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Multiplicative interactions to enhance gabapentin to treat neuropathic pain

Ken-ichiro Hayashida and James C Eisenach

Department of Anesthesiology, Wake Forest University School of Medicine,, USA

Abstract

We previously reported that gabapentin activates the bulbospinal-spinal noradrenergic-cholinergic pathway to produce analgesia in rats after nerve injury. Also, gabapentin interacts synergistically with a cholinesterase inhibitor donepezil to produce analgesia. Duloxetine, a serotonin/noradrenaline re-uptake inhibitor, has been used for the treatment of neuropathic pain and should amplify the noradrenergic mechanisms recruited by gabapentin. In the present study, we determined the interaction between duloxetine and gabapentin with and without donepezil when administered by the clinically preferred oral route in rats after spinal nerve ligation. The ED₅₀ value of gabapentin, donepezil, and duloxetine to reduce mechanical hypersensitivity after nerve injury was 45, 3.7, and 32 mg/kg, respectively. In the examination of two drug combinations, oral duloxetine with either gabapentin or donepezil were additive to reduce hypersensitivity. The combination of all three drugs yielded a synergistic interaction with an observed ED₅₀ at 1/4th the predicted dose of additivity, likely due to the gabapentin-donepezil interaction. This three drug combination did not affect motor coordination or show signs of sedation in the rotarod test. Analgesia by the combination of these three drugs was reversed by intrathecal injection either of the α_2 -adrenoceptor antagonist idazoxan or by the muscarinic receptor antagonist atropine. These results suggest that the combination of these drugs, which stimulate and augment the bulbospinal-spinal noradrenergic-cholinergic pathway, lowers the dose requirement for each drug to reduce hypersensitivity after nerve injury without sedative effects. The current study provides the rationale for clinical study of the combination of gabapentin, donepezil and duloxetine to treat neuropathic pain.

Keywords

Neuropathic pain; descending inhibition; gabapentin; donepezil; duloxetine

1. Introduction

Neuropathic pain following nerve injury, whether from physical, metabolic, infection or other causes, often responds poorly to traditional analgesics. The anti-epileptic agent gabapentin has demonstrated uniform analgesic efficacy in animal models of neuropathic pain (Chen and Pan, 2005; Hayashida et al., 2007b; Pan et al., 1999; Tanabe et al., 2005) and in patients with chronic pain (Laird and Gidal, 2000). Recently, we and others have shown that gabapentin acts on supraspinal structures to stimulate a bulbospinal-spinal noradrenergic-cholinergic pathway in

Corresponding author: Ken-ichiro Hayashida, Department of Anesthesiology, Wake Forest University School of Medicine, Medical center blvd., Winston Salem, NC 27157, USA. Tel. 1-336-716-2743, Fax. 1-336-716-6744, E-mail: khayashi@wfubmc.edu.

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rodents after peripheral nerve injury (Hayashida et al., 2007b; Takasu et al., 2006; Tanabe et al., 2005). Stimulation of α_2 -adrenoceptors by spinally released noradrenaline results not only in inhibition of spinal nociceptive neurons (Jones, 1991), but also in activation of cholinergic circuits (Eisenach, 1999). In accordance with this noradrenergic-cholinergic circuit, both systemic and intracerebroventricular administration of gabapentin produce analgesia which is blocked by intrathecal injection of either α_2 -adrenoceptor or muscarinic receptor antagonists (Hayashida et al., 2007b; Takasu et al., 2006). These observations may be clinically relevant, as demonstrated by the ability of orally administered gabapentin to increase noradrenaline concentration in the cerebrospinal fluid and to decrease morphine requirement after surgery in patients with chronic pain (Hayashida et al., 2007a).

