Original Article

Part 2. Long term *in vivo*/*in vitro* evaluation of the Cholecystokinin antagonists: *N*-(5-methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-*N'*-phenylurea MPP and carboxamide MPM

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ABSTRACT: The mixed CCK antagonist *N*-(3-oxo-2,3-dihydro-1*H*-pyrazol-4-yl)-indole-carboxamide MPP with a binding affinity of 25 nM/20 nM and the CCK_1 selective 3-oxo-1,2-diphenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-*N'*-phenyl-urea MPM (IC_{50} = 25 nM) represent the best two compounds of an amide and a urea pyrazoline series, which were previously evaluated in mice (Part 1) for their CNS activity.

The long term *in vivo* and *in vitro* evaluation is described in this part. Stress was induced for a 4 week period daily. A dose of 0.5 mg/kg of MPP and MPM showed a significant antidepressant effect in the foced swim test in rats, which was in enhanced within a 4 week test period. The mixed CCK antagonist MPM only occurred anxiolytic properties in the elevated X-maze in rats at a 0.5 mg/kg dose. For the stress induced rats, the MPP and MPM treatment reversed the effects of stress on the dendritic atrophy in hippocampal CA3 pyramidal neurons. A reduction of organ weight was reversed for the adrenal gland, when the animals were treated with the CCK antagonists MPP and MPM over a period of 4 weeks.

Keywords: CCK-antagonists, *N*-(3-oxo-2,3-dihydro-1*H*-pyrazol-4-yl)-indole-carboxamides, 3-Oxo-1,2-diphenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-*N'*-phenyl-urea, Forced swim test, Elevated plus-maze, Hippocampal CA3 pyramidal neurons

1. Introduction

Cholecystokinin (CCK) is a peptide neuromodulator and/or neurotransmitter. It was originally discovered from the gastrointestinal system, and is extensively and abundantly distributed within the central nervous system (CNS). CCK was initially isolated as 33 amino acid peptide from the porcine duodenum (*1*). Species specific molecular variants of the CCK have also been identified (CCK-58, CCK-39, CCK-22, sulfated CCK-8, unsulfated CCK-8, CCK-7, CCK-5 and CCK-4) in pig, monkey, rat, cat, dog, chicken and man (*2*).

Receptors for CCK were divided into two subtypes, CCK_A (CCK_1) and CCK_B (CCK_2), which reflected their initial localization in the gastrointestinal tract and the brain, respectively (*3*). However, the presence of CCK_A receptors was demonstrated in various regions of the brain, such as the dorsomedial hypothalamic and habenular nuclei. In addition, CCK_B receptors were identified in the gastrointestinal tract. The CCK_A and CCK_B receptors, both belonging to the class of G protein-coupled receptors, were characterized by seven transmembrane domains (*4*).

The biological roles of peripheral CCK_A receptors were well characterized. They included contraction of the gall bladder, stimulation of pancreatic enzymes secretion, and the potentiation of insulin secretion (*5*).

The peripheral CCK_B receptors were primarily responsible for the stimulation of gastric acid secretion. The central CCK_B receptors were involved in the control of nociception (*6*), the development of anxiety (*7*), panic attacks and satiety (*8*).

Since the CCK-discovery in the CNS, anatomical, physiological and pharmacological studies of cholecystokinin continued steadily. During the last decade, more than 1,000 scientific papers were published on CCK. Interestingly, CCK was not only widely expressed in virtually all CNS regions, it was the most abundant neuropeptide system in the brain of several mammals, especially in the human brain (*9*). In the brain, CCK (*10*) was co-localized with many classical neurotransmitters, such as dopamine (*11*),

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GABA and glutamate (*12*). The co-localization of CCK and GABA in some areas of CNS, especially the cortex and the hippocampus proposed possible roles of CCK in many psychiatric disorders (*13*), including anxiety, depression, attention deficit disorder and in the negative symptoms and cognitive deficits of schizophrenia. Considerable interest was devoted to the pharmacology of CCK_B receptors, since administration of selective agonists produced panic-like attacks in human (*14*). Moreover, CCK_B antagonists had been shown to inhibit panic attacks induced in humans by systemic administration of CCK-4 (*15*). These results led to the conclusion that CCK_B receptors were involved in the regulation of anxiety.

One potential role, which was proposed for CCK, was to act as a modulator of pain (*16*). Indeed, studies have shown, that CCK antagonists potentiated opioid analgesia (*17*) and might also have intrinsic analgesic activity (*18*). A study (*19*) showed that CCK antagonists blocked the development of morphine tolerance (Part 1, *20*).

