

# Rotational behaviour produced by intranigral injections of bovine and human $\beta$ -casomorphins in rats

Mario Herrera-Marschitz<sup>1</sup>, Lars Terenius<sup>2</sup>, Lars Grehn<sup>3</sup>, and Urban Ungerstedt<sup>1</sup>

<sup>1</sup> Department of Pharmacology, Karolinska Institutet, Box 60 400, S-104 01 Stockholm, Sweden

<sup>2</sup> Department of Pharmacology, University of Uppsala, Box 591, S-75124 Uppsala, Sweden

<sup>3</sup> Department of Biochemistry, University of Uppsala, Box 591, S-75124 Uppsala, Sweden

**Abstract.** The biological activity of  $\beta$ -casein derived  $\beta$ -casomorphin peptides was evaluated by injecting bovine  $\beta$ -casomorphin-5 (Tyr-Pro-Phe-Pro-Gly), the homologous sequence in human  $\beta$ -casein (Tyr-Pro-Phe-Val-Glu) and the corresponding N-terminal tetrapeptides into the left substantia nigra of rats. Their ability to produce rotational behaviour was compared to that produced by three reference compounds, morphine, D-al<sup>2</sup>D-leu<sup>5</sup>-enkephalin and U50,488H, ligands for  $\mu$ ,  $\delta$  and  $\kappa$  types of opioid receptors, respectively. The relative potencies of  $\beta$ -casomorphins and morphine were compared to those tested in two in vitro assays for opioid activity: (1) inhibition of the electrically induced contraction of the isolated myenteric plexus-longitudinal muscle of the guinea-pig ileum and (2) displacement of <sup>3</sup>H-dihydromorphine binding to brain membranes. The same ranking order of potency was found in all three assays, the peptides from human  $\beta$ -casein being about 10-fold less potent than those from bovine  $\beta$ -casein. The effects of both morphine and bovine  $\beta$ -casomorphin-5 in producing rotational behaviour were antagonized by naloxone; however, approximately 10-fold more naloxone was required to antagonize the  $\beta$ -casomorphin-5 effect than that of morphine. The present data are discussed in the light of the recent observation that high concentrations of  $\beta$ -casomorphin-like peptides are found in the cerebrospinal fluid and plasma of women with postpartum psychosis.

**Key words:** Postpartum psychosis – Basal ganglia –  $\beta$ -Casein – Opioid receptors – Rotational behaviour – Rat brain

Henschen et al. (1979) first isolated a peptide with opioid activity from an enzymatic digest of bovine  $\beta$ -casein. The peptide, Tyr-Pro-Phe-Pro-Gly-Pro-Ile, was named  $\beta$ -casomorphin. Shorter fragments, such as the N-terminal pentapeptide ( $\beta$ -casomorphin-5), were also found to be active, in fact more active than  $\beta$ -casomorphin itself. The primary structure of human  $\beta$ -casein was not determined until recently (Greenberg et al. 1984). The homologous sequence in the human protein corresponding to  $\beta$ -casomorphin is Tyr-Pro-Phe-Val-Glu-Pro-Ile. The pharmacologic profile of the  $\beta$ -casomorphin peptide family is similar to that of morphine and thought to be mediated mainly through  $\mu$ -receptors (Brantl et al. 1981). Human (h)  $\beta$ -casomorphin-5 and

-4, respectively, have been synthesized and found to have only about 1/10 the potency of the bovine homologues in the electrically stimulated guinea-pig ileum myenteric plexus-longitudinal muscle preparation, which is a  $\mu$ -receptor mediated response (Brantl 1984). High levels of  $\beta$ -casomorphin-like opioid peptides have been found in the cerebrospinal fluid and plasma of women with so-called postpartum psychosis (Lindström et al. 1984). This finding led to the hypothesis that the opioid activity might derive by abnormal proteolysis of  $\beta$ -casein in mother's milk of these women.  $\beta$ -casomorphin fragments may cross the blood-brain barrier and bind to opioid receptors in the brain.

