (μ , κ , δ)	[Ref.]
β -Casein exorphin (1-7)	β -Casein (90-96)	RYLGYLE	Agonist	μ / \ll	[62,98]
β -Casein exorphin (2-7)	β -Casein (91-96)	YLGYLE	Agonist	μ /	[62]
Casoxin D	$\text{s}_{1(\text{H})}$ -Casein (158-164)	YVPFPPF	Antagonist	μ /	[64]
$\text{s}_{1(\text{H})}$ -Casomorphin (1-5)	$\text{s}_{1(\text{H})}$ -Casein (158-162)	YVPFP	Agonist/Antag.	μ / $\ll\ll$	[99]
$\text{s}_{1(\text{H})}$ -Casomorphin (1-5) -NH ₂	$\text{s}_{1(\text{H})}$ -Casein (158-162)	YVPFP-NH ₂	Agonist/Antag.	μ \ll /	[99]
β - Casomorphin (1-7)	β - Casein A2 (60-66)	YFPFGPI	Agonist	μ > >>	[60, 65, 85, 100, 101]
Morphiceptin	β - Casein A2 (60-63)	YFPF - NH ₂	Agonist	μ >> >>	[69, 87, 88]
Neocasomorphin	β - Casein (114-119)	YPVEPF	Agonist	(μ)	[71]
w_β - Casomorphin (1-8)	w_β - Casein (60-67)	YFPFGPIP	Agonist	μ >> ,	[66,89,100]
h - Casomorphin (1-5)	h - Casein (51-55)	YPFVE	Agonist	(μ / >>)	[67,68,100]
h - Casorphin	h - Casein (41-44)	YPSF - NH ₂	Agonist	(μ)	[61,70]
Valmuceptin	h - Casein (51-54)	YPFV - NH ₂	Agonist	μ >	[61,68]
N.N. h - Casein fragment	h - Casein (59-63)	YGFLP	Agonist	(μ)	[61,70]
Casoxin A	β - Casein (35-41)	YPSYGLN	Antagonist	(μ)	[72]
Casoxin C	β - Casein (25-34)	YIPIQVLSR	Antagonist	μ	[72]
Casoxin-5	β - Casein (34-38)	RYPYSY-OCH ₃	Antagonist	μ >	[73]
Casoxin-6	β - Casein (33-38)	SRYPYSY-OCH ₃	Antagonist	μ >	[72,73,90]
β -Lactorphin	β -Lactalbumin (50-53)	YGLF - NH ₂	Agonist	(μ)	[61, 70]
Des-NH ₂ - β -Lactorphin	β -Lactalbumin (50-53)	YGLF	Agonist	(μ)	[91]
h -Lactorphin	h -Lactalbumin (50-53)	YGLF - NH ₂	Agonist	(μ)	[61,70]
Des-NH ₂ - h -Lactorphin	h -Lactalbumin (50-53)	YGLF	Agonist	(μ)	[91]
β -Lactorphin	β -Lactoglobulin(102-105)	YLLF - NH ₂	Agonist	(μ)	[61,70]
Des-NH ₂ - β -Lactorphin	β -Lactoglobulin(102-105)	YLLF	(Agonist?)	(μ)	[91]
Lactoferroxin A	(H) Lactoferrin (318-323)	YLGSGY -OCH ₃	Antagonist	μ	[73]
Lactoferroxin B	(H) Lactoferrin (536-540)	RYYGY -OCH ₃	Antagonist	μ	[73]
Lactoferroxin C	(H) Lactoferrin (673-679)	KYLGPPQY -OCH ₃	(Antagonist?)	(μ ?)	[73]
Gluten exorphin A5	High molecular weight glutenin (15 repeats)	GYYP	Agonist	μ $\ll\ll$	[77,92,102]
Gluten exorphin B5		YGGWL	Agonist	μ \ll	[77,92]
Gluten exorphin C5	Glutenin HMW chain 1By9 precursor (624-628)	YPISL	Agonist	μ <	[78,103]
Gliadorpin (1-7)	-Gliadin (43-49)	YPPQPPF	Agonist		[80,93]
Oryzatensin (1-9)	Allergen RA5B precursor (47-55)	GYPMYPLPR	Agonist	μ	[76,94,104]
Oryzatensin (5-9)	Allergen RA5B precursor (51-55)	YPLPR	Agonist	(μ ?)	[76,94,104]
Serorphin	Bovine serum albumin (399-404)	YGFQNA	Agonist	μ <	[75]
Hemorphin (1-4)	Bovine hemoglobin / α -chain (34-37)	YPWT	Agonist	μ / >	[81,107]

(Table. 3) contd....