Specific and highly potent CCK antagonists for both receptor subtypes were developed and suggested to have much pharmacological and therapeutic potential. The discovery of asperlicin (*21*) was the initial point for this new discovery programme. CCK_A antagonists, such as the amino acid derivatives lorglumide and loxiglumide (*22*), the benzodiazepines devazepide (*23*) and FK-480 (*24*) have been developed. Moreover, the pharmacological properties of the potent selective CCK_A antagonists, TP-680 (*25*) and T-0632 (*26*), have been reported. Some CCK_B receptor antagonists such as L-365,260 reached clinical trials and had clinical utility as anxiolytics (*27*), antipsychotics (*28*) or analgesics (*29*). Although various CCK antagonists were produced and studied continuously, toxicity, lack of efficiency and poor pharmaceutical properties of the substances made new compounds still be needed. We have reported the antinociceptive, anxiolytic and antidepressant effects of our *N*-(5-methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-*N'*-phenylureas and carboxamides in Part 1.

It is focused in this part of the publication on the long term evaluation of two pyrazoline based antagonists, a CCK_A selective amide and a mixed phenyl ureido-antagonist.

2. Materials and Methods

2.1. *Animals*

Experiments were conducted in male IRC mice obtained from the Animal House, Faculty of Medicine, Khon Kaen University. Each experimental group consisted of 6 animals and the treatment procedures were approved by the ethical committee, Faculty of Medicine, Khon Kaen University.

Mice were intraperitoneal injected with test compounds dissolved in 5% DMSO and not more than 0.2 mL/animal. After 30 min animals were tested, as described in the following sections.

2.2. *Antidepression test*

The forced swim test: The forced swim test was carried out in a glass cylinder filled with water and the water temperature was approximately 25-28℃. Rats were gently placed into the water and the immobility time was recorded by an observer during the period of 5 min. Immobility was defined as absence of all movement and rats remained floating passively in the water with its head just above the water surface.

2.3. *Anxiolytic activity test*

The elevated plus-maze: The elevated plus-maze consisted of two open arms without any walls, two enclosed arms, an end wall and the central arena interconnecting all of the arms. The maze was elevated from the floor. At the beginning of the experiment the rat was placed in the central arena facing one of the enclosed arms. During a 5 min interval, the time rats spent in the open arms of the plus-maze was recorded. The rat was considered to be in the open part when it had clearly crossed the line between the central arena and the open arm with its 4 legs.

2.4. *Effect of the CCK antagonists **MPM** and **MPP** in the stress model*

Male Sprague-Dawley rats, weighting 250-300 g at the beginning of the experiment, were housed in groups of three. They were kept in a 12 h light/dark cycle and given food and tap water ad libitum. Rats were divided into 2 conditions, stress (s) and non-stress (ns) and 6 rats/group were used for each test. Stress groups of rats were subjected to chronic restraint stress over a period of 28 days. On each day, rats were individually restraint in wire mesh cages for 6 h (10 am - 4 pm). Prior to the restraint sessions, the rats received either 5% DMSO or the synthetic CCK antagonists at a dose of 0.5 mg/kg BW orally at 9:00 am. On day 1, 7, 14, 21 and 28, the animals were evaluated in the elevated plus maze and the forced swim tests for studying behavioural changes under stress and non- stress conditions.

At the end of the treatment period, rats were deeply anesthetized with thiopental sodium 60 mg/kg intraperitoneally. The adequacy of anesthesia was monitored by checking for the absence of corneal reflexes and the flexor withdrawal response. Anesthetic rats were transcardially perfused with 0.1 M phosphate buffer followed by 4% paraformaldehyde in 0.1 M phosphate buffer. After the fixative perfusion, the brain was removed rapidly and cut into 2 sides, which

were subsequently used for the Golgi-Cox method and immunohistochemistry.

2.5. *Golgi-Cox method*

Preservative perfused slices were cut into 4-5 mm thick slices with a sharp razor blade and impregnated in the Golgi-Cox solution for 20-30 days in the dark. The impregnated blocks of tissue were embedded in paraffin before sectioning. The coronal sections, 100 µm thick were cut on a microtome. The sections were put on a clean drop of water on glass slides. Subsequently the sections were spread at 40°C on a hot plate. They were dried at 40°C in the oven for 1 h, rinsed in xylene, covered with mounting media, which was slipped. In order to be selected for analysis, golgi impregnated neurons had to possess the following characteristics:

(i) Location within the CA3 region of the dorsal hippocampus
(ii) Dark and consistent impregnation throughout the extent of all of the dendrites
(iii) Relative isolation from neighboring impregnated cells, which could interfere with the analysis

From each animal, 8-10 pyramidal cells from CA3 were selected. Each selected neuron was traced at 10× magnification, using a light microscope with a camera lucida drawing tube attached. From these drawings, the number of dendritic branch (bifurcation) points tree was determined for each selected neuron within a 100 µm thick section of each dendritic.