If gabapentin analgesia depends on the activation of a spinal noradrenergic-cholinergic circuit, its potency should be enhanced by amplifying the effects of these neurotransmitters. We recently reported that oral administration of the cholinesterase inhibitor donepezil reduces hypersensitivity by spinal muscarinic receptor activation (Clayton et al., 2007), and that oral gabapentin and donepezil interact in a strongly synergistic manner after peripheral nerve injury in rats (Hayashida et al., 2007b). Duloxetine, an approved serotonin / noradrenaline re-uptake inhibitor for the treatment of diabetic peripheral neuropathic pain (Goldstein et al., 2005), should also enhance the noradrenergic-cholinergic circuit activated by gabapentin. Unlike other antidepressants such as amitriptyline, duloxetine lacks significant affinity for muscarinic, histamine-1, α_1 -adrenoceptor, dopamine, 5-hydroxytryptamine (HT)₁, 5-HT₂, opioid receptors and sodium channels (Bymaster et al., 2001). Systemic administration of duloxetine reduces hypersensitivity after nerve injury and formalin-induced inflammatory pain in rats (Bomholt et al., 2005; Iyengar et al., 2004). Like the descending noradrenergic pathway, the descending serotonergic pathway may play an antinociceptive role, but pro-nociceptive effects have also been observed, likely due to actions on multiple 5-HT receptor subtypes. Depletion of spinal serotonin by an intrathecal injection of 5,7di-hydroxytryptamine reduces thermal and mechanical hypersensitivity after peripheral nerve injury in rats (Rahman et al., 2006). Blockade of 5-HT₃ receptors by an intrathecal injection of ondansetron, a 5-HT₃ receptor antagonist, also reduces hypersensitivity in rats (Suzuki et al., 2004a) and produces analgesia in patients with neuropathic pain (McCleane et al., 2003), suggesting that endogenous serotonin may play predominantly a facilitatory rather than inhibitory role in neuropathic pain states.

Since gabapentin, donepezil, and duloxetine are clinically available, the interaction of these three drugs to produce analgesia is highly clinically relevant. The purpose of the present study was to determine the type and degree of interaction between duloxetine and gabapentin, as well as with donepezil when administered by the clinically preferred oral route in rats after spinal nerve ligation. We also tested the α_2 -adrenergic and muscarinic receptor dependency of the gabapentin/donepezil/duloxetine combination. Finally, we tested whether this three drug combination induces sedation or affects motor coordination as measured by the rotarod test in normal and SNL animals.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN, USA), weighing 230-300 g, were used. All studies were performed under Wake Forest University Guidelines on the ethical use of animals and Animal Care and Use Committee approval. Animals were housed under a 12-h light-dark cycle, with food and water *ad libitum*.

2.2. Surgical preparations

2.2.1. Spinal nerve ligation (SNL)—As previously described (Kim and Chung, 1992), animals were anesthetized with inhalational 2% isoflurane in oxygen, the lateral laminae of the L6 and S1 vertebrae were exposed, the right L6 transverse process was removed and the right L5 and L6 spinal nerves were tightly ligated using 6-0 silk suture. Animals were allowed to recover for 2 weeks.

2.2.2. Intrathecal catheterization—Animals were anesthetized with 2% isoflurane and intrathecal catheterization was performed as previously described (Yaksh and Rudy, 1976). A small puncture was made in the atlanto-occipital membrane of the cisterna magnum and a polyethylene catheter (ReCathCO LLC, PA USA), 7.5 cm, was inserted so that the caudal tip reached the lumbar enlargement of the spinal cord. After implantation of the intrathecal catheters, rats were housed individually with free access to food and water. Animals were allowed at least 5 days to recover from the surgery.

2.3. Behavioral tests

2.3.1. Randall-Selitto test—Withdrawal threshold to pressure applied to the hind paw, expressed in grams, was measured using an analgesimeter (Ugo Basile, Comerio, Italy) as previously described (Randall and Selitto, 1957). The device applies increasing pressure to the hind paw. When the animal withdrew the paw or vocalized, the pressure was immediately released and the withdrawal threshold read on a scale. Training of animals for this test was performed for 3-5 days before the drug treatment. A cut-off of 250 g was used to avoid potential tissue injury.

2.3.2. von Frey test—Hypersensitivity to light touch following SNL was tested by means of calibrated von Frey filaments (Stoelting, Wood Dale, IL, USA) applied to the plantar surface of the hind paw. Filaments were applied to the bending point for 5 s, and a brisk paw withdrawal was considered as a positive response. Withdrawal threshold was determined using an up-down statistical method (Chaplan et al., 1994).

2.3.3. Rotarod test—Sedation and motor coordination were tested using the accelerating rotarod (model 47700, Ugo Basile) in which rats were required to walk against the motion of rotating drum with the speed accelerating from 4 to 40 rpm/min over 300 s. The time on the rod from the start of acceleration until the animal fell from the drum onto the counter-trip plate was recorded. A 300 s cut-off was used. One training period per day was performed for two days before the drug treatment. Animals were acclimated to the device and habituated to handling in order to avoid stress during testing.