Food Protein Derivatives (Compounds / Group Representatives)	Protein Fragment	Sequence	Agonist / Antagonist	Opioid Receptor Selectivity (μ , δ , κ)	[Ref.]
Valorphin (1-7) (= VV-Hemorphin (1-5))	Bovine hemoglobin / α -chain (32-38)	VVYPWTQ	Agonist	$\mu >$	[83, 105]
VV-Hemorphin (1-7)	Bovine hemoglobin / α -chain (32-40)	VVYPWTQRF	Agonist	$\mu / ?$	[82,106]
LVV-Hemorphin (1-7)	Bovine hemoglobin / α -chain (31-40)	LVVYPWTQRF	Agonist	$\mu / ?$	[82,106]
Rubiscolin (1-6)	RUBISCO (D-Ribulose-1, 5- biphosphate carboxylase / oxygenase) (103-108)	YPLDLF	Agonist	$\mu <<<$	[84]

As representatives of the group of wheat protein derivatives, gluten exorphins A5, B5 and C5 were chosen as gluten derivatives and gliadorphin (1-7) as a gliadin derivative (Table 3). Whereas opioid receptor selectivities had not been determined for gliadorphin (1-7), tests on guinea pig ileum and mouse vas deferens preparations as well as receptor binding assays conducted with the gluten exorphins showed that they were all δ -selective opioid receptor ligands; the most δ -selective peptide was gluten exorphin A5, whereas the most potent one was gluten exorphin B5 [77, 78, 92]. Gliadorphin (1-7) was shown to bind specifically to opioid receptors on peripheral blood mononuclear cells and to inhibit leukocyte migration, an effect, which proved to be naloxone antagonizable [80, 93]. As-rice albumin-derived opioid receptor ligands the oryzatensins (1-9) and (5-9) have been listed in Table 3. Although either peptide elicited naloxone-antagonizable analgesia upon intracerebroventricular administration in mice [94], opioid receptor selectivities and potencies remain to be clarified [76, 94]. Since Oryzatensin (1-9) has no free N-terminal Tyr residue, the enzymatic liberation of oryzatensin (5-9) presenting this structural feature might be a prerequisite for its interaction with opioid receptors.

Blood protein-derived compounds, i.s. bovine serum albumin and bovine hemoglobin fragments, also have been shown to display opioid agonist properties. The albumin derivative, serorphin, turned out to be a δ -selective opioid receptor ligand of weak potency [75]. The opioid receptor selectivities of the hemoglobin fragments, hemorphins, are not quite clear as yet, although hemorphins appear to display more affinity for μ - than for δ -opioid receptors [81, 82, 83, 105, 106, 107]; in addition, an opioid antagonistic effect has been reported for hemorphin (1-4) [108]. As far as tested, the potencies of the hemorphins appear to be rather weak. Besides opioid effects, these peptides as well as further fragments of hemoglobin display additional activities such as ACE inhibition and many hemoglobin fragments synthesized or isolated from human or animal tissues have been tested for those activities [95, 96, 97]. Efforts to isolate hemorphins from a variety of human [109] or animal tissues [110] led to more sophisticated techniques of isolation, e.g. "on line registration" of the peptides released from hemoglobin during hydrolysis [95, 111].

Finally, fragments of spinach D-ribulose-1,5-biphosphate carboxylase / oxygenase (RUBISCO), rubiscolin (1-5)

and (1-6), proved to be highly selective δ -opioid receptor ligands of moderate opioid agonist potency [84].

FOOD PROTEIN-DERIVED OPIOID RECEPTOR LIGANDS: FOOD HORMONES?

Bioactive compounds derived from food have been termed "food hormones" [20] or, in a short-cut version, "formones" [11]. This term implies that these compounds elicit effects like drugs, but that they are administered by nature in the frame of a function supplementary to, and, thus, equally essential like those of the food-recipient's organism. Compounds, which such a role is ascribed to, should fulfil certain criteria, before they can be accepted as food-derived bioactives or food hormones.

