INTRODUCTION

Melatonin (MT) is a neurohormone synthesized and secreted by the pineal gland. MT plays an important role in the regulation of physiological and neuroendocrine functions, such as in seasonal reproduction and circadian rhythms regulation (1, 2). Furthermore, melatonin’s chronobiologic role is complementary with the pharmacological properties, including the sedative, antioxidative, anxiolytic, antidepressive, and analgesic activity (3-7). Recent data points at the antioxidative properties of melatonin, which may suggest it being more efficient than the classic antioxidants (8-10).

Three subtypes of melatonin receptors have been identified. The mechanisms of action of melatonin include the involvement of membrane receptors (MT1, MT2) and cytosolic binding site (MT3). Recent studies have provided evidence that the MT3 melatonin receptor is actually a cytosolic quinone reductase 2 (QR2), detoxifying and antioxidant enzyme. Luzindole acts as an antagonist on both membrane receptors (MT1 and MT2) and it has been used in numerous studies. MT3 can be blocked by prazosin (also an alpha-1-adrenoceptor antagonist) (11, 12).

The early clinical results suggested that there is a connection between melatonin’s action and nociception processes - the patients who suffered from chronic pain had significantly lowered the blood and urine concentrations of melatonin in comparison to the healthy volunteers (13). There was also observed the regularity that the patients with fibromyalgia had the abnormal melatonin’s secretion and lowered concentrations of melatonin’s precursors - L-tryptophan and serotonin (14, 15). The preclinical evidence is pointing also at the time-dependent antinociceptive properties of melatonin in mice and rats (17, 18). Because of the fact that there is the circadian rhythm of melatonin concentration in the blood, it was discovered that the maximal analgesic effect of melatonin appears in the late evening and surgical removal of the pituitary gland inhibits this effect (16). The occurrence of the systematic daily changes in the pain-threshold was described in humans and certain rodent species (17).

The exact pathways of antinociceptive action of melatonin remain unclear. Therefore, further investigations are needed to establish the exact molecular mechanisms of melatonin’s actions in different forms of pain and subsequently to compile the brand new antinociceptive therapeutic strategies. Since there is a lack of strong evidence regarding melatonin’s influence on neuropathic pain, in our study we used the chronic constriction injury model (CCI model), proposed by Bennett (19).
observed in the rats with CCI model, with a duration between 2
days up to 2 months after surgery (19). The symptoms were
similar to those observed in patients with peripheral
neuropathies.

MATERIALS AND METHODS

Animals

All of the procedures described below were performed in the
Pathophysiology Department of the Jagiellonian University in
Cracow. In the procedures the following were used: 78 male
Wistar rats, weighing ca 200–250 g. The animals were kept in a
light- and temperature-controlled (20–22°C) room on a 12:12 h
light-dark cycle with the ad libitum access to water and a
standard lab rodent food. The experimental protocol was
approved by the Ethics Committee for Animal Research of
Jagiellonian University.

Drugs

Naloxone (Polfia) and prazosin (Sigma Aldrich) dissolved in
saline, melatonin (Sigma Aldrich) in 0.5% ethanol-water,
luzindole (Sigma Aldrich) in 1% DMSO-water, flumazenil
(Sigma Aldrich) in 1% DMSO-water, and picrotoxin (Sigma
Aldrich) in 1% ethanol-water were used in this study. All agents
were subdivided into several groups (Table 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham</th>
<th>CCI</th>
<th>Drug</th>
<th>Von Frey test</th>
<th>Hargreave's test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>saline i.p.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+</td>
<td>saline i.p.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>+</td>
<td>MT (100 mg/kg ip)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>+</td>
<td>NX (1 mg/kg ip) + MT (100 mg/kg ip)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>+</td>
<td>PZ (10 mg/kg ip) + MT (100 mg/kg ip)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>+</td>
<td>LZ (10 mg/kg ip) + MT (100 mg/kg ip)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>+</td>
<td>PC (1 mg/kg ip) + MT (100 mg/kg ip)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>+</td>
<td>FL (0.1 mg/kg ip) + MT (100 mg/kg ip)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

CCI – chronic constriction injury model; MT – melatonin; PZ – prazosin; NX – naloxone; LZ – luzindole; PC – picrotoxin; FL –

Table 1. Experimental procedures.
Luzindole: in the sixth group (n=6) luzindole, MT1/MT2 receptors antagonist was administered (10 mg/kg; Sigma Aldrich) intraperitoneally. After 10 minutes melatonin was injected intraperitoneally and 30 minutes after its administration the von Frey and Hargreaves' tests were once more conducted.

