The Novel Reversible Fatty Acid Amide Hydrolase Inhibitor ST4070 Increases Endocannabinoid Brain Levels and Counteracts Neuropathic Pain in Different Animal Models

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ABSTRACT

The effect of the enol carbamate 1-biphenyl-4-ylethenyl piperidine-1-carboxylate (ST4070), a novel reversible inhibitor of fatty acid amide hydrolase (FAAH), was investigated for acute pain sensitivity and neuropathic pain in rats and mice. Brain enzymatic activity of FAAH and the endogenous levels of its substrates, anandamide (AEA) and N-arachidonoylthanolamine, were measured in control and ST4070-treated mice. ST4070 (10, 30, and 100 mg/kg) was orally administered to assess mechanical nociceptive thresholds and allodynia by using the Randall-Selitto and von Frey tests, respectively. Neurpathy was induced in rats by either the chemotherapeutic agent vincristine or streptozotocin-induced diabetes, whereas the chronic constriction injury (CCI) model was chosen to evaluate neuropathic pain in rats and mice. ST4070 inhibited FAAH activity and increased the brain levels of AEA and PEA, without affecting that of 2-AG. The administration of ST4070 generated long-lasting pain relief compared with pregabalin and the FAAH inhibitors 1-oxo-1-(2-pyridyl)-2-yl)-7-phenylheptane (OL135) and cyclohexylcarbamoyl acid 3’-carbamoyl-biphenyl-3-yl ester (URB597) in CCI neuropathic mice. The antiallodynic effects of ST4070 were prevented by pretreatment with cannabinoid type 1 and cannabinoid type 2 receptor antagonists and by the selective peroxisome proliferator-activated receptor α antagonist [(2S)-2-[[12]-1-methyl-3-oxo-3-[4-(trifluoromethyl)phenyl]-1-propenylamino]-3-[4-[2-(5-methyl-2-phenyl-4-oxazolyl)ethoxy]phenyl]-propyl]carbamoyl acid ethyl ester (GW6471). The administration of ST4070 generated long-lasting neuropathic pain relief compared with pregabalin and the FAAH inhibitors OL135 and URB597. Taken together, the reversible FAAH inhibitor ST4070 seems to be a promising novel therapeutic agent for the management of neuropathic pain.

INTRODUCTION

The analgesic effect of cannabinoid type 1 and type 2 (CB1 and CB2, respectively) receptor agonists such as Cannabis sativa extracts has been known for centuries (Zias et al., 1993). More recently, the Δ9-tetrahydrocannabinol and cannabidiol 1:1 mixture (marketed as the oromucosal spray Sativex, GW Pharmaceuticals, Wiltshire, UK) has proved its efficacy and tolerability in controlled clinical trials where multiple sclerosis-associated disabling symptoms such as pain and muscle spasticity have been evaluated (Nurmikko et al., 2007; Collin et al., 2010). CB receptors are expressed in key areas involved in nociception, including the periaqueductal gray, the dorsal horn of the spinal cord, and dorsal root ganglion
neurons (Tsou et al., 1998). However, as a complementary approach to the direct stimulation of CB receptors, research on pain and inflammation has focused on targeting the endogenous cannabinoid system, attempting to elevate anandamide (AEA; N-arachidonoyl ethanolamine) levels through the pharmacological inhibition of its catalytic enzyme, the fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996; Bisogno et al., 2002). FAAH inhibitors are effective in various rodent models of inflammatory and neuropathic pain, without any apparent motor deficit or other side effects often associated with tetrahydrocannabinol or direct CB receptor agonist administration (Ahn et al., 2009; Schlossberg et al., 2009). Mice lacking the faah gene have increased AEA and N-palmityl ethanolamine (PEA) brain concentrations, are hypoalgesic, and display increased AEA-induced analgesia (Cravatt et al., 2001; Lichtman et al., 2004a; Patel et al., 2005). In addition, the FAAH inhibitors 1-oxo-1-[5-(2-pyridyl)-2-yl]-7-phenylheptane (OL135) and cyclohexylcarbamic acid 3’-carbomoylbiphenyl-3-yl ester (URB597) ameliorate pain behaviors in selected models of inflammatory and/or neuropathic pain in rats (Chang et al., 2006; Jayamanne et al., 2006; Jhaveri et al., 2006; Russo et al., 2007; Kinsey et al., 2009; Naidu et al., 2010). It is noteworthy that FAAH has also been implicated in the antinoceptive effect of paracetamol (Högestätt et al., 2005; Mallet et al., 2008) and other analgesics (Bisogno et al., 2002) that affect prostaglandin production.