Criteria

Although all opioid receptor ligands derived from food proteins are in fact candidates for playing a role as food hormones, for none of them conclusive evidence has been presented as yet for such a role - however, there are quite different levels of probability: The more the experimental design of a study about the function of a food constituent adapts the food ingestion process, the higher the level of evidence for the compound's role as a food hormone.

Thus, whereas about some of the opioid receptor ligands in fact nothing is known except that they are opioid receptor ligands, for most of them at least evidence has been presented that they can be released from their precursor proteins by gastrointestinal enzymes under in vitro conditions (see Table 2). Further, for many food protein-derived opioid receptor ligands a variety of effects in vitro and in vivo, upon oral, intragastric, abomasal, subcutaneous, intraperitoneal or intracerebroventricular administration has been reported, whereby most information is available on the opioid peptides from milk [26, 42], in particular on the δ -casomorphin group [112]. However, findings raised under the conditions of those studies do not meet the relevant criteria by far: For a role as a food hormone, only those effects are relevant, which have been - under our egoistic prevalence of the human species - observed in humans and, further, after oral administration. The closest adaptation to mother nature's design, the "ideal" experiment, would be an experiment, wherein the foodstuff, e.g. milk, is orally

administered in healthy volunteers and the effects and the concentrations of the opioid peptide, e.g. β -casomorphin, in the gastrointestinal tract and in the other compartments of the organism relevant for the peptide's effects are measured. The prevention of the opioid peptide's effects by specific antagonists and specific antibodies should be demonstrated as well as the coincidence of time/ effect and time/concentration courses. Confirmatory data on the mechanism of the peptide's effects would be welcome.

Every kind of experimental design different from this would only provide data on pharmacological effects of the peptide under the specific conditions of that experimental design, i.e. under conditions different from those of the "ideal" experiment (even if evidence is presented that the effects are in fact caused by the peptide). Such different conditions include oral administration of a mixture of foodproteins (e.g., casein), a precursor protein (e.g., the β -casomorphin precursor β -casein), hydrolysates of these proteins or protein mixtures or - even - the peptide itself (e.g., β -casomorphin(1-7)). All these experimental designs may create conditions different from that of mother nature's and, thus, the effects measured under these non-natural conditions might be different from those measured under natural conditions in terms of time shift, enhancement or repression of the peptide's effects. The most dramatic deviation from the above-outlined "ideal" design would be to employ it in the frame of animal studies. Thus, all kinds of data collected under conditions not identical with the natural food ingestion process as well as effects, which are not proven to be directly peptide-related should not just be interpreted in terms of "food hormone" effects relevant for the human species.

Data

Data collected on food protein-derived opioid receptor ligands do not meet the above-set criteria by far. The above-outlined "ideal" experiment has never been performed. Therefore just data about effects of food protein-derived opioid receptor ligands observed upon oral or intragastric administration providing lower levels of evidence for a role as food hormones will be given here. The ranking starts with effects of the peptide itself after oral administration in humans and is continued with effects in animals, followed by the same scale in case of precursor, protein mixture or food administration. Data screening shows that effects after oral or intragastric administration of food protein-derived opioid receptor ligands, their precursor proteins or mixtures or hydrolysates thereof have only been demonstrated for β -casomorphins, cow's milk, casein preparations, gluten exorphins, gluten or gluten hydrolysate.

β -casomorphins

Most information is available about the β -casomorphin group: The role of food hormones may be discussed for β -casomorphins in adults as well as in sucklings. Findings in fact have been raised in either group. The field has been reviewed [26, 113, 114, 115,], but there are some novel data.

In adult humans, upon oral administration of β -casomorphin(1-4)amide, the gastrointestinal transit time was found to be delayed [116]; compatible with this finding was an antidiarrheic effect in calves upon oral administration of β -casomorphin(1-4)-amide [117]. However, naloxone blockade was not tested in these studies. In dogs, a naloxone-antagonizable stimulation of somatostatin [118] and insulin release [119] was observed upon intragastric bovine β -casomorphin instillation; in cows, however, insulin responses to glucose stimulation were lowered upon intra-abomasal bovine β -casomorphin infusion [120]. In rats, bovine β -casomorphin(1-7) stimulated the intake of a high fat diet upon intragastric administration of the peptide [121]; antagonization of the effect by opioid antagonists was not tested.