2.6. *Immunohistochemistry method*

The left side of the brain was postfixed with 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4°C. Tissues were rinsed with phosphate buffer and infiltrated with a 30% sucrose solution in order to provide cryprotection. The specimens were frozen rapidly with deep freeze at -25°C in a cryostat. After freezing, coronal section of 35 µm thick specimens were cut on a cryostat and stored in phosphate buffer. The specimens were stained with monoclonal antibody against choline acetyltransferase (ChAT) enzyme, a marker for cholinergic neurons and the density of immunoreactive neurons was determined in hippocampal areas.

2.7. *Weights of certain organs affected by stress*

After the brain was removed, adrenal glands and the spleen were dissected out. The surrounding fat and extrageneous tissues were removed and the organs were pat dried and weighed using a weighing-machine. The results were expressed as mg/100 g BW.

2.8. *Statistical analysis*

All data were expressed as mean ± SD. Significant difference between control and treatment was determined by using unpaired Student *t*-test. The differences among various groups were compared by ANOVA. Turkey test for pair wise comparison was performed to determine any significant difference at p-value < 0.05.

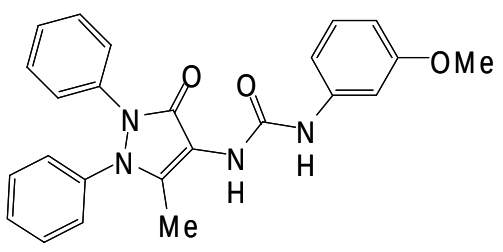
3. Results

The potent CCK_1 selective antagonist **MPP** and the mixed CCK antagonist **MPM** were selected and tested for the long term effects on stress responses in rats. The chemical structures of **MPP** and **MPM** are outlined in Figure 1.

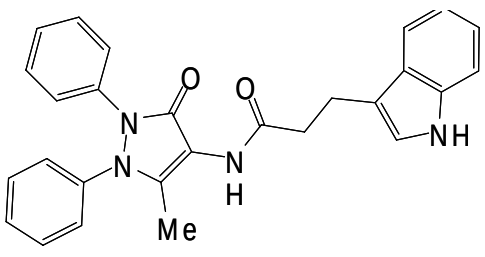
3.1. *Behavioural effects*

In the forced swim test, immobility times (in s) of the control non-stress rats, which received 5% DMSO were 149.15 ± 6.34, 155.99 ± 14.10, 155.09 ± 0.57, 155.31 ± 7.47 and 152.93 ± 6.78, respectively, when tested on day 1, 7, 14, 21 and 28. No significant difference could be observed among the tests carried out at various times in the control non-stress group.

Non-stress rats receiving either **MPM** or **MPP** had a significant decrease in immobility time, when observed on day 7, 14, 21 and 28, but not day 1, compared to the control non-stress group on the same day.



MPM



MPP

Figure 1. Selected structures of *N*-(3-oxo-2,3-dihydro-1*H*-pyrazol-4-yl)-1*H*-indole-carboxamides and ureido-pyrazolines.

Restrained rats (stress group) had a significant increase in immobility time when observed on day 7 and further until the end of treatment, when compared to the control non-stress group on the same day of the test. From day 7 until the end of experiment, stress rats receiving either **MPM** or **MPP** showed a significant reduction of immobility time, when compared to stress rats that received 5% DMSO. In addition, the immobility time of stress rats receiving either **MPM** or **MPP** was found also significantly lower than the non-stress control rats, especially at day 28.

In the elevated plus maze test (Figure 3), no change in time in the open arms and number of entry could be observed in the non-stress rats that received 5% DMSO (as control) until the end of the experiment. On day 21 and 28, non-stress rats, which received 0.5 mg/kg BW of **MPM**, had a significant increase in time spent in the open arms and the number of entry, when compared to the control non-stress group of the same day.

From day 7 until the end of the experiment, stress rats showed a significant reduction of time in the open arms and number of entry, when compared to the control non-stress group tested at the same day. It was observed that **MPM** treatment reduced the anxiogenic effect of stress significantly when tested on day 14, 21 and 28.

