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The periaqueductal gray contributes to bidirectional enhancement of antinociception between morphine and cannabinoids

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Abstract

Co-administration of opioids and cannabinoids can enhance pain relief even when administered on different days. Repeated systemic administration of morphine has been shown to enhance the antinociceptive effect of tetrahydrocannabinol (THC) administered 12 hours later, and repeated microinjection of the cannabinoid receptor agonist HU-210 into the ventrolateral periaqueductal gray (PAG) has been shown to enhance the antinociceptive effect of morphine administered one day later. The primary objective of the present study was to test the hypotheses that this cannabinoid/opioid interaction is bidirectional. Experiment 1 showed that microinjection of morphine into the ventrolateral PAG of male Sprague-Dawley rats twice daily for 2 days enhanced the antinociceptive effect of HU-210 measured one day later. In Experiment 2, twice daily systemic injections of THC enhanced the antinociceptive effect of morphine administered one day later. These results complement the previously mentioned studies by showing that morphine and cannabinoid interactions are bidirectional and that the ventrolateral PAG plays an important role in this effect. In contrast to the PAG, repeated administration of HU-210 or the cannabinoid receptor agonist, WIN 55,212-2, into the RVM had a neurotoxic effect. Rats became ill following repeated cannabinoid administration whether given alone or with morphine. Presumably, this neurotoxic effect was caused by the high cannabinoid concentration following RVM microinjection because rats did not become ill following repeated systemic THC administration. These findings indicate that alternating opioid and cannabinoid treatment could produce a longer lasting and more potent analgesia than either compound given alone.

Keywords

Cannabinoid; Opioid; Periaqueductal gray; Rostral ventromedial medulla; Analgesia; Morphine

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1. Introduction

Opioids such as morphine are the most effective treatment for most types of pain. Unfortunately, the use of opioids is limited by side effects, the potential for addiction, and the development of tolerance (Morgan and Christie, 2011). One promising approach to enhance the antinociceptive effect of opioids is combination drug treatment in which low doses of two different types of analgesics, such as opioids and cannabinoids, are administered. Although the antinociceptive effects of cannabinoids are relatively mild (Frank et al., 2008, Hohmann et al., 1999), pretreatment with a nonantinociceptive dose of tetrahydrocannabinol (THC) has been shown to cause up to a 22-fold enhancement of morphine potency (Smith et al., 1998). This enhanced antinociception has been shown following oral, dermal, intrathecal, intracranial ventricular and systemic injections and with a number of different species, opioids, and cannabinoids (Cichewicz et al., 1999, Cichewicz et al., 2005, Cox et al., 2007, Finn et al., 2004, Smith et al., 1998, Welch and Stevens, 1992, Welch et al., 1995).

In addition to the synergy produced by simultaneous administration of an opioid and cannabinoid (Cichewicz, 2004, Cichewicz and McCarthy, 2003, Cox et al., 2007), the antinociceptive effects of cannabinoids are enhanced even when administered after morphine antinociception has dissipated (Williams et al., 2006). This is particularly true in morphine-tolerant animals. Mice made tolerant to morphine showed enhanced antinociception to oral THC when assessed 12 hours after the last morphine injection (Cichewicz and Welch, 2003, Rubino et al., 1997). The opposite is also true: Repeated microinjection of the CB receptor agonist HU-210 into the ventrolateral periaqueductal gray matter (PAG) enhances the antinociceptive effect of morphine microinjected 16 hours later (Wilson et al., 2008). These findings are particularly interesting because they suggest long lasting and bidirectional enhancement of antinociception between opioids and cannabinoids.

If antinociceptive enhancement between opioids and cannabinoids is bidirectional, then repeated systemic administration of THC should enhance morphine antinociception, and repeated morphine microinjection into the ventrolateral PAG should enhance cannabinoid antinociception. Given that the PAG is part of a descending nociceptive modulatory system that relays in the rostral ventromedial medulla (RVM) (Morgan et al., 2008), cannabinoid/opioid enhancement also would be expected in the RVM. These hypotheses were tested by pretreating rats with an opioid or cannabinoid and then assessing the antinociceptive potency of the other compound one day later.