Picrotoxin: in the seventh group (n=6) the noncompetitive antagonist for the GABA\, receptor, picrotoxin (1 mg/kg) was administered intraperitoneally and after 10 minutes, melatonin was given intraperitoneally. After 30 minutes the behavioral tests were done.

Flumazenil: in the eighth group (n=6) flumazenil, benzodiazepine antagonist (0.1 mg/kg), was administered intraperitoneally. After 10 minutes melatonin was injected intraperitoneally and 30 minutes after melatonin's administration the von Frey and Hargreaves' tests were once more performed.

Our preliminary study showed that none of the used drugs (naloxone, prazosin, luzindole, picrotoxin nor flumazenil) affected the nociceptive threshold when administered alone (n=15).

Evaluation of nociception

Mechanical allodynia was determined by using von Frey’s hairs (Dynamic Plantar Aesthesiometer; Ugo Basile, Italy). The pinched pressure to the ipsilateral (right) hind paw in ascending order of force (0–26 g) of the rats with CCI, was defined as a noxious stimulation and recorded as paw withdrawal threshold (PWT) in grams. The measurements were repeated twice and the arithmetical average was calculated as a final result.

Evaluation of thermal hyperalgesia was assessed using the Hargreaves’ model of thermal hyperalgesia. A Plantar Test (Ugo Basile Italy) was used to measure the withdrawal latencies of the hind paws from a radiant heat stimulus. The measurements were repeated twice and the arithmetical average was calculated as a final result.

All behavioral tests were conducted between the hours: 8:00 a.m. and 2:00 p.m. to maintain the same pattern of animals’ daily activity. No differences in baseline paw withdrawal thresholds were noted during these hours.

Data analysis and statistics

The results are shown as the mean values ±standard deviation (S.D.); one-way analysis of variance (ANOVA), followed by Tukey’s test, were used to compare differences between treatments. Statistical significance was considered to be achieved when p<0.05.

RESULTS

Bennett’s neuropathic pain model was evaluated by two behavioral tests: von Frey’s test and Hargreaves’ test. The obtained results were calculated from the preliminary data and shown (Table 2 and 3) as the mean values ±S.D. (for six animals per group).

All animals in the CCI group developed neuropathic pain-like behavior in the right hind paw within 7 days, while there

### Table 2. Mechanical allodynia (von Frey test).

<table>
<thead>
<tr>
<th></th>
<th>7th day</th>
<th>14th day</th>
<th>21st day</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Von Frey test (g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>24.9±1.47</td>
<td>25.2±1.3</td>
<td>25.7±1.7</td>
</tr>
<tr>
<td>CCI</td>
<td>13.1±2.4 P&lt;0.05 (vs. sham)</td>
<td>10.4±2.3 P&lt;0.001 (vs. sham)</td>
<td>12.1±2.9 P&lt;0.05 (vs. sham)</td>
</tr>
<tr>
<td>CCI+MT</td>
<td>17.4±3.6 P&lt;0.05 (vs. CCI)</td>
<td>16.8±2.6 P&lt;0.001 (vs. CCI)</td>
<td>17.2±2.3 P&lt;0.05 (vs. CCI)</td>
</tr>
<tr>
<td>CCI+MT+PZ</td>
<td>18.2±4.2 P&lt;0.05 (vs. CCI+MT)</td>
<td>15.6±4.6</td>
<td>16.9±1.6</td>
</tr>
<tr>
<td>CCI+MT+NX</td>
<td>14.1±4.2 P&lt;0.05 (vs. CCI+MT)</td>
<td>11.2±2.1 P&lt;0.05 (vs. CCI+MT)</td>
<td>12.1±3.4 P&lt;0.05 (vs. CCI+MT)</td>
</tr>
<tr>
<td>CCI+MT+LZ</td>
<td>12.2±3.4 P&lt;0.05 (vs. CCI+MT)</td>
<td>11.4±2.1 P&lt;0.05 (vs. CCI+MT)</td>
<td>13.1±2.4 P&lt;0.05 (vs. CCI+MT)</td>
</tr>
<tr>
<td>CCI+MT+PC</td>
<td>12.9±3.9 P&lt;0.05 (vs. CCI+MT)</td>
<td>14.1±4.46 P&lt;0.05 (vs. CCI+MT)</td>
<td>10.7±4.04 P&lt;0.05 (vs. CCI+MT)</td>
</tr>
<tr>
<td>CCI+MT+FL</td>
<td>12.8±5.8 P&lt;0.05 (vs. CCI+MT)</td>
<td>13.2±6.1 P&lt;0.05 (vs. CCI+MT)</td>
<td>14.1±5.95 P&lt;0.05 (vs. CCI+MT)</td>
</tr>
</tbody>
</table>

CCI – chronic constriction injury model; MT – melatonin; PZ – prazosin; NX – naloxone; LZ – luzindole; PC – picrotoxin; FL – flumazenil.