The results showed that restraining stress could produce depression and anxiety in rats, which could be observed as early as 7 days of restraint. Oral treatment with the mixed CCK antagonist **MPM** and the CCK_A selective antagonist **MPP** reduced depression and **MPP** reduced the anxiogenic effect of stress in rats in our experiments.

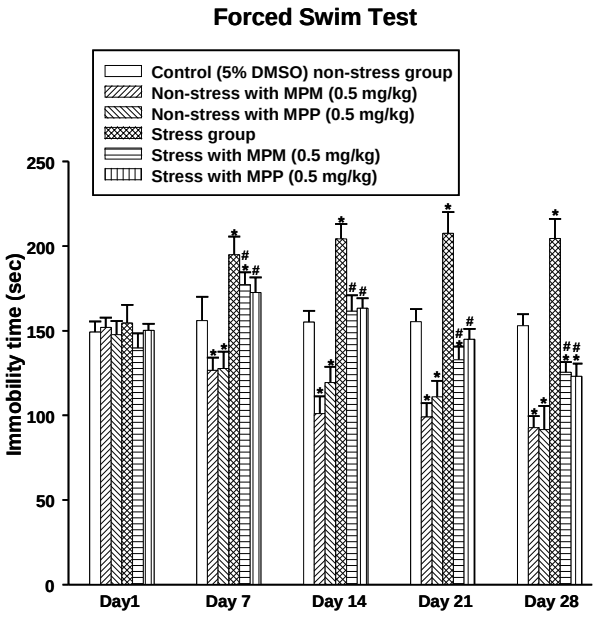


Figure 2. Effects of **MPM** and **MPP** on immobility time of non-stress and stress rats tested in the forced swim test. *P*-value < 0.05; * compared to the control non-stress (5% DMSO); # compared to the stress group on the same day of the test.

3.2. *Effects of **MPP**/**MPM** and stress on hippocampal CA3 pyramidal neurons*

Figure 4 showed the hippocampal CA3 pyramidal neurons impregnated with Golgi-Cox solution for 20-30 days from various treatment groups. In the control non-stress group, both basal and apical dendritic trees were highly branched (Figure 4A). Pyramidal neurons from rats, which were restrained for 28 days, showed atrophic changes of dendrites especially in the apical branches (Figure 4B). **MPM** (Figure 4C) and **MPP** (Figure 4D) treatment reversed the effect of stress on dendritic atrophy and the neurons appeared normal.

Each selected neuron from the sections was drawn on paper with a 10 µm sector from the centre (neuronal cell body) using a camera lucida drawing tube, attached to the microscope under 10× objective magnification and the drawings were shown in Figure 5.

The total number of branch points and the length of the dendrites, as estimated by the radius of the field, were

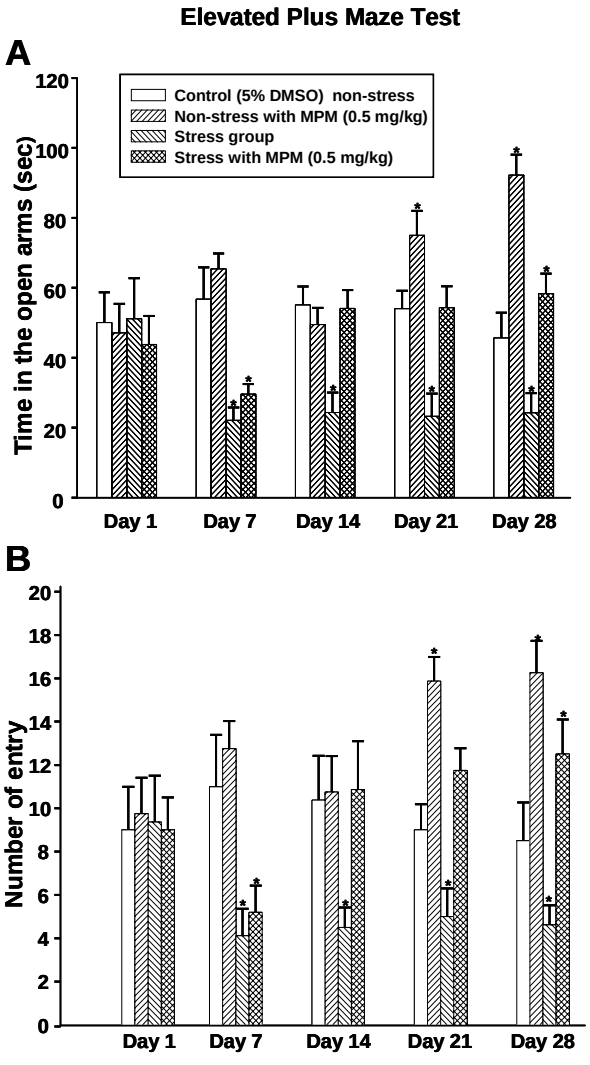


Figure 3. Effects of **MPM** on time spent in the open arms (A) and the number of entries (B) of non-stress and stress rats tested in the elevated plus maze test. *P*-value < 0.05; compared to the control non-stress (5% DMSO).

