ISSN 0370-2731/2002 Proceedings of the New Zealand Society of Animal Production 62: 368-371

Reproductive state differences in analgesic related systems and their influence on learning behaviours

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ABSTRACT

Kappa-opioids exert a more powerful analgesic effect in females than in male animals. In females, this is further enhanced close to and immediately post parturition. Learning behavior is also altered in the female around this period. Recently it was demonstrated that changes in a brain receptor subunit (part of the glutamate NMDA receptor present in all mammalian brains), termed NR2B, accompanied these periods of apparent learning enhancement. In this paper, evidence is presented for a dependence of this increase in NR2B and learning ability on Kappa-opioid systems. Pharmacological antagonism of the Kappa-opioid system during the peri-parturient period prevents both an increase in NR2B and in learning behaviour. Application of Kappa-opioids at other periods increased NR2B numbers and learning behaviour in female animals and in male castrates co-treated with oestrogen. Intact males were unaffected. The data suggests a new concept linking pain perception and processing systems to reproductive state behaviours and learning abilities.

Keywords: steroidal hormones; pain; NMDA receptor; opioids.

INTRODUCTION

Clinically, opioids that influence mu (m) receptors are regarded as better analgesics than those primarily influencing Kappa (K) receptors (Bhargava, 1994; Reisine & Pasternak, 1996). Kappa opioids, however, are far less suppressive of general behaviour than m-opioids (Bhargava, 1994). Gender-related differences in analgesic effect have been reported in humans (Gear *et al*., 1996) and in sheep (Cook, 1997) with K-opioids showing greater efficacy in females, m-opioids in intact males, and with wethers demonstrating an intermediate point between the females and intact males. This difference appears further enhanced by reproductive state (Cook, 1999) with ewes exhibiting greater analgesic effects to K-opioids during oestrus, late-pregnancy and the immediate post-parturient period. These reproductive state differences have also been observed in female rats and mice (Cook, in press).

Behavioural indices, particularly food consumption and gathering, exploratory activity and fearlessness are also increased during oestrus and post-parturient periods as is spatial task learning ability (Cook, in press; Cook, unpublished data).

Work in rodents (Tang *et al*., 1999) has demonstrated that transgenic over-expression of the NR2B subunit, part of the glutamate NMDA receptor complex expressed at excitatory synapses throughout the central nervous system of all mammals (Collingridge & Bliss, 1995), markedly increased performance on learning and memory related behavioural tasks. However, overexpression of NR2B also produces an enhancement of inflammatory pain (Wei *et al*., 2001). Cook (in press) recently demonstrated that in non-transgenic animals the NR2B sub-unit expression falls off in neonates post-natally, reaching its nadir around 21 days of post-natal age in rats and mice, and that this level was predictive of adult learning performance. This final obtained level can be altered by manipulation during the first 21 days of postnatal life, decreased by painful stimuli and increased by K-opioid (but not μ-opioid) analgesics (Cook, in press).

Further work (Cook, unpublished data) has demonstrated that in adult female rats, NR2B expression varies with reproductive state increasing at both oestrus, late pregnancy and post-parturition times at which the K-opioid systems appears most sensitive. No marked variations were seen in male rats over a 6 month period.

The purpose of the study described here was to examine if:

(i) antagonism of the K-opioid system during the post-parturient period reduced both NR2B expression and the apparent state linked improvement in learning performance
(ii) administration of K-opioids increased NR2B and learning performance during non-oestrus and non-pregnant/post-parturient states, and;
(iii) if (ii) was dependent on the presence of oestrogens.

METHODS

Animals

Adult Sprague-Dawley rats of similar age (females live weights 290-350 g, intact males live weights 320-390 g, castrate males live weights 280-360 g) were housed as doublets with *ad libitum* access to both water and pelleted feed. Housing was environmentally controlled at 21°C with a 12 h:12 h light: dark cycle. Animals were regularly handled and well familiarised with experimenter. Females were familiarized with vaginal swabbing and methylene blue staining of vaginal cells was used to establish oestrus cycles (Rugh, 1990). A group of females that had given birth to pups and were lactating were also used.

Drug administrations

Non-pregnant, non-lactating animals (females, intact and castrate males).

On day 1 of experimentation all animals received a 0.5 ml intraperitoneal injection of sterile physiological saline. These animals were then behaviourally tested (see below) on day 2. In non-pregnant, non-lactating females

these procedures were undertaken during dioestrus.