### Table 3. Thermal hyperalgesia (Hargreaves test).

<table>
<thead>
<tr>
<th></th>
<th>7th day</th>
<th>14th day</th>
<th>21st day</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hargreaves test (sec)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>10.1±0.7</td>
<td>9.9±0.5</td>
<td>10.9±0.6</td>
</tr>
<tr>
<td>CCI</td>
<td>5.6±0.6 P&lt;0.05 (vs. sham)</td>
<td>6.1±0.4 P&lt;0.05 (vs. sham)</td>
<td>6.2±0.5 P&lt;0.05 (vs. sham)</td>
</tr>
<tr>
<td>CCI+MT</td>
<td>6.8±0.4 P&lt;0.05 (vs. CCI)</td>
<td>6.7±0.5</td>
<td>6.5±0.3</td>
</tr>
<tr>
<td>CCI+MT+PZ</td>
<td>6.3±0.9</td>
<td>6.9±1</td>
<td>6.1±1.2</td>
</tr>
<tr>
<td>CCI+MT+NX</td>
<td>6.3±1.3</td>
<td>6.3±1.1</td>
<td>6±1.4</td>
</tr>
<tr>
<td>CCI+MT+LZ</td>
<td>5.7±0.8</td>
<td>5.9±0.9</td>
<td>6.1±0.9</td>
</tr>
<tr>
<td>CCI+MT+PC</td>
<td>7.1±1.2</td>
<td>7.3±0.5</td>
<td>7.6±1.1</td>
</tr>
<tr>
<td>CCI+MT+FL</td>
<td>8±1.2</td>
<td>8.2±0.8</td>
<td>8.5±1.2</td>
</tr>
</tbody>
</table>

CCI – chronic constriction injury model; MT – melatonin; PZ – prazosin; NX – naloxone; LZ — luzindole; PC – picrotoxin; FL – flumazenil.
was no change in paw withdrawal thresholds in the sham group, as assessed by the plantar aesthesiometer and the plantar test. In the neuropathic pain model there was shown mechanical allodynia in the damaged paw in comparison to the sham operated (p<0.05) and also thermal hyperalgesia in comparison to the sham operated (p<0.05). 14 days after surgery the withdrawal threshold in the plantar aesthesiometer decreased from 25.2±1.3 g in sham group to 10.4±3.3 in CCI group. Likewise, the withdrawal latency in response to defined radiant heat stimuli in the plantar test was reduced from 9.9±1.4 s in sham group to 6.1±2.6 s in CCI group, 14 days after surgery. The presence of allodynia was also inferred from the nocifensive responses evoked by standing on an innocuous metal floor during the time of a single test procedure and by the rats’ persistence in holding the hind paw in guarded position after the nociceptive stimuli used in the tests’ procedures during their lasting. The most severe mechanical allodynia was observed after 2 weeks from the right sciatic nerve ligation procedure (Fig. 1), whereas the most significant severe thermal hyperalgesia occurred after 1 week from the surgical CCI procedure (Fig. 2). There were no differences between baseline, 7 days, 14 days and 21 days after the ligation in sham-operated rats, confirming the absence of thermal hyperalgesia or mechanical allodynia in these animals. Intraperitoneal administration of melatonin resulted in the increase in mechanical stimulus pain threshold reaction (Fig. 1), that points on the antinociceptive effects of melatonin. In comparison, the Hargreaves’ test showed a minimal increase in thermal pain threshold reaction after melatonin administration (Fig. 2) and only after 1 week from

Fig. 1. Time course of mechanical allodynia in sham operated and neuropathic rats (CCI). Effects of melatonin (100 mg/kg i.p.) on nociceptive response in CCI rats. Mechanical threshold measured by Dynamic Plantar Aesthesiometer is expressed as grams. Data represent mean ±S.D. of 6 rats. The most severe mechanical allodynia was observed after 2 weeks from the right sciatic nerve ligation procedure. CCI – chronic constriction injury model; MT – melatonin. # p<0.05, ## p<0.001, (sham vs. CCI); * p<0.05, * p<0.001, (CCI vs. CCI+MT).