2.4. Drugs and administrations

For oral administration, gabapentin solution (Neurontin® 50 mg/ml, Parke-Davis, New York, NY, USA) was diluted in 0.5% carboxymethylcellulose solution, duloxetine hydrochloride (Cymbalta®, Eli Lilly, Indianapolis, IN) and donepezil hydrochloride (Aricept®, Pfizer, New York, NY, USA) were crushed with a mortar and dissolved in 0.5% carboxymethylcellulose solution, and then administered by a feeding tube (5mL/kg). For the combination study, gabapentin and duloxetine were administered 2 h and donepezil was administered 1 h prior to the measurement, using a fixed ratio (gabapentin: donepezil: duloxetine = 12: 1: 9). For intrathecal administration, atropine sulfate (Sigma Chemical CO, St. Louis, MO, USA) and idazoxan hydrochloride (Sigma) were dissolved in saline and were injected in a volume of 10 µl followed by 10 µl of saline 30 min prior to the measurement. Animals were studied up to three times on different days. Drugs and doses were randomly assigned and no animal received

same treatment twice. Experiments were separated by at least 5 days. The person performing the behavioral test was blinded to drug and dose.

2.5. Statistical analyses

Unless otherwise stated, data are normally distributed and presented as mean $\pm$ S.E.M. Differences among groups for withdrawal threshold were determined using one or two-way analysis of variance (ANOVA). To calculate the effective dose to produce a 50% maximum effect (ED₅₀) of each drug, the response threshold data were converted to a percentage of return to pre-surgery threshold according to the following formula: % return to pre-surgery threshold = (post drug threshold - baseline pre-drug threshold)/(pre-SNL threshold - baseline pre-drug threshold) $\times$ 100. Pre-drug threshold was the withdrawal threshold after SNL. ED₅₀ was determined using linear regression for each drug. Based on the ED₅₀ obtained from the single drug dose response and a fixed ratio (gabapentin: donepezil: duloxetine = 12: 1: 9), the theoretical additive ED₅₀ value for each drug in two [three] drug combination studies was calculated using the following equation: (Theoretical ED₅₀ of Drug A)/(Single drug ED₅₀ of Drug A) + (Theoretical ED₅₀ of Drug B)/(Single drug ED₅₀ of Drug B) + [(Theoretical ED₅₀ of Drug C)/(Single drug ED₅₀ of Drug C)] = 1. To compare the calculated theoretical additive and experimental observed ED₅₀, isobolographic analysis was performed by z-test as described previously (Tallarida et al., 1989). Significance level was set at P < 0.05.

3. Results

SNL strongly decreased the withdrawal threshold of the hindpaw ipsilateral to SNL from 148 $\pm$ 16 g to 71 $\pm$ 14 g (mean $\pm$ S.D., P < 0.0001, n = 78) in the Randall-Selitto test. All animals in the current study showed at least 40 g reduction in the withdrawal threshold of the ipsilateral hindpaw after SNL. We also observed that the withdrawal threshold in the contralateral hindpaw was slightly but significantly decreased from 150 $\pm$ 18 g to 120 $\pm$ 26 g (mean $\pm$ S.D., P < 0.0001, n = 78), similar to our previous reports (Hayashida et al., 2007b; Hayashida et al., 2006; Hayashida et al., 2008).

3.1. Dose-response of gabapentin, donepezil, and duloxetine

Orally administered gabapentin, donepezil, and duloxetine reduced mechanical hyperalgesia in the hindpaw ipsilateral to SNL in a dose- and time-dependent manner (Fig. 1). Gabapentin showed significant analgesic effects from 50 to 100 mg/kg compared to vehicle (Fig. 1A, P < 0.05). The peak effect of oral gabapentin was observed 2 h after administration. The ED₅₀ value (95% confidence interval: CI) of gabapentin calculated at the 2 h time point was 45 mg/kg (13-77 mg/kg). Donepezil showed significant analgesic effects from 2.5-10 mg/kg compared to vehicle (Fig. 1B, P < 0.05) and the ED₅₀ value of donepezil was 3.7 mg/kg (1.4-6.0 mg/kg) at the 1 h time point. The peak effect of oral duloxetine was observed 2-3 h after administration. Duloxetine showed significant analgesic effects from 25-50 mg/kg compared to vehicle (Fig. 1C, P < 0.05) and the ED₅₀ value of duloxetine calculated at 2 h was 32 mg/kg (12-56 mg/kg). Based on these ED₅₀ values and time courses, we tested a fixed ratio for the gabapentin: donepezil: duloxetine combination of 12: 1: 9 and the timing of behavioral testing (2 h after gabapentin and duloxetine and 1 h after donepezil administration) for the following studies.