Neither in human neonates nor in new-born animals, studies for assessment of effects after oral or intragastric β -casomorphin administration have been conducted as yet. Compatible with the findings described for adults (see above) would be a prolongation of gastrointestinal transit time as demonstrated in rat pups for a synthetic β -casomorphin analogue stable against enzymatic degradation; in this investigation, β -casomorphin(1-4)amide apparently was degraded and had no effect [122].

Casein, Casein Hydrolysate and Milk

Opioid effects observed upon the oral or gastrointestinal administration of caseins, casein hydrolysates or milk, in principle, can be interpreted in terms of release of each of the opioid peptides or peptide groups derived from milk proteins as listed in Table 2 or Table 3. Although there are quite a few studies showing the presence of β -casomorphins or β -casomorphin immunoreactive materials in the gut or elsewhere in the organism after oral administration of casein preparations or milk, this could be due to the fact that other milk protein-derived opioid peptides just have not been searched for in those studies.

No data are available on opioid effects of casein, casein hydrolysate or milk in adult humans, which could be reduced to the presence of milk protein-derived opioid receptor ligands.

In dogs, stimulation of insulin release was observed upon intragastric administration of a casein hydrolysate [119]. Upon intragastric administration of casein in dogs gastrointestinal motility was reduced [123] and abomasal casein infusion in steers had the same effect [124]. In rats satiety was demonstrated to be influenced by casein or casein hydrolysate [125]. In dogs, milk induced the release of somatostatin [118] and in ewes, LH secretion during the early suckling period appeared to be inhibited by milk constituents [126]. All effects observed in these animal studies were antagonizable by opioid antagonists.

In neonates, reduction of crying due to blood collection via heel lance has been reduced to the oral administration of milk [127]; naloxone has not been tested. In preterm infants, the reduction of gastrointestinal transit time upon oral administration of a protein hydrolysate formula was considered to be caused by degradation of milk protein-

derived opioid receptor ligands [128], which appear to slow down the gastrointestinal transit, when they are released from intact precursor proteins in the gastrointestinal tract (see above); however, naloxone antagonism of the effect has not been tested in this study [128].

Prolongation of gastric emptying rate and gastrointestinal transit time was observed in rat pups after intragastric administration of casein [122]; this effect could be blocked by naloxone. Oral administration of milk induced naloxone-antagonizable antinociception in new-born and in 10-day-old rats [129, 130]. However, naloxone-antagonizable antinociception was also observed upon oral administration of corn oil and polydose in 10-day-old rats [131]. Thus, opioid effects measured after oral administration of opioid peptides or their precursor proteins must not necessarily be elicited by the opioid peptide itself interacting at any site of the recipient's organism with opioid receptors; even in case of oral administration of the opioid peptide itself a non-opioid effect could be elicited in the gastrointestinal tract by the peptide, which could trigger a series of processes in the organism, one of which could be a naloxone-antagonizable interaction of an endogenous opioid with opioid receptors. Only an "ideal" experiment, wherein a coincidence of time/concentration and time/effect curves, etc., etc. as outlined above has been demonstrated could provide evidence for an opioid effect elicited by the opioid peptide itself at any site in the consumer's organism..

Nevertheless, there are a few interesting studies showing the presence of opioid peptides or opioid peptide immunoreactive materials in the gastrointestinal tract or in the cardiovascular compartment after oral administration of precursor proteins or precursor hydrolysates. In adult humans, β -casomorphins have been shown to be released into the gastrointestinal lumen after ingestion of cow's milk [132] and in minipigs a β -casomorphin was demonstrated in the gastrointestinal lumen after ingestion of bovine casein [86]. Whereas, thus, β -casomorphins showed up in the gastrointestinal tract of adults after oral administration of their precursor, they apparently were not able to pass over to the cardiovascular compartment. Neither after the ingestion of milk or milk products in adult humans [133], nor after feeding adult dogs with bovine β -casein [134] β -casomorphins were found in the plasma of humans or dogs, respectively. In new-born mammals, however, β -casomorphin precursor fragments were demonstrated not only in the gastrointestinal tract, but also in the cardiovascular compartment after oral administration of milk or casein. These materials were demonstrated in the gut of prurinant calves after skim milk ingestion [135], in the plasma of new-born dogs after canine milk or bovine β -casein ingestion [134] and in the plasma of new-born calves after cow's milk intake [136]. There is even indication for further permeation of β -casomorphins from the cardiovascular compartment into the central nervous system in human neonates [137].

