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Histidyl-Proline, a Rapidly Degraded Metabolite of Thyrotropin Releasing Hormone, Has Behavioural Activity

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COGGINS, P. J., A. SAHGAL, J. R. McDERMOTT, C. R. SNELL, A. B. KEITH AND J. A. EDWARDSON. *Histidyl-proline, a rapidly degraded metabolite of thyrotropin releasing hormone, has behavioural activity.* PHARMACOL BIOCHEM BEHAV **24**(5) 1229–1233, 1986.—The effects of intracerebroventricular (ICV) administration of histidyl-proline (His-Pro; 0, 1, 10 and 100 μg/rat), a rapidly degraded metabolite of thyrotropin releasing hormone (TRH), were assessed using three behavioural paradigms. Locomotor activity was significantly increased over 30, but not 90, min following administration of the highest dose. This stimulatory effect was more apparent when naive rats were tested in an open field without any prior exposure to the apparatus; the effect was attenuated on a second exposure. His-Pro treated subjects also tended to prefer the area near the walls of the apparatus. The effects of the dipeptide on visual perception were studied, using a two-choice task. A low but not high (10, versus 100 μg/rat) dose of His-Pro increased response switching, but did not affect response repetition, latencies or percent correct performance. The results indicate that His-Pro can alter behaviour by affecting motor output, as well as emotional processes.

Histidyl-proline Locomotor activity Open field behaviour Visual discrimination Rat

THYROTROPIN releasing hormone (TRH, pGlu-His-Pro-NH_2) gives rise to two main metabolites on incubation *in vitro* with brain subcellular fractions: deamidated-TRH (TRH-OH) and cyclo(His-Pro), and there is evidence that both are present *in vivo* [13,18]. TRH-OH is produced by the action of proline endopeptidase, while cyclo(His-Pro) is formed by the action of pyroglutamyl aminopeptidase via an unstable intermediate, His-Pro-NH_2, which spontaneously cyclises at neutral pH. The two metabolites have been reported to have a number of biological effects, both related to [1–4, 23] and distinct from [1–4, 6, 16, 17, 23] those of TRH itself. It has been proposed that cyclo(His-Pro) is an active neuropeptide. However, unlike other neuropeptides and neurotransmitters, cyclo(His-Pro) has a relatively even distribution [18] and no demonstrable receptors in the central nervous system (CNS).

A third degradation product of TRH *in vitro* is the dipeptide histidyl-proline (His-Pro), which appears to be a product of the combined action of proline endopeptidase an pyroglutamyl aminopeptidase on TRH [9,10]. Additionally, specific binding sites for [^{3}H]-His-Pro may be present in rat brain membranes [10,11]. In this investigation, the biological activity of His-Pro was studied using three behavioural paradigms: locomotor activity, open field behaviour and visual discrimination. These provide indices of motor activity, emotionality and perceptual ability, thereby assessing effects on behavioural mechanisms ranging from sensorimotor to cognitive processes.

GENERAL METHOD

Subjects and Surgery

Male Wistar or PVG rats, weighing 180–200 g, were obtained from Bantin and Kingman, Hull, UK, and, following surgery, were individually housed under diurnal conditions (lights on 0700–1900 hr). Water was available at all times, and, where necessary, (experiment 2) food deprivation was based on a 23 hr fasting schedule.

Each rat had a 23-gauge stainless steel cannula (Clark Electromedical Instruments, Pangbourne, UK) implanted into the left lateral ventricle under general Nembutal/ketamine anaesthesia; coordinates from bregma were AP −1, LV +1.3, HV −4.5 mm. These placements were subsequently verified by injection of Evans's blue dye via the cannula and inspection of gross brain sections.