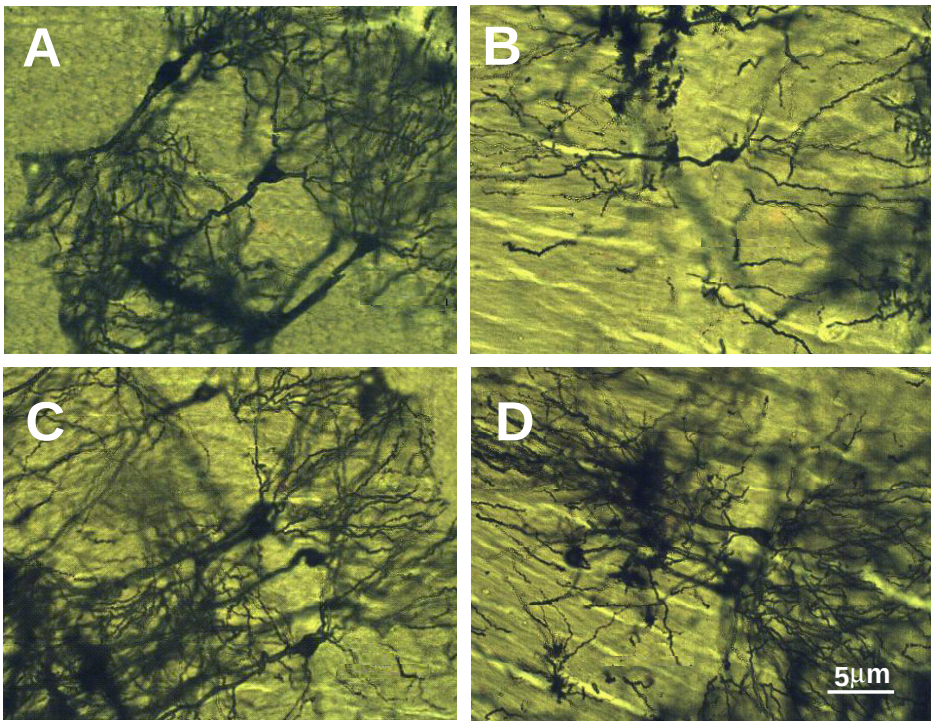


Figure 4. The Golgi-impregnated CA3 pyramidal neurons from rat's hippocampus at 20× magnification. A: the control, non-stress group; B: stress group; C: stress with **MPM** group; D: stress with **MPP** group.