Between 5 and 7 days later, when females were again in dioestrus, one of several drug treatments was administered. The drug treatments were (i) sterile saline (ii) sterile saline containing 5 mg per kg live weight of the m-opioid agonist DAMAGO, (D-ala^2, N-Me Phe^4, Gly-ol^5)-enkephalin (iii) sterile saline containing 5 mg per kg live weight of the K-opioid agonist GR89696 (4-[(3,4-dichlorophenyl)acetyl]-3-(1-pyrrolidinynlmethyl)-1-piperazine carboxylic acid methyl ester fumurate (iv) sterile saline containing 5 mg per kg live weight soluble aspirin (v) sterile saline containing either (ii) or (iii) and 1 mg per kg live weight 17-β-oestradiol or, (vi) sterile saline containing 17-β-oestradiol only. All injected volumes were 0.5 ml. Injections were randomised amongst animals. On the day following injection all animals undertook behavioural testing. Upon completion of this, half the animals in each group were euthanised by CO_2 asphyxiation and brain levels of NR2B measured (see below).

Approximately one week later when remaining females were again in diestrous, behaviour testing was undertaken on all remaining animals. Half of these remaining animals (i.e. 1/4 of the original number) were euthanised and NR2B levels measured. Approximately four weeks after this testing, again correlating in time to a diestrous in the females, remaining animals received a second injection as above, again randomised but excluding their first treatment. Protocol was as above, but with all animals euthanised following the behavioural testing.

Lactating animals

On day two post-parturition, lactating females received one of the following drug treatments: (i) sterile saline, (ii) sterile saline containing 5 mg per liveweight DAMAGO, (iii) sterile saline containing 5 mg per liveweight GR 89696, (iv) sterile saline containing the specific m-opioid antagonist cyprodime HBr, or (v) sterile saline containing the specific K-opioid antagonist nor-binaltorphimine HCL.

On day three all animals undertook behaviour testing and half the animals in each group euthanised with NR2B measurements subsequently made.

Remaining animals undertook a repeat of this protocol, with a different drug treatment from (i) to (iv) above, on day 10 post-parturition. Following behavioural testing on day 11, all animals were euthanised and NR2B measurements subsequently undertaken.

Behavioural testing

All animals undertook behavioral training for 2 weeks prior to commencement of experimentation.

Two tests were employed.

(i) Novel object recognition task

All rats were individually habituated to an open-box (60x60x30 cm high). During training sessions, two novel objects were placed into the open box and the animal allowed to explore for 5 min. This was repeated fives times in the first week. The time spent exploring each object was recorded. During subsequent retention tests (three prior to start of experiment and then on each day following drug treatments) animals were placed back in the box, with one of the familiar objects from training and one unfamiliar novel object, for 5 min. A preference index, a ratio of the amount of time spent exploring any one of the two objects over total time spent exploring objects was used to assess recognition memory. Rats and mice following training in this protocol show retention of preferences for familiar objects.

(ii) Water-maze task

The water-maze apparatus is a circular pool (1.2 m diameter). Training procedures have been well described previously (Tsien *et al*., 1999). Training protocol consisted of 8 sessions over 2 weeks prior to start of experiment. Each training session consisted of 4 trials. The animal searches for a submerged platform in the pool and upon successful location the platform mechanically lifts the animal clear of the water. Visual cues in the maze guide the animal and the number of attempts (each 2 min duration in pool) to success indicates learning/retention on this model.

NR2B measurement

Brain tissue was removed postmortem and the forebrain cut into 40mm sections.

In situ hybridization, as has been described (Monyer *et al*., 1994) was used. Briefly antisense oligonucleotide probes, constructed against cDNA sequences for NR2B were used. The oligonucleotides were 3' end labeled using terminal deoxynucleotidyl transferase and [alpha-^{35}S] dATP. Sections were hybridised overnight at 42°C in 50% formamide, 0.6 M NaCl, 0.06 M sodium citrate, 10% dextran sulfate and 1 pg/ml of the probe.

Sections were washed in 1x SSC at 60°C for 20 min. Sections were then exposed to Kodak XAR-5 film for 21 days.

Data Analysis

Results are expressed as mean ± SEM. Statistical comparison was made using repeat measure analysis of variance (RMANOVA) with post-hoc Scheffe F-test for NR2B immunocytochemistry and the Student-Newmann-Keuls test in behavioural analysis. In all cases, $P<0.05$ was considered significant.