Peptide

His-Pro·2HBr (Bachem, Bubendorf, Switzerland) was

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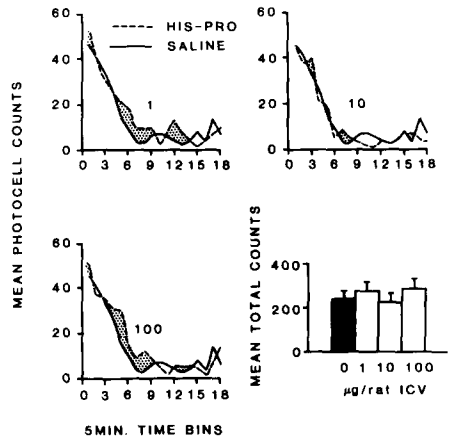

FIG. 1. Locomotor activity data, experiment 1. Saline control data are shown plotted against each dose (1, 10 and 100 μg/rat), and the stippled areas illustrate when His-Pro treatment exceeded control counts. A significant increase was recorded only if data from bins 1–6 (first 30 min) were analysed. The histogram illustrates total counts, when data across bins 1–18 (90 min) are collapsed.

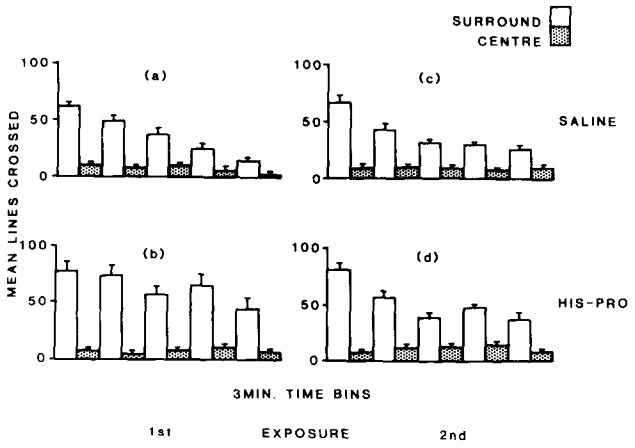

FIG. 2. Open field data, experiment 1. (a) and (b) refer to the 1st exposure, SAL and His-Pro data respectively; similarly, (c) and (d) illustrate scores on the 2nd exposure. Note that there were a greater number of opportunities to cross lines in the surround (16 squares), as opposed to central (9 squares), areas; see text for further detail.

dissolved in 0.9% saline (SAL) to give the required dose in 10 μl for intracerebroventricular (ICV) injection immediately prior to test. The solution was freshly prepared, and peptide purity was assessed using high performance liquid chromatography (HPLC).

Control and Data Acquisition

Acorn System III microcomputers (Acorn Computers, Cambridge, UK), running on ONLIBASIC [15] software were used.

Statistics

In all cases, parametric 2- or 3-factor analysis of variance (ANOVA; repeated or mixed measures, depending on experimental design) was used [24]. Latin square analysis followed the procedure described by Bliss [7]. Dunnett's *t*-test [24] was used to compare control with treatment means. Transformations (logarithmic or arcsin) of data followed the procedures described by Winer [24], and a 0.05 level of significance was selected.

EXPERIMENT 1: THE EFFECTS OF HIS-PRO ON LOCOMOTOR ACTIVITY AND OPEN FIELD BEHAVIOUR

Locomotor activity counts, using photocell cages, provide a simple estimate of effects on general motor activity. This method was used to investigate the effects of His-Pro since TRH, the parent neuropeptide, has been shown to increase locomotion under certain circumstances [1], possibly via an interaction with the mesolimbic dopaminergic system [4].

Different groups of subjects were also studied in a relatively large open field. This apparatus also provides an index of activity, for example in terms of number of lines (drawn on the floor) crossed. In addition, other types of behaviour such as grooming and rearing can be simultaneously assessed by the experimenter. Moreover, the paradigm provides a simple method for studying emotional factors [5,19]: subjects tend to stay near the walls of the apparatus and, at least during initial stages, to hesitate from entering the central zone, which induces anxiety. Thus, anxiolytic drugs may alter patterns of behaviour, by hastening entry into the centre and *vice versa*. A preliminary, unpublished, study indicated that His-Pro affected emotional processes, and it was therefore decided to investigate the effects of the dipeptide on open field behaviour.