2. Material and Methods

2.1 Subjects

Male Sprague-Dawley rats (210–340 g; Harlan, Livermore CA) were housed 8 per cage (61 × 43.5 × 21.5 cm) with *ad libitum* food and water except during injection and testing procedures. Rats were maintained on a reverse 12 hr light/dark cycle (on at 19:00) so testing could be conducted during the active dark phase. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Washington State University.

2.2 Surgery

Rats (210–321 g; Harlan, Kent WA) were anesthetized with equithesin (60 mg/kg, i.p.) and stereotactically implanted with a 23 gauge (0.573 mm) stainless steel guide cannula aimed at the ventrolateral PAG (9 mm long, AP +2.3 mm; ML –0.6 mm; DV –4.6 mm from lambda)

or RVM (12 mm long, AP -2.2 mm; ML 0.0 mm; DV -8.8 mm from lambda). The guide cannula was held in place with dental cement affixed to two screws in the skull. Following surgery, a removable stainless steel stylet was inserted into the guide cannula, and the rat was housed individually with food and water available *ad libitum*. Lights were maintained on a reverse 12 h light/dark cycle so all injections and testing occurred during the dark active phase. Rats were handled daily before and after surgery. Behavioral testing began one week following surgery.

2.3 Microinjections

The day before the first microinjection an injection cannula that extended 2 mm beyond the guide cannula was inserted, but no drug was administered. This procedure habituates rats to the injection procedure and reduces potential confounds on the test day resulting from mechanical damage to neurons.

Drugs were injected into the PAG or RVM through a 31-gauge (0.226 mm) injection cannula that extended 2 mm beyond the tip of the guide cannula. The injection cannula was connected to a 1 µl syringe (Hamilton Co., Reno, NV) with PE20 tubing filled with sterile water. All microinjections were administered in a volume of 0.4 µL over 40 s while the rat was gently restrained. The injection cannula remained in place an additional 20 s after the injection to minimize backflow of the drug up the cannula tract. Following the injection, the stylet was reinserted into the guide cannula and the rat was returned to its home cage.

2.4 Drugs

Drug doses and concentrations were selected based on previous studies (Finn et al., 2003, Morgan et al., 2005). For systemic injections, morphine sulfate (a gift from the National Institute on Drug Abuse) was dissolved in saline. The cannabinoid receptor agonist delta-9-tetrahydrocannabinol (THC) was dissolved in 50% ETOH, 25% DMSO and 25% saline. Drugs were injected subcutaneously in a volume of 2 ml/kg, and control groups received equivalent injections of vehicle (50% ETOH, 25% DMSO and 25% saline, s.c.). For microinjections, the CB1 receptor agonists HU-210 (Tocris, St. Louis, MO) and WIN 55,212-2 (Cayman Chemical, Ann Arbor, MI) were dissolved in ethanol, Tween 80, and saline in a 1:1:8 concentration. Control groups received microinjections of saline or vehicle (ethanol: Tween 80: saline) depending on the experiment. Each rat was used for only one experiment, and all rats within an experiment received the same number of injections regardless of treatment.

2.5 Behavioral Tests

Nociception was assessed using the hot plate test. The hot plate test measures the latency for a rat to lick a hind paw when placed on a 52.5°C surface. The rat was removed from the hot plate immediately following a response, or after 50 s if no response occurred. The experimenter conducting the behavioral tests was blind to the pretreatment condition.

2.6 Experiment 1 - PAG

Our previous research showed that repeated microinjection of the CB receptor agonist HU-210 into the ventrolateral PAG enhanced morphine antinociception measured one day later. The objective of the present experiment was to test the opposite hypothesis: Do repeated morphine microinjections into the ventrolateral PAG enhance the antinociceptive effect of microinjecting HU-210 one day later?

Morphine (5 g, N = 9) or saline (N = 7) was microinjected into the ventrolateral PAG twice daily (0900 and 1600 h) for two days (Trials 1–4). This repeated administration protocol

produces tolerance to morphine (Morgan et al., 2006). Hot plate latency was assessed 30 after the first injection, but not after subsequent microinjections to limit the development of behavioral tolerance (Gamble and Milne, 1989; Lane & Morgan, 2005). On the third day (Trial 5), cannabinoid potency was assessed by cumulative microinjections of HU-210 into the ventrolateral PAG resulting in cumulative doses of 5, 10, and 20 $\mu\text{g}/0.4 \mu\text{L}$. HU-210 was injected every 20 min, and the hot plate test was conducted 15 min after each microinjection. Problems with HU-210 solubility prevented the use of concentrations higher than 10 $\mu\text{g}/0.4 \mu\text{L}$.