RESULTS

Pre-drug treatment behavioural testing

Over the course of two weeks, males (both intact and castrate) and females (other than pregnant) showed statistically similar results on both water-maze and novel object testing.

All animals (other than pregnant) showed similar time exploring both familiar objects and more time exploring the familiar object when a novel object was present during the 2-week training period. In the water maze a decreased latency to find the submerged platform was seen.

Pregnant animals significantly out-performed all other animals in learning the water-maze.

Table 1 summarises this data. Non-pregnant females showed a non-significant trend ($p<0.08$) towards

TABLE 1: Learning performance* on water-maze and novel object recognition

	Males	Females		
	Entire	Castrate	Non-pregnant	Pregnant
Water-maze				
Latency to success (s)	10.4 ± 4.1	12.3 ± 3.6	9.7 ± 4.4	3.2 ± 1.5
Number of Trials to success	2.1 ± 1.0	1.9 ± 1.4	1.9 ± 1.2	1.1 ± 0.8
Total time exploring objects	129 ± 11	108 ± 21	133 ± 15	181 ± 24
Ratio of time between objects	1:1.3 (familiar: familiar)	1:1.1 (familiar: familiarl)	1:1.1 (familiar: familiar)	1:1.2 (familiar: novel)
	1:0.7 (familiar: novel)	1:0.5 (familiar: novel)	1:0.5 (familiar: novel)	1:0.3 (familiar: novel)

*Performance estimated over the final two sessions of the two week familiarization period
Data is presented as mean
± SEM, except for ratio of time, which is the mean result only.
n = 100 for each group

TABLE 2: Effect of treatment on behavioural performance and NR2B expression.

Treatment	K-opioidGR 89696	17-β-oestradiol	GR89696 + 17-β-oestradiol
Castrate Males			
Water-maze			
Latency (s)	9.3 ± 1.4	11.1 ± 1.3	5.2 ± 1.3
No. of trials	1.4 ± 0.7	1.9 ± 0.8	1.0 ± 0.4
Novel object ratio (familiar:novel)	1.0:0.7	1.0:0.8	1.0:0.4
NR2B* expression	159 ± 14	138 ± 12	178 ± 12
Females			
Water-maze			
Latency (s)	5.2 ± 1.9	9.4 ± 1.3	3.1 ± 2.9
No. of trials	1.1 ± 0.5	1.4 ± 0.6	1.1 ± 0.4
Novel object ratio	1.0:0.6	1.0:0.8	1.0:0.4
NR2B* expression	173 ± 11	158 ± 14	249 ± 20

All other treatments did not show significant differences. The pool of this other data gave: Water-maze latency 15.5 ± 3.6s, number of trials to success 2.7 ± 0.8, Total time exploring objects 115 ± 29s, ratio of time between familiar:novel objects 1.0:1.1.
*NR2B is presented as a % compared to the pool of non-significant treatments (100% ± 7%).

improved performance on water maze and higher investigation of open box objects during proestrus and oestrus than during metoestrus and dioestrus over the two week pre-drug training period.

Drug Treatment Comparisons

Non-lactating,non-pregnant females and males.

Both behavioural testing outcomes and NR2B expression levels did not differ significantly between saline only, saline plus DAMAGO, and saline plus aspirin treatments in both males (intact and castrates) and females (in dioestrus) and intact males administered saline plus GR 89696, saline plus 17-β-oestradiol with or without either DAMAGO or GR 89696 treatments. However in all of these treatment groups, independent of treatment and treatment order, there was a small fall-off in performance on the behavioural testing over the course of experiment. This fall-off is consistent with that seen in non-treatment animals with a week between testing. Table 2 presents a summary of the above data.

In castrate males, treatment with saline plus GR89696 a small significant increase in both performance and NR2B expression was seen. In females this same treatment produced significant increases.

In both castrate males and females treatment with both 17-β-oestradiol and GR 89696 produced significant increases in both performance and NR2B expression. In females treatment with 17-β-oestradiol alone produced lesser, but still significant, increases (Table 2).

Lactating Females

On day 2, post-natally treatments with the K-opioid antagonist nor-binaltorphimine decreased both NR2B expression and behavioural performance compared to other treatment groups. Treatment with GR 89696 increased both measures relative to other treatment groups. All other treatment groups were not significantly different in effect on either parameter.

Treatments on day 10 post-natally did not differ significantly in effect on either parameter. Table 3 summarises this data.