3.2. Effects of the combination of duloxetine, gabapentin, and donepezil

In the two drug combination studies, gabapentin or donepezil combined with duloxetine in a fixed ratio produced dose-dependent analgesia in the paw ipsilateral to SNL (Fig. 2A, 3A). In the presence of duloxetine, ED₅₀ values (95% CI) of gabapentin and donepezil were 25 mg/kg (11-44 mg/kg) and 1.9 mg/kg (0.9-3.0 mg/kg), respectively. Isobolographic analysis indicated that duloxetine interacted additively with either gabapentin or donepezil (Fig. 2B,

3B). In the three drug combination study, we observed a dose-dependent analgesia (Fig. 4A). There were significant differences between the observed ED₅₀ values ($\pm$ S.E.M.) of the test drugs (gabapentin 3.8 ± 0.1 mg/kg, donepezil 0.3 ± 0.01 mg/kg, duloxetine 2.9 ± 0.1 mg/kg) and the theoretical ED₅₀ value of additivity (gabapentin 15 ± 2.9 mg/kg, donepezil 1.2 ± 0.2 mg/kg, duloxetine 11 ± 2.1 mg/kg), indicating a synergistic interaction (Fig. 4B, $P < 0.05$ by z-test). We also confirmed the efficacy of the three drug combination on mechanical allodynia after SNL using punctate stimuli with von Frey filaments. Withdrawal threshold to light touch by a von Frey filament in the paw ipsilateral to SNL markedly decreased from 17.8 ± 1.6 g to 1.6 ± 0.4 g ($P < 0.001$, $n = 12$). Consistent with the result in the Randall-Selitto test, the combination of gabapentin (6 mg/kg), donepezil (0.5 mg/kg), and duloxetine (4.5 mg/kg) significantly increased paw withdrawal threshold in response to von Frey filaments (from 1.5 ± 0.6 g to 9.4 ± 2.4 g, $n = 6$, $P < 0.05$) compared to the vehicle (from 1.7 ± 0.5 g to 2.3 ± 0.7 g, $n = 6$) in SNL animals.

Intrathecal administration of the α_2 -adrenoceptor antagonist idazoxan (30 μ g) or the muscarinic antagonist atropine (30 μ g), neither of which affected withdrawal threshold alone, completely blocked the analgesia by the combination of gabapentin (6 mg/kg), donepezil (0.5 mg/kg), and duloxetine (4.5 mg/kg), consistent with stimulation of a spinal noradrenergic-cholinergic pathway (Fig. 5).

3.3. Motor coordination

In the rotarod test, neither vehicle nor the combination of gabapentin (6 mg/kg), donepezil (0.5 mg/kg), and duloxetine (4.5 mg/kg) affected performance in either normal or SNL animals (Fig. 6). In SNL animals, the combination at the higher dose (gabapentin 18 mg/kg, donepezil 1.5 mg/kg, and duloxetine 13.5 mg/kg) did not affect performance.

4. Discussion

Despite decades of research, only few drugs including gabapentin, the noradrenaline re-uptake inhibitor duloxetine, and the noradrenaline -mimetic clonidine have been approved to treat chronic neuropathic pain. Interestingly, these drugs activate, augment, or mimic the descending noradrenergic pathway, resulting in spinal α_2 -adrenoceptor stimulation and subsequent release of acetylcholine, which produces muscarinic analgesia (Eisenach, 1999; Obata et al., 2005; Xu et al., 1997). Thus, clinical evidence supports the concept that activation of noradrenergic-cholinergic pathway is a key strategy to treat neuropathic pain. The current study demonstrates that activation and augmentation of descending noradrenergic-cholinergic pathway by gabapentin, duloxetine and donepezil results in synergistic effects to reduce mechanical hypersensitivity in rats after peripheral nerve injury without sedative effects, and provides a powerful rationale for examining this multi-drug approach clinically.