METHOD

Apparatus

Locomotor activity was measured in black Perspex cages measuring 40×20×20 cm. Each cage was fitted with 2 photocells connected to a computer; details are provided elsewhere [1].

The open field was constructed out of white Perspex, and measured 100×100×50 cm high. Lines were drawn on the floor such that the area was subdivided into 25 squares. The 16 squares adjacent to the walls were designated the "surround" zone, and the remaining 9 were termed the "centre." Behaviour was measured using a hand held 8-key pad connected to a computer, which stored a record of each line crossed within or directed towards the surround or central zones; the time at which each event occurred was also simultaneously recorded. Other behaviours such as grooming, rearing and sniffing were also recorded when the appropriate key was depressed, but none were substantially altered by the dipeptide.

Procedure

All testing took place in the afternoon (see [1]) in a room where white noise (70 dB) was present. In the activity cage procedure, 7 male Wistar rats were habituated to the cages for 90 min on 3 consecutive days, and the computer recorded total beam interruptions within successive 5 min time bins (18 bins) for each rat. On the fourth day, saline (SAL, 10 μl)

was administered, and this was repeated on the following day, which became the first data collection day (dose "0"). Testing continued with a 1 day interval between sessions, using a "reverse block" design with doses of 1, 10 and 100 μg His-Pro, ICV; thus, a subject could have received the following sequence of doses: 0, 10, 1, 100, 100, 1, 10, 0 μg. This design attenuates the effects of gradual reduction of activity across days since, for example, "0" is administered both when activity could have been at its highest, as well as its lowest; average values would therefore be reasonably representative.

For the open field experiment, two groups of rats (N=8 per group) were individually housed, and maintained under conditions of minimal handling. Each test session for each rat lasted 15 min. On the first test day, 8 animals received 100 μg His-Pro (ICV), and 4 saline; on day 2, the remaining 4 received saline. Days 3 and 4 repeated days 1 and 2 respectively, and the saline data from days 1 and 2, and from days 3 and 4 were pooled as the control for the first and second exposures respectively.

RESULTS AND DISCUSSION

Activity Cage Data

The data (Fig. 1) were subjected to 2-factor analysis, and this showed that there was no dose effect over the whole 90 min time period, $F(3,18)=2.11$, $0.25>p>0.1$. The time bin factor was highly significant, $F(17,102)=28.30$, $p<0.01$, as is usually found to be the case.

At first sight, these results suggest that the dipeptide had no effect on locomotor activity. However, inspection of the data (Fig. 1, stippled areas) suggests that a stimulatory effect exists, but that it is transient, lasting about 30–40 min. This fact may simply reflect the rather rapid metabolism of His-Pro, suggesting that the 90 min session was excessive. Accordingly, data for the *first 30 min period only* were reanalyzed; now, the data reached significance, $F(3,18)=3.32$, $p<0.05$, with the highest dose increasing counts relative to control ($p<0.05$).

Open Field Data

His-Pro significantly increased activity, relative to saline controls, following both exposures to the apparatus (3-factor analysis, $F(1,14)=10.08$ and 4.83, $p<0.01$ and 0.05 respectively); these results are shown in Fig. 2. Activity gradually decreased within a session, and this was highly significant, at the $p<0.01$ level, for all 4 groups (Fig. 2). Similarly, all groups showed a highly significant preference for the surround, as opposed to the central zone. In order to represent this effect numerically, the surround/centre ratio (average number of lines crossed in the surround, divided by the average number crossed in the central zone) was calculated for each group. For His-Pro the values were 8.03 and 4.07 (first and second exposure, respectively); the corresponding values for SAL were 4.54 and 3.78. Thus, the dipeptide group preferred the surround zone, at least on the first day's exposure, suggesting that His-Pro had *anxiogenic*, in addition to motor stimulatory effects. Finally, His-pro induced occasional "wet-dog shakes" of the type observed after TRH and TRH-OH, but not cyclo(His-Pro) injection ([23], and Coggins *et al.*, unpublished). However, both the frequency and duration of this behaviour was less than after injection of the parent peptide (TRH).