2.7 Experiment 2 – Systemic

Repeated oral administration of morphine has been shown to enhance the antinociceptive effect of THC administered 12 hours later (Cichewicz and Welch, 2003). The objective of the present experiment was to test the opposite hypothesis: Do repeated systemic injections of THC enhance the antinociceptive effect of morphine administered one day later?

Rats ($N = 8/\text{group}$) received twice-daily injections of either THC (20 mg/kg) or vehicle (injections given at 0900 and 1600) for two consecutive days. Nociception was assessed before and 60 min after the first injection. No additional behavioral testing was done until Day 3 to minimize behavioral tolerance caused by repeated testing on the hot plate (Gamble and Milne, 1989, Lane and Morgan, 2005). The effect of chronic THC exposure on morphine antinociception was tested 18 hours after the last pretreatment injection using a cumulative dosing procedure. Cumulative doses of morphine were given 20 min apart resulting in quarter log doses of 1.8, 3.2, 5.6, 10.0, and 18.0 mg/kg. Nociception was assessed using the hot plate test 15 min after each injection.

2.8 Experiment 3 – RVM

The RVM is part of the descending nociceptive modulatory system, receiving input from the PAG and projecting to the spinal cord (Morgan et al., 2008). Both opioids and cannabinoids produce antinociception when injected into the RVM (Barbaro et al., 1986, Heinricher et al., 1994, Martin et al., 1998, Meng and Johansen, 2004). Thus, the objective of this experiment was to test the hypothesis that repeated cannabinoid injections into the RVM will enhance morphine antinociception measured one day later as has been reported in the ventrolateral PAG (Wilson et al., 2008).

Rats were injected with the cannabinoid receptor agonists HU-210 (5 $\mu\text{g}/0.5 \mu\text{L}$, $N = 16$), WIN 55,212-2 (5 $\mu\text{g}/0.5 \mu\text{L}$, $N = 21$), vehicle ($N = 37$), morphine (5 $\mu\text{g}/0.5 \mu\text{L}$, $N = 17$), or a morphine/CB1 receptor agonist combination (either HU-210 or WIN 55,212-2, $N = 21$). Injections were given twice a day (0930 & 1600) for two days (Trials 1 – 4). Rats receiving combined opioid/cannabinoid injections received both drugs in a single injection. Nociception was assessed using the hot plate test 30 min after the first injection on Day 1. No testing was conducted following injections on Trials 2 – 4. The antinociceptive effect of cumulative doses of morphine microinjected into the RVM was assessed 18 hrs after the last pretreatment injection.

2.9 Histology

Rats were euthanized following testing by exposure to a lethal dose of Halothane. For rats with an intracranial cannula, the brain was removed and placed in formalin (10%). At least 2 days later, the brain was sectioned coronally (100 μm) and the location of the injection site was identified (Paxinos and Watson, 2005).

2.10 Data Analysis

ANOVA (SPSS) was used to compare mean differences in baseline hot plate latency, open field activity, and RVM lesion size. Dose-response curves were generated for hot plate data using nonlinear regression (Graph Pad Prism). The half-maximal effective dose (D_{50}) for antinociceptive was calculated for each condition (Tallarida, 2000). Differences in potency between groups were compared using ANOVA and 95% confidence intervals. Statistical significance was defined as a probability of less than .05.