Gabapentin has shown analgesic properties in a wide range of animal pain models (Chen and Pan, 2005; Hayashida et al., 2007a; Hayashida et al., 2007b; Pan et al., 1999; Tanabe et al., 2005) and effectively reduces chronic pain in patients (Laird and Gidal, 2000). Although gabapentin relies on interaction with $\alpha_2\delta$ subunits of calcium channels (Li et al., 2006), the location(s) of gabapentin action and the circuits involved are under investigation. Systemic or centrally administered gabapentin relies on activation of spinal α_2 -adrenoceptors and muscarinic receptors (Hayashida et al., 2007b; Takasu et al., 2006) but interestingly does not affect spinal serotonin release (Takeuchi et al., 2007). Similarly in humans, oral gabapentin increases spinal noradrenaline release in patients with chronic pain (Hayashida et al., 2007a), although its effects on serotonin release in humans have not been studied. These findings argue that activation of descending bulbospinal noradrenergic pathway is one of the pivotal mechanisms of gabapentin analgesia. Although the mechanisms by which gabapentin activates the descending noradrenergic pathway are unknown, gabapentin reduces synaptic transmission

of γ -aminobutyric acid in the locus coeruleus neurons through presynaptic mechanisms (Takasu et al., 2008), consistent with gabapentin-induced disinhibition. Peripheral nerve injury may paradoxically enhance the capacity of analgesia from the spinal noradrenergic-cholinergic circuit activated by gabapentin. Peripheral nerve injury induces noradrenergic axon sprouting in the spinal dorsal horn by a mechanism dependent on brain-derived neurotrophic factor (Hayashida et al., 2008) and also up-regulates inhibitory M₂ muscarinic receptors in injured and uninjured primary sensory neurons (Hayashida et al., 2006). This plasticity of spinal noradrenergic-cholinergic circuits after nerve injury may enhance the analgesic efficacy of gabapentin in neuropathic pain states.

Donepezil has been safely used for the treatment of Alzheimer's dementia and a secondary analysis of its use to reduce opioid induced sedation in cancer patients suggests it may produce analgesia for chronic pain (Slatkin and Rhiner, 2003). We recently reported in rats after peripheral nerve injury that oral donepezil produces analgesia by spinal muscarinic receptor activation and its efficacy was maintained over two weeks without leading to desensitization of muscarinic receptor-coupled G-proteins in the brain and spinal cord (Clayton et al., 2007). We also reported that oral gabapentin and donepezil interact in a strongly synergistic manner after peripheral nerve injury in rats (Hayashida et al., 2007b). In the current study, we extended these observations by testing whether inhibition of noradrenaline re-uptake further increases gabapentin and/or donepezil analgesia using a clinically available serotonin /noradrenaline re-uptake inhibitor duloxetine.

Duloxetine interacted in an additive, rather than synergistic manner, with gabapentin and donepezil in the current study, contrary to our expectations. We speculate that this lack of synergy may reflect enhanced serotonergic as well as noradrenergic signaling in the spinal cord after duloxetine administration. Serotonin is released in the spinal cord from descending serotonergic axons which mainly originate from the nucleus raphe magnus in the rostral ventromedial medulla and can produce antinociceptive and pro-nociceptive effects depending on the spinal 5-HT receptor subtype activated (Millan, 2002). Although earlier studies mostly focused on the inhibitory function of descending serotonergic pathways, recent studies have shifted attention to excitatory influences of serotonin which contribute to the development and maintenance of persistent pain (Suzuki et al., 2004b). Tonic activation of descending facilitation from the rostral ventromedial medulla was shown to be involved in the maintenance of neuropathic pain (Porreca et al., 2002). The depletion of spinal serotonin (Rahman et al., 2006) or blockade of spinal 5-HT₃ receptors (Suzuki et al., 2004a) reduces hypersensitivity in rats after peripheral nerve injury. In neuropathic pain patients, selective serotonin re-uptake inhibitors exhibit lower efficacies than those of less selective serotonin /noradrenaline re-uptake inhibitors (Sindrup et al., 2005). Thus, enhanced facilitation from serotonin release after duloxetine may have limited its ability to interact synergistically with gabapentin or donepezil, which rely essentially on noradrenaline release.