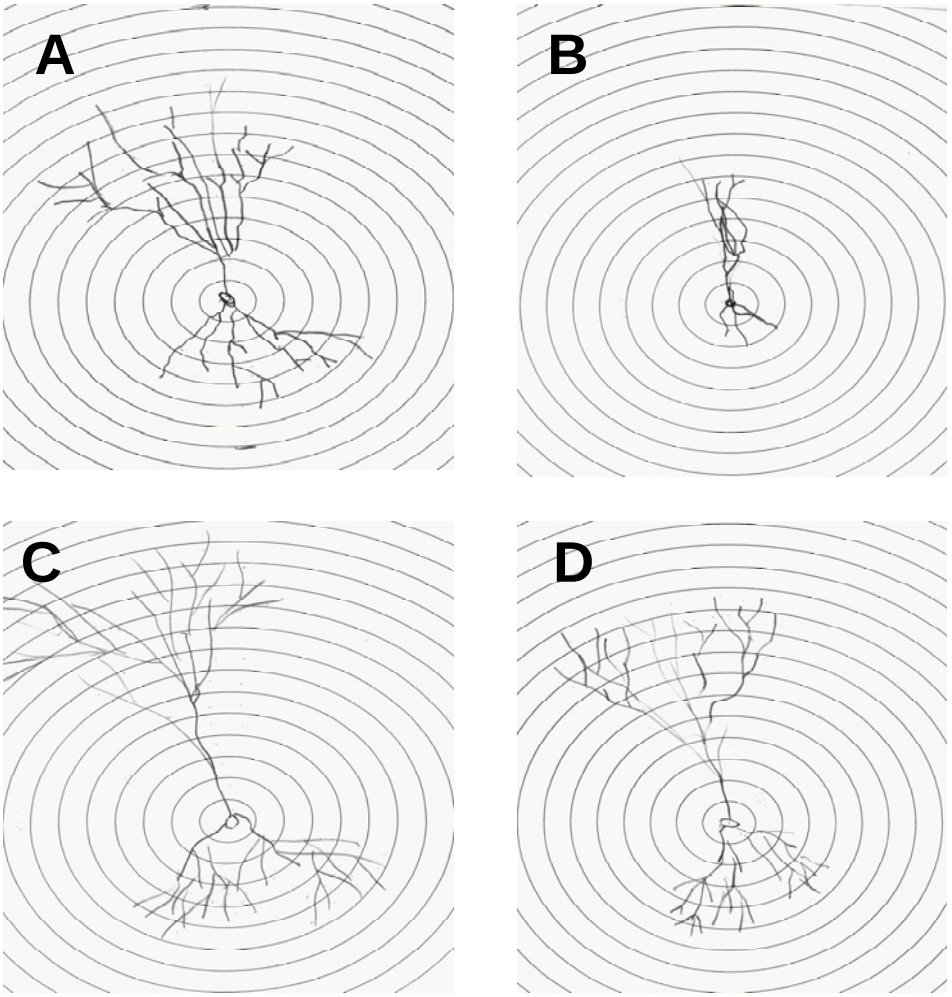


Figure 5. Camera lucida drawings of Golgi-impregnated CA3 pyramidal neurons from rat's hippocampus. Each sector of the drawing was equal to 10 µm. A: Control, non-stress group; B: Stress group; C: Stress with **MPM** group; D: Stress with **MPP** group.

determined from the drawings (10 neurons were traced from each group). In the stress group, both the number of branch points (Figure 5) and the radius (Figure 6) of the dendritic field of the apical tree, but not basal tree, were found significantly reduced compared to the control non-stress group. Stress rats, which received either **MPM** or **MPP**, showed no difference from the control group in both observed parameters. This suggested that both antagonists, **MPM** and **MPP** were able to antagonize the induced dendritic atrophy caused by stress.

3.3. *Effects of **MPM**/**MPP** and stress on organ weight*

Normally, the adrenal glands and the spleen are two of many organs affected by stress conditions. The weights of the adrenal glands and the spleen, expressed as mg/100 g BW, were outlined in Figure 8 for various groups of rats. Non-stress rats receiving 5% DMSO, served as control and the wet weights of the adrenal glands and the spleen were recorded as 20.00 ± 2.97 and 324.25 ± 18.49 mg/100 g BW, respectively. No effect of either **MPM** or **MPP** treatment on the weights of the two organs was observed in non-stress rats. Restraining the rats for 28 days increased the wet weights of the adrenal glands significantly, without having any effect on the weight of the spleen. Treatment with either **MPM** or **MPP**, at a dose of 0.5 mg/kg BW/day, antagonized in stress rats the effects of stress on the wet weights of the adrenal glands, which was found comparable to the control group after this 28 day treatment period.

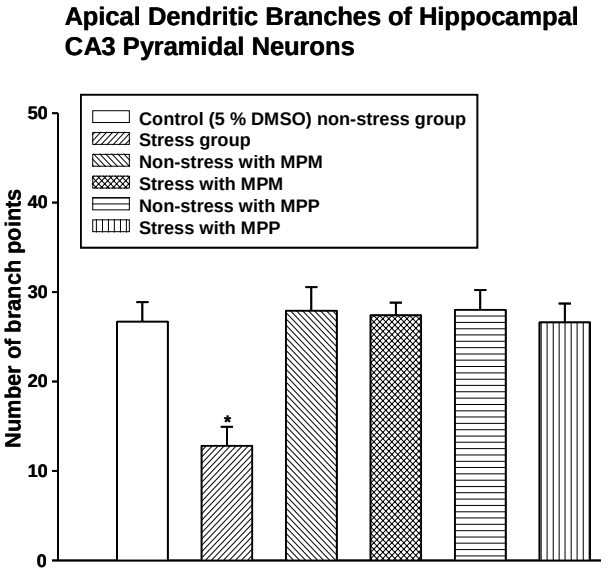


Figure 6. Effects of **MPM** and **MPP** on the number of apical dendritic branch points observed in hippocampal CA3 pyramidal neurons for non-stress and stress rats. * *P*-value < 0.05 when compared to the control non-stress group.