The substantia nigra, a strategic nucleus gating the inputs and outputs of the basal ganglia, contains high concentrations of opioid receptors (Herrera-Marschitz 1986). It is therefore possible that  $\beta$ -casomorphins penetrating into the brain may exert stimulation of this region, resulting in behavioural imbalance. This prompted the present study, where the behavioural effects produced by unilateral injections of  $\beta$ -casomorphins into the substantia nigra of rats were evaluated. The potencies of the  $\beta$ -casomorphins producing rotational behaviour were compared to those evaluated in two in vitro opioid assays: (1) inhibition of the electrically induced contraction of the isolated myenteric plexus-longitudinal muscle of the guinea-pig ileum (Kosterlitz and Watt 1968) and (2) displacement of <sup>3</sup>H-dihydromorphine binding to brain membranes (Terenius 1974). The effects of  $\beta$ -casomorphins on rotational behaviour were also compared to those produced by three different types of opioid agonists, i.e., morphine (a  $\mu$ -receptor agonist), D-al<sup>2</sup>D-leu<sup>5</sup>-enkephalin (a  $\delta$ -receptor agonist) and U50,488H (a  $\kappa$ -receptor agonist) (see Herrera-Marschitz 1986).

A preliminary account of this work has been presented elsewhere (Herrera-Marschitz et al. 1985).

## Material and methods

### Rotational behaviour

**Animals.** Sprague-Dawley (Alab, Stockholm, Sweden) male rats with access to food and water ad lib. were used in the rotational behaviour experiments. They were maintained in a temperature controlled environment on a 12 h light/dark cycle when not in experimental sessions.

**Intracerebral injection experiments.** An acute intracerebral injection procedure (see Herrera-Marschitz 1986) was used

to administer the peptides into the substantia nigra. In short, rats were anesthetized with a mixture of air and halothane and placed in a Kopf stereotaxic frame. An injection cannula, conically shaped with a tip diameter less than 0.15 mm, was implanted into the left substantia nigra, pars reticulata (SNR) (coordinates: A 1.8, L -2.0 and V -2.6, according to the atlas of König and Klippel 1963). The drugs or saline were then injected in a total volume of 0.2  $\mu$ l at a rate of 0.2  $\mu$ l/min. The anaesthesia was turned off as soon as the injection had been performed. The injection cannula was maintained in position for an additional minute and carefully retracted. The rats were then placed in a rotometer in order to record their behaviour. Each rat was used only once for intracerebral injections.

**Evaluation of rotational behaviour.** Rotational behaviour was recorded in a modified version of the original rotometer (Ungerstedt and Arbuthnott 1970) that allowed the continuous recording of left or right turns by a detector utilizing infrared photocell beams (see Herrera-Marschitz 1986). The rotational behaviour of each animal was expressed as the number of 360° turns/min for the entire duration of the behavioural response (total rotation) and as turns/10 min (maximum intensity). Since rotometers are sensitive to 180° right or left movements, counts are recorded whether the rat complete 360° turns or moves 180° to the left or the right. Therefore, direct observation was used to differentiate between asymmetric and symmetric motor behaviour, since the latter also resulted in an increase of the total counts. The term *rotation* was ascribed to any asymmetric motor behaviour in counts/min, to the left or right.

**Histology.** On completion of the experiments, histological analysis was performed to confirm the location of the intracerebral injections as reported elsewhere (Herrera-Marschitz 1986).

#### *Bioassay on guinea-pig ileum*

Pigmented, male guinea-pigs of 300–500 g (Sahlins, Stockholm, Sweden) were used. The myenteric plexus-longitudinal muscle was prepared (Kosterlitz and Watt 1968) and mounted in an organ bath of 3 ml. Bath fluid was a Krebs solution (120 mM NaCl, 4.75 mM KCl, 2.54 mM CaCl<sub>2</sub>, 1.20 mM MgSO<sub>4</sub>, 1.19 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 11 mM D-glucose) kept at 35° C and continuously aerated with 95% oxygen, 5% carbon dioxide giving a pH of 7.4. The tissue was given supra-maximal rectangular pulses of 60 V and 1 ms duration at 0.1 Hz. Contractions were measured isometrically. After stabilisation of the twitch, a normorphine dose-response curve was run. Dose response curves for each test compound included at least five different concentrations. It was assumed that all dose-response curves were parallel to each other (full dose-response curves were not obtained with the most inactive peptides due to solubility limitations) and a dose giving 50% suppression of the twitch was taken as the IC<sub>50</sub>.