EXPERIMENT 2: THE EFFECTS OF HIS-PRO ON A TWO-CHOICE VISUAL DISCRIMINATION TASK

Experiment 1 indicated that His-Pro affected both responsivity (activity) as well as other cognitive mechanisms, including emotionality. Observations of the animals' behaviour in the open field also suggested that they were more alert, in that they tended to explore the "surround" zone of the apparatus more than control animals. This may simply be the result of heightened anxiety; however, perceptual mechanisms could also have been affected. It was therefore decided to investigate the effects of His-Pro on a task developed to assess visual attention and discrimination ability [3,20].

METHOD

Apparatus

Skinner (operant) boxes (Campden Instruments, London, UK), connected to microcomputers, were used. Each box was fitted with 2 retractable levers, stimulus, house and magazine lights (all 24 V, 2.9 W), and a food pellet dispenser.

Procedure

Nine food restricted PVG rats were trained to press, within 5 sec, the lever above which a brief (0.5 sec) light appeared, for food reward; details of the procedure are given elsewhere [3]. Each day, 50 trials were given, with 25 left and 25 right stimuli, presented in a different random order every day. Animals were trained to a stable performance level of 85% correct.

Three groups of 3 rats were then tested, using a latin square design in order to minimise dose-order effects, with SAL (0), 10 and 100 μg/rat his-Pro (ICV). Each drug day was preceded by a test, but drug free, day and followed by a rest day.

The computer recorded the nature and outcome of each trial, together with response latency. Sequential analysis of response patterns was undertaken and two "responsivity" indices computed: (a) P(rep), the probability of response repetition, defined as the number of times the animal responded on a lever given that the preceding response was on the same lever, divided by the total number of trials minus 1; (b) Index X, which is based on the algebraic sum of the conditional probabilities (p) of repetition and switching errors; it equals [p(repetition) − p(switching) + 1]/2. Inappropriate switching occurs when a response is switched when it should have been repeated, and *vice-versa*. Switching and repetition are the 2 types of errors of commission that can occur in this task; missed opportunities (when the subject fails to respond within the allocated time, in this case 5 sec) are errors of omission, and these were negligible. Further details and mathematical rationale for these indices are provided elsewhere [20].

RESULTS AND DISCUSSION

His-Pro had no effect on performance, as measured by proportion corrects, $F(2,14)=0.27$, $p>0.25$; Fig. 3, and in addition neither correct nor incorrect latencies, $F(2,14)=0.36$ and 0.13 respectively, $p>0.25$, nor the P(rep) index of responsivity were affected, $F(2,14)=2.52$, $0.25>p>0.1$. On the other hand, Index X was very significantly, albeit slightly, reduced relative to SAL scores,

TABLE 1
LATENCY AND REPETITION INDICES, EXPERIMENT 2

Dose (μg/rat)	Latency (sec) Correct	Incorrect	P(rep)
0	1.14	1.36	0.51
10	1.19	1.28	0.45
100	1.22	1.29	0.51

Correct and incorrect latencies in sec, and P(rep) values. These indices were not significantly affected by His-Pro.

$F(2,14)=6.86$, $p<0.01$, but only following treatment with the (low) 10 μg dose of His-Pro (Table 1 and Fig. 3).

These results suggest that His-Pro does not markedly affect visual attention ability, but instead potentiates inappropriate response switching behaviour. The latter could reflect a general, non-specific increase in activity directed towards different parts of the test environment, manifested as switching from one lever to the other. However, this effect was slight; a substantial increase in inappropriate switching would have significantly disrupted (percent correct) performance. The significant reduction in Index X at 10, but not 100, μg may be indicative of an "inverted-U" dose-response curve. Such non-monotonic curves have been detected in several investigations of peptide effects [12,21] and have also been found to hold for amphetamine (AMP) where 1.6 mg/kg induces switching, with repetition gaining prominence at 3.2 mg/kg and higher doses [14]. A simple explanation for this effect would be that both His-Pro and AMP first *stimulate* activity, leading to inappropriate switching, and then, at higher doses, induce stereotypic behaviour manifested by response repetition. The present results do not allow us to conclude that His-Pro induces such repetition, but the general trend of the data suggest that it may mimic some of the behavioural effects of AMP, albeit in a much weaker manner.