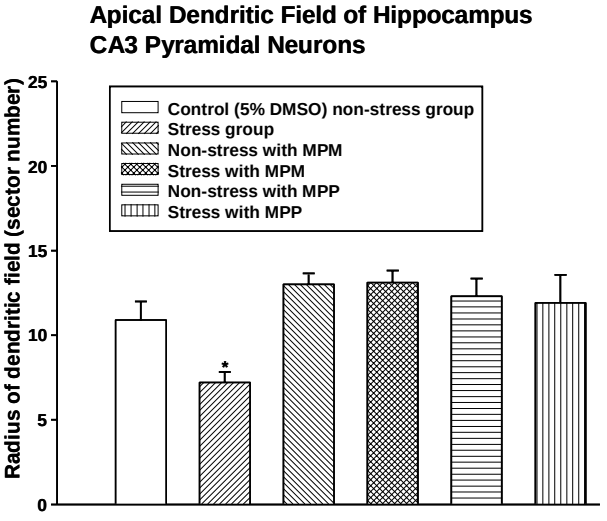


Figure 7. Effects of **MPM** and **MPP** on the radius of apical dendritic fields observed in hippocampal CA3 pyramidal neurons in non-stress and stress rats. * *P*-value < 0.05 when compared to the control non-stress group.

4. Discussion

In the present 4 week-study in rats, the antidepressant-like and anxiolytic-like long term effects of a mixed (**MPP**) and CCK_1 selective antagonist (**MPM**) were further evaluated, using effective and reliable animal models, such as the Porsolt swim test and the elevated X-maze.

The antagonistic effects against stress on rat's behaviours and the hippocampal neurons were clearly determined for the previously found active dose of 0.5 mg/kg of both CCK antagonist. Animal models of anxiety and depression, based on emotional reactivity, have been designed and proven to be bidirectional sensitive to stressful manipulations (*30*) and after the determination of effective doses in part 1 it was now investigated, what long term effects were observed when used at an effective dose. By simple, rapid and inexpensive ways of evaluating an animal's conditions, the forced swim test was used for testing antidepressant-like effects, whereas the elevated plus maze was used

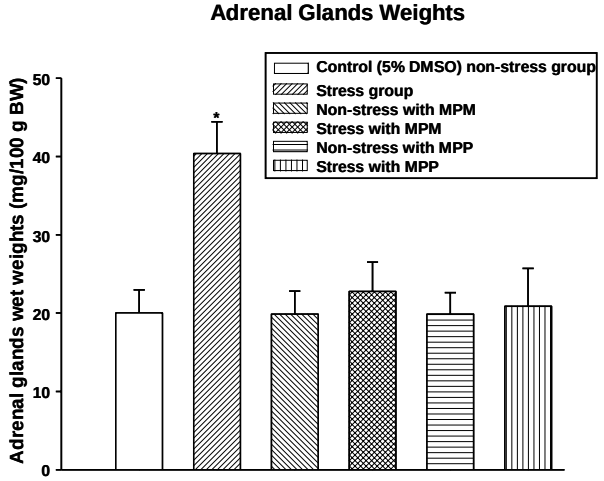


Figure 8. Effects of **MPM** and **MPP** on the wet weights of adrenal glands of non-stress and stress rats. Data were presented as mean ± SD. * *P*-value < 0.05 when compared to the control non-stress group.

for anxiolytic-like effects showing better effect when used long term. The aim was to investigate, if tolerance or further improvement, was observed towards the positive biological effects. Among the experimental models used for testing the antidepressant-like effect of the compounds, the forced swim test (also known as the Porsolt swim test) is one of commonly used and best model. The test is easily to perform and there is no need to use any expensive instruments. In our study, the forced swim test was found sensitive and reliable in detecting the antidepressant-like of the CCK_1 selective amide **MPP** and the mixed antagonist **MPM**.

The elevated plus maze and the light and dark box tests are also classified as a test, suitable for studying the acute stress effects. The elevated plus maze test, a well-validated animal model has become the most widely used model for the study of drug effects on anxiety (*31*) and only **MPM** showed anxiolytic effects.

The tail flick and the hot plate tests are widely used in pain assessment in animals and considered to be supraspinally integrated responses to heat (*32*) and the antinociceptive effects were discussed in part one of this series of publications (*20*).