3. Results

3.1 Experiment 1 - PAG

Only rats with microinjection placements in or immediately adjacent to the ventrolateral PAG were included in data analysis (Figure 1). Microinjection of morphine ($5 \mu\text{g}/0.4 \mu\text{l}$) into the ventrolateral PAG (Trial 1) increased hot plate latency to the cut-off threshold of 50 s in 8 out of 9 rats. Mean hot plate latency was 48.4 ± 1.6 s compared to 13.4 ± 1.6 s for saline injected rats. Rats continued to receive morphine or saline injections on Trials 2 – 4, but were not tested. Baseline hot plate latencies on Trial 5 were not significantly different for morphine (17.3 ± 1.6 s) and saline-pretreated (15.0 ± 1.7 s) groups ($t(14) = 2.14$, $p = 0.34$). Cumulative microinjections of HU-210 in salinepretreated rats produced mild antinociception (Figure 2). The highest dose of HU-210 (20 g) resulted in a mean hot plate latency of only 22.3 s (20.9% of the maximum possible effect; MPE). In contrast, microinjection of the same dose of HU-210 into the PAG of rats given morphine on the previous two days produced a significantly higher mean hot plate latency (36.8 ± 5.4 s; MPE of 60.0%) ($t(14) = 1.76$, $p = 0.04$).

3.2 Experiment 2 – Systemic

Injection of THC (20 mg/kg, s.c.) on Trial 1 produced hot plate latencies that were similar to rats injected with an equivalent volume of vehicle (11.4 ± 0.8 vs. 9.9 ± 0.9 s; $t(14) = 1.274$, $p = 0.22$). Rats also were injected with THC or vehicle on Trials 2 – 4, but were not tested. On Trial 5, systemic injections of morphine caused a dosedependent increase in hot plate latency in both THC and vehicle-pretreated rats (Figure 3). Morphine potency was greater in rats injected with THC ($D_{50} = 3.7$ mg/kg) on the previous two days compared to rats pretreated with saline ($D_{50} = 7.7$ mg/kg). This leftward shift in the morphine dose-response curve for THC pretreated rats was significantly different than for vehicle-pretreated rats ($F(1,76) = 21.83$, $p < 0.0001$).

3.3 Experiment 3 – RVM

Repeated microinjection of the cannabinoid agonists HU-210 or WIN 55,212-2 produced profound catalepsy, illness, and lethargy in many animals. These effects interfered with nocifensive behavior and led to the removal of many animals from the study. In total, 59% of the rats did not complete all four of the pretreatments with the cannabinoid. In contrast, only 7% of the rats receiving repeated microinjections of morphine or vehicle into the RVM did not complete the full test sequence (Table 1). Histological examination of the microinjection sites in some of these rats revealed significantly larger lesions in cannabinoid-pretreated rats than in vehicle-pretreated rats ($t(9) = 1.83$, $p < .05$). Similar neural damage was not evident in Experiment 1 above or in our previous study in which rats received repeated injections of the same dose of HU-210 into the ventrolateral PAG (Wilson *et al.*, 2008). The behavioral problems associated with these lesions prevented assessment of changes in morphine antinociception.

Trial 1 data were analyzed for the subset of rats that made it to Trial 5 without behavioral confounds (WIN 55,212-2 (N = 6), HU-210 (N = 4), vehicle (N = 7), morphine (N = 6), morphine+HU-210 (N = 2), morphine+WIN 55,212-2 (N = 3)). Acute microinjection (Trial 1) of either 5 µg of a CB1 receptor agonist (HU210 and WIN 55,212-2) or morphine were insufficient to produce an antinociceptive effect that was greater than vehicle alone. The mild antinociceptive effect of morphine microinjection into the RVM (compared to the PAG) is consistent with previous findings (Morgan and Whitney, 2000). However, rats that received the combination CB1 receptor agonist/morphine injection demonstrated significant antinociception (Figure 4). The antinociceptive effect of co-administration of the CB1 receptor agonists and morphine was greater than that of injecting either drug alone ($F(3,22) = 9.5, p < 0.05$).

4. Discussion

Previous research shows that systemic THC administration enhances the antinociceptive effect of morphine when administered 12 hours later (Cichewicz and Welch, 2003) and repeated microinjection of a cannabinoid agonist into the ventrolateral PAG enhances morphine antinociception measured one day later (Wilson et al., 2008). The present data extend these findings by showing that both of these effects are bidirectional (Table 2). That is, systemic administration of THC enhances the antinociceptive effect of morphine measured one day later, and repeated morphine microinjection into the ventrolateral PAG enhances the antinociceptive effect of microinjecting a CB1 receptor agonist into the ventrolateral PAG. Whether opioids and cannabinoids interact in a similar manner in the RVM is not clear because repeated microinjections of CB1 receptor agonists into the RVM made rats ill as a result of RVM lesions.