Fig. 2. Time course of thermal hyperalgesia in sham operated and neuropathic rats (CCI). Effects of melatonin (100 mg/kg i.p.) on nociceptive response in CCI rats. Withdrawal latency to heat measured by plantar test is expressed as seconds. Data represent mean ±S.D. of 6 rats. The most severe thermal hyperalgesia occurred after 1 week from the surgical CCI procedure. CCI – chronic constriction injury model; MT – melatonin. # p<0.05, (sham vs. CCI); * p<0.05, (CCI vs. CCI+MT).
the right sciatic nerve ligation procedure the obtained results were of statistical importance.

After intraperitoneal administration of naloxone, a non-selective opioid receptor antagonist and then melatonin, there we observed the inhibition of antinociceptive action of melatonin in the von Frey’s test that suggests that melatonin takes its antinociceptive action via the opioid system activation (Fig. 3). There was no such effect observed in the thermal hyperalgesia evaluation. Prazosin, the MT3 receptor antagonist, does not play a statistically important role in melatonin’s antinociceptive action potential. Intraperitoneal administration of luzindole completely inhibits the antinociceptive effects of melatonin, especially in the von Frey’s test, and this leads to the conclusion that melatonin plays its role via the MT1 and MT2 receptors pathway (Fig. 4). After intraperitoneal administration of picrotoxin (a noncompetitive antagonist for the GABA_A receptor), as well as flumazenil (benzodiazepine’s antagonist), the inhibition of melatonin’s antiallodynic effects was observed that suggests the benzodiazepine - GABA-ergic mechanism of its action (Fig. 3). Intraperitoneal application of flumazenil and picrotoxin alone had no effect on tactile allodynia in CCI rats (n=6; our preliminary study, data not shown).

**DISCUSSION**

Our results confirm prior study data that the exogenous melatonin has antinociceptive effects (16, 17). Those effects are seen mainly in the case of the mechanical pain response threshold, whereas no changes are seen in the process of thermal hyperalgesia. The experimental results suggest that melatonin can influence the pain threshold via the modulation of the opioid system. Naloxone, a nonselective opioid receptors’ antagonist, almost completely inhibited the antinociceptive effects of melatonin, causing the decrease in the pain threshold and the increase of the mechanical pain sensitivity. However this effect was not observed in the thermal hyperalgesia. Prazosin, a MT3 receptor inhibitor, does not affect the antinociceptive properties of melatonin. While the intraperitoneal administration of luzindole almost completely inhibited the antinociceptive effect of melatonin, especially in the test evaluating the mechanical allodynia, which leads us to conclude that melatonin plays its antinociceptive role via the MT1 and MT2 receptors’ activation pathway. The MT1/MT2 receptor’s agonists could become a potential therapeutically important option in the pain treatment strategies.

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**Fig. 3.** Behavioural response to von Frey filaments (g) in various experimental groups in 14th day. The influence of naloxone (1 mg/kg i.p.), picrotoxin (1 mg/kg i.p.) and flumazenil (0.01 mg/kg i.p.) on melatonin-induced antiallodynic effects. Data represent mean ±S.D. of 6 rats. Statistical differences between groups were tested using one-way ANOVA, followed by Tukey’s test. Statistical significance was considered to be achieved when p<0.05.

CCA – chronic constriction injury model; MT – melatonin; NX – naloxone; PC – picrotoxin; FZ – flumazenil. ## p<0.001; * p<0.05; ** p<0.001.

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**Fig. 4.** Behavioural response to von Frey filaments (g) in various experimental groups in 14th day. The influence of prazosin (10 mg/kg i.p.) and luzindole (10 mg/kg i.p.) on antiallodynic effects of melatonin. Data represent mean ±S.D. of 6 rats. Statistical differences between groups were tested using one-way ANOVA, followed by Tukey’s test. Statistical significance was considered to be achieved when p<0.05.