Despite many findings, however, conflicting results concerning the types of CCK receptors involved in those mood disorders have been reported. The stimulation of CCK_1 or CCK_2 receptors was implicated in the physical and psychological responses of CCK to stress. Furthermore, several selective CCK_2 agonists produced anxiogenic-like effects, while CCK_2 antagonists induced anxiolytic-like effect in several models of anxiety (*33*). However, there was evidence indicated, that CCK_1 receptors were involved in the mediation of anxiolytic-like effects in the light and dark box model of exploration in mice (*34*). In the same model CCK_2 antagonists also showed an anxiolytic-like effect (*35*). Thus, both CCK_1 and CCK_2 receptors could have roles in the modulation of anxiety-related behaviour in animal models (*36*) as seen for **MPM**. The anxiolytic-like effect of only the mixed CCK antagonists is rather complex, as discussed by Hendrie *et al*., 1993. It has been reported, that CCK through CCK_1 receptor could potentiate the effect of amines, while CCK_2 receptor could inhibit the amine release (*37*). It might be the case, that the optimal ratio of the binding affinity among CCK_1 and CCK_2 receptors reflects best the results on mood disorders, as seen here with **MPM**.

As mood disorders are the abnormal behaviours, mostly found as response to stress conditions, it is interesting to see the effects of CCK antagonists in antagonizing the effects of stress. In the present study, 28 days of chronic restraint stress produced significant hippocampal dendritic atrophy, especially in the CA3 area, as previously shown (*38*). Atrophic changes (*39*) were clearly seen in apical, but not basal dendrites. Changes in basal dendrites were reported with prolonged stress (*40*). The effects of stress on hippocampal neurons were suggested to mediate through many mechanisms including glucocorticoid (*41*), glutamate (*42*), serotonin (*43*) and GABA (*44*). Glutamate, as an excitotoxin, might be a very important pathway in the hippocampal damage by stress, by acting through NMDA receptors. Serotonin released by stress may interacted pre-or post-synaptically with glutamate release and also potentiate NMDA receptor binding *via* $5\text{-}HT_2$ receptors.

Restraint stress also showed effects on the adrenal glands, but not the spleen (*45*). The enlargement of adrenal glands, observed after restraint stress, might indicate an increase in glucocorticoid synthesis / release in response to stress. However, it is still not known, whether the enlargement was due to hypertrophy or cellular hyperplasia and if the findings were sub-region specific or not.

The spleen size was not changed by stress in this study. Although a lower number of spleen cells were present, which correlate with a decreased number of lymphocytes in the circulation (*46*), the changes in cell numbers may not be detectable by measuring the wet weight of the organ.

MPM and **MPP**, prevented the effects of stress on mood changes, hippocampal dendrites and adrenal gland weight. The anti-stress effects of CCK antagonists could possibly act at many sites. The interaction of CCK-8S with glutamate was studied in the hippocampal CA3 and suggested, that excitatory amino acids may be enhanced by CCK-8S (*47*). Moreover, CCK was also able to regulate the limbic hypothalamo-pituitary-adrenal (LHPA) axis, acting on both, its central and peripheral parts.

CCK stimulated aldosterone secretion *via* CCK_1 and CCK_2 receptors in zona glumerulosa cells in the adrenal cortex and therefore, enhanced glucocorticoid secretion from zona fasciculata-reticularis cells *via* an indirect mechanism, involving a CCK_2 receptor mediated stimulation of ACTH release (*48*). Accordingly, CCK antagonists might antagonize stress effects through both types of receptors at hippocampus, pituitary and adrenal glands and break the LHPA axis in response to stress. As suggested earlier, the effects of CCK antagonists against stress may need the proper ratio of the effect against CCK_1 and CCK_2 receptors, since that receptor could inhibit and stimulate corticosteroid secretion, respectively (*49*).

5. Conclusions

Significant antidepressant-like effects were clearly observed and improved over time in rodents, treated with **MPM** or **MPP** in the forced swim-tests.

Anxiolytic-like effects were determined in rodents treated with **MPM**. The effects could be seen best in the elevated plus maze and no tolerance was observed.

MPM and **MPP** at a dose of 0.5 mg/kg BW in rats, antagonized all the effects of chronic restraint stress *in vivo* and *in vitro*. The CCK antagonists antagonised mood disorders (depression/anxiety) in rats *in vivo* and antagonised the stress induced hippocampal dendritic atrophy and an increased in adrenal glands weight *in vitro* over a 4 week period. These non-chiral, readily available agents, such as **MPM**, will play an exciting new role as novel substances in clinical trials for mood disorders and/or, in combination with morphine in various types of pain (part 1 and part 2).

Acknowledgement

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