GENERAL DISCUSSION

The experimental results suggest that the TRH metabolite His-Pro has mild and short-lived, but reliable, motor stimulatory effects, and that it also affects emotional/motivational mechanisms, possibly by increasing anxiety. The transient nature of the effect on locomotor activity was strikingly evident in experiment 1 (Fig. 1); when data over 90 min—which is a period of time often chosen when working with classical, and relatively stable, stimulant drugs such as amphetamine—were analysed, no significant dose-response relationship emerged. However, analysis of the first 30 min of data indicated that the dipeptide increased responding. Although such *post hoc* selection may appear questionable on statistical grounds, there are very good reasons in its favour. Unlike drugs such as AMP, many peptides are prone to rapid metabolism by endogenous enzymes, and a case can therefore be made for relatively short test periods. Inspection of the His-Pro data indicates that the behaviourally effective duration is, at most, about 40 min post injection (ICV).

In certain respects, the observed effects appear to be rather similar to those produced by low doses of the indirect

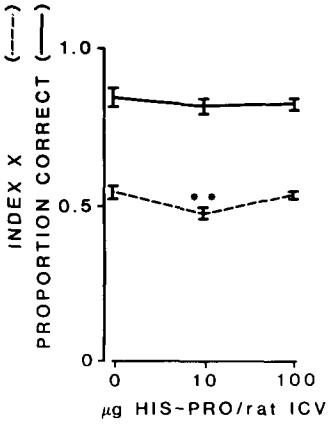

FIG. 3. Mean (±SEM) proportion corrects and Index X scores, experiment 2. The significant result is marked by asterisks ($p<0.01$).

dopamine agonist amphetamine. However, the dipeptide did not induce stereotypy at the doses studied and had no effect on purely perceptual processes. Because of the apparent anxiogenic properties of His-Pro, it would be informative to assess its effects on formal tests of anxiety, such as responding in a conflict situation. Certain benzodiazepines, many of which are powerful anxiolytics, can displace [^{3}H]-(3MeHis)-TRH from central binding sites [22]; TRH, and its metabolites, may therefore be involved in behavioural mechanisms concerned with novel, stressful and other anxiety-invoking situations.

The fact that P(rep) failed to detect the change in response patterns (experiment 2) once again demonstrates its inferiority over the more recently developed Index X [20], which gave highly significant results. Although both indices indicate a reduction in repetition at the lower dose, the excessive variability of P(rep) scores resulted in a non-significant statistic.

It has been proposed that the effects of TRH, the parent molecule, are mediated via specific receptors in the CNS, and the effects of cyclo(His-Pro) via as yet undiscovered cyclo(His-Pro) receptors [18]. There is growing evidence to indicate that *both* His-Pro and cyclo(His-Pro) are major products of TRH degradation [8,10]. However, there is also evidence to suggest that His-Pro, but not cyclo(His-Pro), specifically binds to brain membranes [10]. In addition, both TRH and His-Pro are rapidly degraded, whereas cyclo(His-Pro) appears completely stable [9]. Although His-Pro can be produced from TRH, and can be degraded by CNS peptidases *in vitro*, its existence *in vivo* awaits demonstration.

In conclusion, the results suggest that the behavioral effects of His-Pro are both broadly similar to, and in certain respects different from, those of TRH. Thus, both peptides tend to increase locomotor activity [1], but His-Pro increases response switching, whereas TRH may induce repetition, as measured by P(rep) [3]. However, Index X [20] had not been formulated when the earlier, TRH, study was undertaken, and hence subtle alterations in patterns of switching errors could have been obscured. At present it is prudent to conclude that although His-Pro has reliable stimulatory effects on motor behaviour, these effects are, dose for dose, weaker than those observed after TRH injection.

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