Gluten Exorphins

In rats, the oral administration of the gluten exorphins A5 or B5 increased postprandial plasma insulin levels; these effects were reversed by naloxone [138].

Gluten and Gluten Hydrolysate

In healthy volunteers, gluten hydrolysate was shown to cause a prolongation of gastrointestinal transit time; this effect was blocked by naloxone [139]. Gluten hydrolysate also produced a naloxone-reversible increase in plasma somatostatin activity, which may have been responsible for the transit delay [139]. In dogs, intragastric instillation of gluten or gluten hydrolysate induced a rise in postprandial plasma insulin levels; these effects could also be blocked by naloxone [140].

Conclusions

Obviously, the data presented do not meet the above-set criteria by far. Nevertheless, although hard evidence is lacking, there appears to exist sort of a hard core in the middle of a field of relatively soft information, which might justify the use of terms like "food hormones" when talking about food protein-derived opioid receptor ligands. There is a promising coincidence of some findings raised by different groups in different species based on different experimental designs. In particular, a reduction of intestinal motility upon oral or intragastric administration of β -casomorphins or casein has been observed in adult humans [116], in calves [117], in dogs [123], in steers [124] and in rat pups [122]; it may have been caused by β -casomorphins demonstrated to be present in the gastrointestinal lumen of humans [132] or animals [86] after milk or casein ingestion. A naloxone-antagonizable prolongation of gastrointestinal transit time has also been observed upon oral administration of gluten hydrolysate in humans [139]. These effects are compatible with a classic opioid effect, obstipation, and they are also compatible with direct effects of β -casomorphins on the gastrointestinal wall as demonstrated by *in vitro* studies [141, 142, 143, 144, 145, 146, 147], whereby gastrointestinal functions may be influenced in opposite directions by opioid peptides as dependent on the animal species [148]. Thus, a gastrointestinal role of food protein-derived opioid receptor ligands is likely.

Further, compatible with our knowledge about the properties of the gastrointestinal wall in mammals is the information that neither in adult humans nor in adult dogs β -casomorphin immunoreactive materials were found in the plasma after ingestion of bovine β -casein or milk [133, 134], whereas a β -casomorphin precursor apparently passed over from the gastrointestinal lumen into the cardiovascular compartment in new-born dogs as well as in new-born calves after oral milk intake [134, 136]. Moreover, whereas central effects after casein or milk ingestion have not been observed in adults, they have been reported in new-born mammals; it should be emphasized, however, that central opioid-like effects in new-born mammals as seen after oral milk administration require further confirmation by studies showing that the effects in fact have been elicited by a food protein-derived opioid receptor ligand present in the central nervous system. Also for opioid effects on the endocrine system seen in adult animals after oral or intragastric administration of food-derived opioid peptides it is not quite clear, whether they have been elicited by primary interaction of a food protein-derived opioid peptide with opioid receptors in the gastrointestinal tract.

Hypotheses

Food-derived Opioid Peptides: Low Affinities for Opioid Receptors - High Gastrointestinal Concentrations

Endogenous opioid receptor ligands display high affinities for opioid receptors with K_D values in the nanomolar range [32]; therefore they are able to elicit opioid effects despite of relatively low peptide concentrations determined in various compartments of the organism. In contrast, food-derived opioid peptides have much lower affinities with K_D values exceeding those of their endogenous counterparts by two orders of magnitude or even more [100]. However, in case, the whole amount of food protein as ingested is processed to release opioid peptide in a 1 : 1 proportion, really high concentrations of opioid receptor ligands are to show up in the gastrointestinal lumen. Under this condition low peptide affinity would be compensated by high peptide concentration and, in fact, effects on the gastrointestinal tract have been observed as outlined above.