There are numerous reports of enhanced antinociception following coadministration of opioids and cannabinoids (Welch and Stevens, 1992). This enhanced antinociception occurs following oral, dermal, intrathecal, intracranial ventricular and systemic injections (Cichewicz et al., 1999, Cichewicz et al., 2005, Cox et al., 2007, Finn et al., 2004, Smith et al., 1998, Welch and Stevens, 1992, Welch et al., 1995). Isobolographic analysis reveals that the antinociception produced by co-administration of opioids and cannabinoids is synergistic (Cichewicz and McCarthy, 2003, Roberts et al., 2006, Tham et al., 2005). The main difference between these studies and the present data is that here, morphine and cannabinoid receptor agonists were injected on different days, which suggests the effects are due to long-lasting adaptations in ventrolateral PAG neurons. Nonetheless, it is possible that the enhanced antinociception in the present study is caused by the presence of residual drug from the previous day. However, enhanced antinociception was evident even when HU-210 was microinjected into the ventrolateral PAG 16 hours after the last morphine injection. Microinjection of morphine into the ventrolateral PAG produces antinociception that lasts approximately 90 minutes (Bobeck et al., 2009), so the presence of residual morphine in the PAG 16 hours after a microinjection of 5 g of morphine is unlikely.

The fact that this enhancement of antinociception occurs following systemic or PAG administration suggests that neural adaptations in the ventrolateral PAG at least partially underlie this enhancement. Although the mechanism for this enhanced antinociception is not known, our recent anatomical analysis showing that CB1 receptors are found on approximately one third of the PAG neurons with mu-opioid receptor labeling (Wilson-Poe et al., 2012) suggests that cannabinoid/opioid interactions are mediated by adaptations to intracellular signaling pathways in neurons expressing both receptors. Both CB1 and mu-opioid receptors activate potassium channels, inhibit Ca⁺⁺ channels, inhibit cAMP activation, and alter other downstream signaling pathways such as c-Jun N-terminal kinase (Connor and Christie, 1998, Fan et al., 2003, Howlett, 1995, Howlett et al., 2010, Ingram et

al., 1998, Pertwee, 1997, Rueda et al., 2000, Vaughan and Christie, 1997). Thus, it is plausible that chronic drug treatment-induced adaptations in one receptor signaling cascade could result in compensatory changes in the signaling cascade of the other receptor.

The role of the PAG in this enhancement is noteworthy in that the antinociceptive effects of cannabinoids microinjected into the PAG are relatively mild both here and in previous studies (Lichtman et al., 1996, Martin et al., 1995). In contrast, the antinociceptive effect of microinjecting morphine into the PAG is profound, but tolerance develops rapidly to morphine antinociception (Morgan et al., 2006, Tortorici et al., 1999). Thus, alternating administration of opioids and cannabinoids could overcome both of these problems by enhancing the cannabinoid potency and reversing morphine tolerance.

Similar long-lasting changes do not appear to occur in RVM neurons. The primary effect of repeated cannabinoid administration in the RVM was cell death. Cell death occurred with repeated administration of either HU-210 or WIN 55,212-2 whether administered alone or with morphine (see Table 1). This finding is consistent with other reports of neurotoxic effects following repeated administration of cannabinoid receptor agonists (Lawston et al., 2000, Scallet, 1991). CB1 receptor activation has been shown to reduce cell proliferation and initiate cell death signaling cascades via mitogen activated protein kinases (Mukhopadhyay et al., 2010, Yang et al., 2010). The cannabinoid system also stimulates the release of tumor necrosis factor-alpha, which can trigger apoptotic cell death (De Laurentiis et al., 2010). Although neurotoxic effects of cannabinoids have not been examined in the PAG or RVM previously, our data indicate that neurons in the RVM are particularly susceptible especially with the high doses neurons are exposed to as a result of intracranial microinjection. However, there was no evidence of illness or lesions following repeated microinjections of the same dose of HU-210 into the ventrolateral PAG (Wilson et al., 2008).