Combination of these three drugs in the current study resulted in an ED₅₀ for each drug less than at 1/4th the predicted dose of an additive interaction and this enhanced analgesia was completely blocked by antagonists of spinal α_2 -adrenoceptors or muscarinic receptors, consistent with our idea that activation of noradrenergic-cholinergic pathway is a key strategy to treat neuropathic pain. The enhanced analgesia by gabapentin/donepezil/duloxetine likely reflects a synergistic interaction of gabapentin with donepezil and an additive interaction of duloxetine with the other two components.

We also demonstrated that the three drug combination, in an effective anti-hypersensitivity dose, did not affect motor coordination or cause sedation in either normal or SNL animals. These results provide a strong hope that such an interaction in patients could yield effective analgesia without therapy limiting side effects of single agents.

In summary, the combination of orally administered gabapentin, donepezil and duloxetine produces synergistic antihypersensitivity after peripheral nerve injury which depends on spinal noradrenergic-cholinergic circuits in rats. Established safety profiles, clinical availability, and the strongly enhanced analgesia observed in this study suggest that clinical trials of this combination should be performed in neuropathic pain patients.

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Fig.1.

Withdrawal threshold to pressure to the paw ipsilateral to SNL after oral treatment with gabapentin (A. 25-100 mg/kg, $n=6$), donepezil (B. 2.5-10 mg/kg, $n=6$), and duloxetine (C. 12.5-50 mg/kg, $n=6-8$) was compared to vehicle by two-way ANOVA. * $P < 0.05$ vs time 0 by one-way ANOVA.

Fig.2.

Interaction between oral gabapentin and duloxetine after SNL in rats. A. Oral gabapentin and duloxetine were administered 2 h prior to the determination of withdrawal threshold with a fixed ratio dose (gabapentin: duloxetine = 3:2, n=6). Data expressed as the percentage return to pre-surgery withdrawal threshold (see Method in detail). *P<0.05 vs pre-drug value for each dose by one-way ANOVA. B. Isobologram of the gabapentin-duloxetine interaction. The open symbols represent ED₅₀ values with 95% confidence intervals for each agent alone. The line connecting these two points is the theoretical line of additivity. The closed circle represents the experimentally observed ED₅₀ point with 95% confidence interval for the drug combination calculated from data from panel A.

Fig.3.

Interaction between oral donepezil and duloxetine after SNL in rats. A. Oral donepezil was administered 1 h and duloxetine was administered 2 h prior to the determination of withdrawal threshold with a fixed ratio dose (donepezil: duloxetine = 1:9, n=6). Data expressed as the percentage return to pre-surgery withdrawal threshold (see Method in detail). * $P < 0.05$ vs pre-drug value for each dose by one-way ANOVA. B. Isobologram of the donepezil-duloxetine interaction. The open symbols represent ED₅₀ values with 95% confidence intervals for each agent alone. The line connecting these two points is the theoretical line of additivity. The closed circle represents the experimentally observed ED₅₀ point with 95% confidence interval for the drug combination calculated from data from panel A.

Fig.4.

Interaction between oral gabapentin, duloxetine and donepezil after SNL in rats. A. Gabapentin and duloxetine were administered 2 h and donepezil was administered 1 h prior to measurement of withdrawal threshold, using a fixed ratio dose (gabapentin: donepezil: duloxetine = 12:1:9, n=6-8). * $P < 0.05$ vs pre-drug value for each dose by one-way ANOVA. B. Theoretical ED₅₀ of additivity and experimentally determined combination ED₅₀ calculated from data from panel A. * $P < 0.05$ vs theoretical value for each drug by z-test.

Fig.5.

Effects of intrathecal idazoxan and atropine on oral gabapentin-donepezil-duloxetine combination analgesia after SNL in rats. Gabapentin (6 mg/kg) and duloxetine (4.5 mg/kg) were administered 2 h and donepezil (0.5 mg/kg) was administered 1 h prior to the measurement. Intrathecal idazoxan (30 μ g), atropine (30 μ g) or saline were injected 30 min prior to the measurement. * $P < 0.05$ vs pre-drug by t-test. # $P < 0.05$ vs p.o. 3 drugs comb + i.t. saline by one-way ANOVA.

Fig.6.

Effect of oral gabapentin-donepezil-duloxetine combination on motor coordination in normal and SNL rats. Gabapentin and duloxetine were administered 2 h and donepezil was administered 1 h prior to the rotarod test, using a fixed ratio dose (gabapentin: donepezil: duloxetine = 12:1:9, n=6). Data expressed as the performance time on the rod (see Method in detail).