Food-derived Opioid Peptides: Concerted Actions

The effects of endogenous opioid peptides are not restricted to the opioidergic systems, but include also effects on non-opioid systems, e.g. in the frame of interactions with the immune system [44]. This holds also for activities of exogenous opioid peptides, e.g. the effects of δ -casomorphins on the cardiovascular system [112]. Apparently, different fragments of the peptide are able to interact with different receptors, opioid and non-opioid receptors; such different receptor populations serving as targets for opioid peptides can even be present on the same cell [44]. Besides this possibility of a dual interaction displayed by one single peptide, a further dualism displayed by the group of food protein-derived opioid receptor ligands would be the opposite actions of opioid receptor ligands with opioid agonist or with opioid antagonist activity (see Table 3); the presence of opioid antagonists among the food-derived opioid receptor ligands, most of which are opioid agonists, might provide sort of a buffer system against an overshoot of opioidergic influence on the gut under certain conditions [10]. Thus, from one food protein, not only different fragments with quite different [7-14] or even opposite (Table 3) bioactivities can be released; different effects on different systems of the organism can be even elicited by one single peptide. It is tempting to speculate that the effects of food protein-derived opioid receptor ligands are essential parts of the "concerted effects" of a variety of food protein-derived bioactive peptides on the food consumer's organism.

Food-derived Opioid Peptides: Phylogenetic Symbiosis

The detection of food protein-derived opioid receptor ligands gave rise to a number of interesting speculations trying to answer the question, whether exorphin-containing food has been integrated into our human / agricultural co-development due to the rewarding properties of those opioid receptor ligands [149]. It was suggested that daily opioid peptide intake facilitated the behavioral changes and subsequent growth of civilisation by increasing people's tolerance of living in crowded sedentary conditions and of

playing a subservient role in a vast hierarchical social structure. However, data and conclusions presented in this review do not support those suggestions as yet.

FOOD PROTEIN-DERIVED OPIOID RECEPTOR LIGANDS: NUTRACEUTICALS?

Over the last decade considerable interest in the potential economic significance of food-derived bioactive peptides has developed [8, 23, 150] and therefore it might be worth taking a look at food protein-derived opioid receptor ligands under this point of view. Although information about biological effects of food-derived opioid peptides under really relevant conditions, which only count in this context, i.e. effects observed upon oral administration of the compounds in humans, is more than meagre as outlined in this review, it should also be mentioned that an overwhelming bulk of data exists about all kinds of effects observed upon non-oral administration of those peptides [26, 56, 112]. A couple of these effects might also be observed after oral administration of the opioid peptides in humans when searched for; other ones might be never seen after oral administration due to degradation of the peptide on its way from the gastrointestinal tract to its site of action in the human organism, e.g. the central nervous system.

The latter point, of course, deserves consideration under the condition that the peptides are intended to receive nutraceutical relevance in adult food consumers as well as in neonates or sucklings. In sucklings up to a certain age, not only the opioid peptides, but even their precursors can be assumed to penetrate the gastrointestinal wall; protected against enzymatic attack as long as integrated in the precursor's protein molecule, the peptide might be released from the precursor molecule at any site in the suckling's organism to elicit opioid effects there [26]. This, however, means that oral administration of a food-derived opioid peptide to a neonate or suckling would lead to the peptide's distribution over his whole organism - which indeed is still in a very vulnerable state; in contrast, apparently (see above!) oral administration of the peptide to adult humans would only result in its presence in the gastrointestinal tract. Thus, adults and sucklings up to a certain age do have organisms of quite different complexity and, as economic targets, require quite different degrees of responsibility to be taken over by the respective food or food additive producer.

There are apparently several possibilities to introduce food-derived bioactive peptides, e.g. opioid receptor ligands, into the market, e.g. as dietary supplements or food additives, as constituents of "functional foods" or as constituents of "medical foods", which then allows or requires the respective claims such as "nutrient function claim", "reduced disease risk claim" or "medical benefit claim" [8, 23, 151]. Obviously, the choice of name or claim is not by far as important as the correspondence of the respective claim or name with its promise. In other words, the benefit for the consumer expected from ingestion of a certain bioactive peptide should have been proven before it's claimed. This point has been substantially considered

[23, 152]. At the medical, e.g. the pharmacotherapeutic level, the term "evidence-based medicine" has been introduced to emphasize the need of presenting evidence for the validity of a certain medical intervention, before it's used [153, 154]. Since the term "evidence-based nutrition" is already occupied by dietary support of therapeutic interventions such as the therapy of diabetes, it is tempting to coin the term "evidence-based dietary supplementation", which means nothing else than presenting evidence for the benefit of a food additive claimed by the producer and, consequently, expected by the consumer upon ingestion of nutraceuticals, e.g. opioid receptor ligands derived from food proteins.

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