TABLE 3: Effect of treatment on behavioural performance and NR2B expression in peri-parturient females on day 2 post-natally.

	GR-89696	Binaltorphimine
Water-maze		
Latency (s)	2.2 ± 1.1	16.5 ± 5.1
No. of trials	1.0	2.1 ± 0.8
Novel object ratio (familiar:novel)	1.0:0.2	1.0:0.9
NR2B* expression	485 ± 22	86 ± 22

All other treatment did not show significant differences. The pool of this data gave: Water-maze latency 10.9 ± 1.3 s, number of trials to success 1.4 ± 0.8, Total time exploring objects 147 ± 19 s, ratio of time between familiar:novel objects 1.0:0.9.
There were no significant differences between any treatment given on day 10 post-natal date.
*NR2B is presented as a % compared to the pool of the non-significant treatments normalized to 100% (100% ± 8%).

DISCUSSION

Previous work has suggested three concepts. The first is that in females, and castrate males, K-opioids are more potent analgesics than m-opioids (Gear *et al*., 1996; Cook, 1997). This is not the case for intact males. The analgesic efficacy of K-opioids also varies with reproductive state in females, being more effective around oestrus and in the peri-parturient period (Cook, 1998; 1999; in press). Administration of oestrogens also increase analgesic efficacy for the K-opioids (Cook, 1998). During the peri-parturient period administration of K-opioids also increase maternal care behaviour and food gathering and consumptive behaviours (Cook, 1999). Antagonists of K-opioids reduce these suggesting an active endogenous system. Co-administration of other analgesics does not prevent this reduction, suggesting the behaviours are linked to K-opioid system *per se*, rather than of necessity to states of analgesia. It is important to note on this, however, that K-opioids in females can produce a high level of analgesia without other behavioral suppression compared to achieving that level of analgesia with other agents.

The second concept is that behavioural performance on learning or memory-retention tests is greater in females around oestrus and in the peri-parturient period (Russell *et al*., 2001). Whether increased analgesia allows this to occur is not known.

The third concept is that a particular subunit in the glutamate NMDA receptor of the brain is linked strongly to certain aspects of both cognition and pain (Tang *et al*., 1999). Total expression of this subunit, NR2B appears set in early neonatal life by experiences of the environment including pain and analgesic state (Cook, in press). Although absolute expression is set during this period, during adult life variations in expression can occur and can influence con-current behavioral performance. As expression of this subunit is closely linked to perception of pain, pain clearly inhibits expression, I hypothesised that increases in analgesic state may increase expression to the upper levels set in neonatal life. In studies not presented in this paper (Cook, unpublished data), healthy entire males showed no increased in NR2B expression with administration of analgesics. However, males suffering from chronic pain, showed a decrease in NR2B expression that was reversible by any form of analgesia, provided a suitable analgesic state was achieved. In females similar results were seen, however in addition, administration of K-opioids alone, without obvious pain, increased NR2B expression. Castrate males appeared somewhat in-between the two genders and in both castrates and females, co-administration of oestrogens increased the effects of K-opioids.

In the study presented herein, the animals were not in any known (or identified) form of pain and analgesic state was not assessed. This lack of assessment limits, somewhat, interpretation of results.

However, the results strongly support the concept that K-opioids increase both NR2B expression and behavioural performance in females, but not entire males. Castrate males are somewhat influenced by the K-opioids and share with females an increase in this influence by co-administration of oestrogen.

Peri-parturient females show evidence of endogenous K-opioid system activation as antagonists of K-opioids decreased both NR2B expression and performance. Interestingly however, no effects were seen in mid-lactation.

To my knowledge, linking concepts of behavioural performance during different reproductive states and between genders to a known analgesic system is a unique finding. Care in this linkage must be taken as a number of questions must be clearly addressed. These include: (i) the dependence on actual analgesic state *per se* versus simple multiple involvement of K-opioids in diverse (not necessarily linked) behaviours, (ii) the importance (or not) of certain female behaviours (and performance) in successful reproduction and rearing and (iii) the hormonal differences (particularly testosterone) that may underlie gender differences.

Irrespective of outcome, the speculative linking of cognitive performance to a physical unit (the NMDA receptor subunit NR2B) influenced by analgesic state, reproductive state and gender is a tantalizing and interesting concept that may offer broad biological significance.

ACKNOWLEDGEMENTS

I acknowledge funding from the New Zealand Royal Society Marsden Fund for this work.

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