CCA – chronic constriction injury model; MT – melatonin; PZ – prazosin; LZ – luzindole. ## p<0.001; * p<0.05; ** p<0.001.
Ulugol et al. showed the opposite results concerning the antinociceptive properties of melatonin (20). According to their data melatonin was efficient in the thermal hyperalgesia, whereas had no such effect in the mechanical allodynia. The possible explanation could be the fact, that the neuropathic pain experimental model in that case was different (Seltzer’s model) (21), or the fact that the experimental animals were mice. However, it is interesting that the other authors (22) did not observe the thermal hyperalgesia but only the mechanical allodynia using the same as Ulugol et al. model of neuropathic pain. Furthermore, our results agree with evidence showing that oral and intrathecal administration of melatonin is able to reduce tactile allodynia in a different model of neuropathic pain (23). The significant influence of melatonin on the mechanical allodynia process, the absence of such an effect on the process of thermal hyperalgesia, and lack of influence of naloxone on those processes, can implicate that the different pathomechanisms of melatonin’s action may be involved. The potential explanation is that the influence of melatonin on the process of thermal hyperalgesia is based rather on its anti-inflammatory effect than on the opioid system activation, like in the process of mechanical allodynia. The anti-inflammatory effects of melatonin are well defined and proved (9, 10, 24). Melatonin inhibits the synthesis of the pro-inflammatory cytokines, such as TNF-α and also inhibits the cyclooxygenase pathway (25). Recent data suggests that the main pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, are important mediators of the processes of the allodynia and hyperalgesia, as being the main symptoms of neuropathic pain (26). Moreover in Bennett’s model, in the ligated sciatic nerve, after 7 days there was increased expression of IL-1β and IL-6 and after 14 days the increased expression of TNF. Then, TNF together with IL-1 and IL-6 maintains the nociceptive processes (27). The presence of the peripheral mechanisms of melatonin’s action points at the fact that its systemic administration (like in our experiment) and not only the central one, is diminishing the hyperalgesia process (17, 28).

Neuropathic pain evolves as a result of impaired nociceptors’ functions and severed nociceptors’ pathways due to injury to the peripheral nerves, nerves roots, plexus, or changes in the central nervous system. (26). As a cause of continuously conducted peripheral stimuli secondary changes in the spinal cord are observed, the central sensitization that leads to the increased intensification and consolidation of the pain process in the spinal cord. In the process of the spinal sensitization, NMDA receptor (that are responsible for the pain consolidation and its opioid poor sensitivity) activation is of main importance. Melatonin’s antinociceptive effects could also arise as a result of its interaction with the NMDA receptors and the NO pathway (29). In the dorsal horn of the lumbar and thoracic segments of the spinal cord there was shown the presence of the MT1 and MT2 receptors (30).

The opioid and GABAergic systems also play a role in the antinociceptive properties of melatonin. Our results indicate that melatonin-induced systemic antinociception can be blocked by intraperitoneal administration of the non-selective opioid receptor antagonist naloxone. It was reported that melatonin does not take its action directly via the opioid receptors (31). There is also evidence suggesting that one of the main mechanisms of the analgesic effects of melatonin is the increase of the release of the endogenous β-endorphins in the central nervous system (31). Yu et al. (17) demonstrated that melatonin’s administration induces the release of beta-endorphin in periaqueductal gray in rats. Then, β-endorphin could bind to μ or κ opioid receptors in order to produce its effect. (31) The endogenous opioid system is involved in the regulation of the experience of pain, and in the action of analgesic opiates drugs. The present study suggests an important role of the opioid naloxyne-sensitive pathway in melatonin-induced antinociception.

Melatonin could also exert its central action via the modulation of the GABA A receptors in the spinal cord (4, 32). Some reports have shown that melatonin modulates function of benzodiazepine binding site on the GABA A receptor complex (4). We demonstrated that antialloednic effect of melatonin is reversed by flumazenil and picrotoxin, indicating that melatonin’s antinociceptive action involves GABA A-benzodiazepine receptors. Melatonin may act directly on MT2 subtype of melatonin’s receptor, playing its analgesic role by modulating the GABA A receptor functions as demonstrated by Wan and co-workers (33). On the other hand, Dhanraj et al. (34) suggest that melatonin shows GABA A receptor-selective synergism with a benzodiazepine-like agonist action and this pathway seem to be melatonin’s receptors independent. Taking these findings under consideration, it can be proposed that some of melatonin’s actions are present because of the direct interaction with GABA A receptor.

Neuropathic pain is being considered as an important therapeutic problem; there is no efficient therapeutic strategy of its treatment. Moreover, this specific type of pain is usually insensitive to pharmacologic treatment options, including opioids. Besides, the antinociceptive effects of opioids are connected with multiple, serious side-effects that sets measures to their therapeutic possibilities. To reduce those side effects, opioids are often used together with other pharmacologic substances. Melatonin could have its therapeutic value as a potent new efficient co-analgesic.

Classic therapeutic strategies are not efficient in the neuropathic pain management, therefore new options are needed to alleviate pain. Melatonin could potentially be added to this class of drugs with its peripheral (anti-inflammatory) and central (activation of the endogenous opioid system and benzodiazepine-GABAergic pathway) action.

Conflict of interests: None declared.

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