#### *Receptor assay using rat brain membranes*

The membranes used were osmotically shocked crude mitochondrial fractions prepared from whole rat brain excluding the cerebellum (Terenius 1974). <sup>3</sup>H-labelled dihydromorphine (DHM) (specific activity 79 Ci/mmol) (NEN,

Dreieich, W. Germany) was the tracer. Potency to displace <sup>3</sup>H-DHM (0.2 nM) was measured for each compound. In short, brain membrane suspension (equivalent to 0.2 mg protein) was incubated, with the tracer and increasing amounts of non-labelled test compound, in 0.425 ml total volume of a physiological phosphate buffer at pH 7.4. Incubation was at 25° C for 25 min and was terminated by centrifugation in a Beckman Microfuge in the cold. The supernatant was removed in a sling. The pellet was saved, solubilized and the radioactivity counted. Every assay included a DHM standard curve and samples with 1  $\mu$ M unlabelled DHM defining nonsaturable binding. Every experiment was run in triplicates (for details see Terenius 1974).

#### *Drugs*

Naloxone hydrochloride (Endo Laboratories, Garden City, New York, USA) was dissolved in saline and injected IP in a volume of 5 ml/kg body weight 5 min before intracerebral injections. Saline, bovine  $\beta$ -casomorphine-4,  $\beta$ -casomorphin-5 and  $\beta$ -casomorphin-7 (CRB, Cambridge, England), human (h)  $\beta$ -casomorphin-4, h $\beta$ -casomorphin-5 (synthesized and purified by HPLC in our laboratory), morphine sulphate (ACO, Solna, Sweden), D-al<sup>2</sup>D-leu<sup>5</sup> enkephalin (Bachem, California, USA) and U50,488H (The Upjohn Co., Kalamazoo, Michigan, USA) were dissolved in physiological saline and injected into the SNR in a total volume of 0.2  $\mu$ l (doses were calculated as the free base).

#### *Statistics*

Mean values and standard errors of the means (SEM) were calculated. Effects of doses were analysed with F-ANOVA, in such a manner that a significant value reflects changes produced by the doses of each respective drug. A level of  $P < 0.05$  for one-tail test was considered as critical for statistical significance.

## **Results**

#### *Rotational behaviour*

Intranigral injection of  $\beta$ -casomorphin-4, -5, -7, h $\beta$ -casomorphin-4 and -5 produced contralateral rotation that increased, in a dose-dependent manner, in both intensity and duration (Table 1). The rats started to rotate immediately they recovered from the halothane anesthesia, with a maximal peak of rotation (maximum intensity) occurring during the first 10-min period; the intensity of the rotation then diminished rapidly (see Fig. 1). The rotational behaviour was characterized by a combination of locomotor and stereotypic behaviour towards the side contralateral to the injection site. The rotation was accompanied by rapid up and down head movements, wet-dog shakes, chewing, sniffing and grooming.  $\beta$ -casomorphin-5 was the most potent fragment.  $\beta$ -casomorphin-4 and -5 produced contralateral rotation at doses 10-fold lower than those of h $\beta$ -casomorphin-4 and -5 (Table 1). Morphine, D-al<sup>2</sup>D-leu<sup>5</sup> enkephalin and U50,488H produced strong, dose-dependent, contralateral rotation; morphine being the most potent of all tested compounds (Table 1 and Fig. 2).

Naloxone (given IP 5 min before intranigral injection) produced a dose-dependent inhibition of the rotational behaviour produced by intranigral administration of