Acutely however, co-administration of opioids and cannabinoids into the RVM resulted in greater antinociception than administration of either drug alone. Synergistic interactions between opioids and cannabinoids have been reported previously following systemic administration (Cichewicz and McCarthy, 2003, Roberts et al., 2006, Tham et al., 2005). Although morphine/cannabinoid interactions in the RVM have not been examined previously, microinjection of cannabinoid receptor agonists into the RVM have been shown to produce antinociception (Martin et al., 1998, Meng and Johansen, 2004). This antinociception appears to be mediated by inhibition of GABAergic neurons (Meng et al., 1998, Vaughan et al., 1999), as has been reported for morphine antinociception (Heinricher and Tortorici, 1994, Pan et al., 1990). Thus, enhanced GABAergic inhibition in the RVM may be one mechanism involved in the acute synergy between opioids and cannabinoids.

Recent clinical data suggest that combined opioid/cannabinoid therapy is a viable option for efficacious pain relief, particularly in patients already undergoing opioid therapy (Abrams et al., 2011). Determining the neural mechanisms underlying bidirectional enhanced antinociception between opioids and cannabinoids is essential in developing drug and treatment protocols that safely and effectively manage pain. The results of this study demonstrate that in the case of cannabinoids, mechanisms that enhance antinociception and limit neurotoxicity are critical. The present study also demonstrates that the PAG is at least partially responsible for enhanced cannabinoid antinociception in morphine tolerant rats. In contrast, RVM neurons appear to be particularly sensitive to the neurotoxic effects of cannabinoids.

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Abbreviations

PAG	periaqueductal gray
RVM	rostral ventromedial medulla
MPE	maximal possible effect
CB	cannabinoid

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Highlights

- PAG mediated cannabinoid antinociception is enhanced in morphine tolerant rats
- Systemic morphine antinociception is enhanced following repeated THC administration
- Repeated microinjection of cannabinoids into the RVM is neurotoxic
- The enhanced antinociception from opioid/cannabinoid interactions is bidirectional

Figure 1. Location of microinjection sites in and immediately adjacent to the ventrolateral PAG. There is no difference in the location of microinjection sites for rats pretreated with saline (□) or morphine (■). Injection sites are plotted on coronal sections of the midbrain anterior to the interaural line (Paxinos and Watson, 2005).

Figure 2.

Enhanced cannabinoid antinociception in the PAG of morphine tolerant rats. Microinjection of the CB1 receptor agonist HU-210 into the PAG produced minimal antinociception in vehicle-pretreated rats, but significantly greater antinociception in rats pretreated with morphine into the PAG twice a day for 2 days ($*p < 0.05$). Cumulative doses of HU-210 were administered one day after the last morphine injection.

Figure 3. Enhanced morphine antinociception following repeated systemic administration of THC. Administration of cumulative doses of morphine caused a dose-dependent increase in hot plate latency in both pretreatment groups. However, morphine potency was enhanced in THC pretreated rats even though morphine was injected 16 hours after the last of four THC injections.

Figure 4.

Hot plate latency following an acute microinjection of a CB1 receptor agonist and/or morphine into the RVM. Microinjection into the RVM of CB1 receptor agonists (HU-210 or WIN 55,212-2) combined with morphine caused an antinociceptive effect that was greater than administration of either drug alone ($*p = 0.05$). Rats that became sick with repeated injections were not included in this analysis so as not to confound neurotoxic and receptor mediated effects.

Table 1

Number of rats removed from study because of illness

Pretreatment	Total # of Rats	# of Rats excluded	% excluded
WIN 55,212-2	21	10	48%
WIN+Morphine	14	8	57%
HU-210	16	11	69%
HU-210+Morphine	7	5	71%
Total CBI treated rats	58	34	59%
Morphine	17	1	6%
Vehicle	37	3	8%

Table 2

Enhanced antinociception between opioids and cannabinoids is bidirectional

Pretreatment drug	Test drug (12–16 hours later)	Administration site	Citation
Hu-210	Morphine	PAG	Wilson et al., 2008
Morphine	Hu-210	PAG	see Figure 2
Morphine	THC	Oral	Cichewicz and Welch, 2003
THC	Morphine	Subcutaneous	see Figure 3