We have developed a new enol carbamate (1-biphenyl-4-yl ethenyl piperidine-1-carboxylate; ST4070) that was shown to be a potent, reversible, and selective inhibitor of FAAH (Gattinoni et al., 2010). In the present study, after the evaluation of the effects of ST4070 on mechanical nociceptive threshold in naive animals, we ascertained whether ST4070 could produce pain relief in different animal models of neuropathies. Next, we assessed whether ST4070 affects the enzymatic activity of its target and, consequently, endogenous brain levels of the FAAH substrates AEA, PEA, and 2-arachidonoylglycerol (2-AG). The antineoplastic drug vircristine ([3aR,3c1R,4R,5S,5aR,10bR]-methyl 4-acetoxy-3-ethyl-9-[[5S,7S,9S]-5-ethyl-5-hydroxy-9-(methoxy carbonyl)-2,4,5,6,7,9,10-octahydro-1H-3,7-methano]azacycloundecino[5,4-b][indol-9-y1]-6-formyl-5-hydroxy-8-methoxy-3a,3c1,4,5,5c,6,11,12-octahydro-1H-indolizino[8,1-cd]carbazole-5-carboxylate) was administered in rats to induce neuropathic pain, whereas either streptozotocin (STZ) administration or sciatic nerve chronic constriction injury (CCI) was used in mice as models of diabetic neuropathy and neuropathic pain, respectively. Previous studies have provided evidence that both CB1 and CB2 receptor blockade abolished URB597-induced attenuation of thermal hyperalgesia and mechanical allodynia (Russo et al., 2007; Kinsey et al., 2009); more controversial were the effects of the selective antagonism of CB1 but not CB2 receptor on the antiallodynic effects of OL135 treatment (Chang et al., 2006; Kinsey et al., 2009). Because both stimulation of CB1 and CB2 subtypes and the activation of PPARα subtypes are viewed as potential underlying mechanisms indirectly modulated by the FAAH inhibition (Lo Verme et al., 2005; Wallace et al., 2007; Costa et al., 2008; Desroches et al., 2008; Jhaveri et al., 2008; Sagar et al., 2008; Endocannabinoid Research Group et al., 2010) we tested the hypothesis that ST4070-induced analgesia in the CCI model of neuropathy could be antagonized by CB receptor subtypes and PPARα antagonists. Neuropathic pain is hugely detrimental to an individual’s quality of life, and the response of this chronic condition to existing treatments is often inadequate. New compounds are therefore needed to be validated in a therapeutic perspective by translational models.

Materials and Methods

Chemicals and Drugs. Chemicals were of the purest analytical grade. AEA, PEA, and chloral hydrate were purchased from Sigma (St. Louis, MO). 2-AG was from Enzo Life Sciences, Inc. (Farmingdale, NY). AEA-ethanolamine-1-[3H] (60 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waldbheim, MA). d3-AEA, d2-2-AG, and d2-2-AG were from Cayman Chemical (Ann Arbor, MI). ST4070, prepared as reported previously (Gattinoni et al., 2010), was suspended in sterile water with 0.5% sodium carboxymethylcellulose with medium viscosity (Fluka, Buchs, Switzerland) and 0.1% Tween 80 (Merck, Darmstadt, Germany). FAAH inhibitors OL135 and URB597 (Enzo Life Sciences, Inc.) and (S)-3-amino-5-methylhexanoic acid (pregabalin) (Lyrica; Pfizer, New York) were used as reference compounds. N-[1-(4-piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide HCl (Cayman Chemical), N-(1S-endo)-1,3,3,tri-methylbicyclo[2.2.1]heptan-2-yl)-5-(4-chole-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528) (Cayman Chemical), and (S)-2-[2-[[1Z]-1-methyl-3-oxo-3-[4-(trifluoromethyl)phenyl]-1-propenyl-1-amino]-3-[4-[5-(2-methyl-5-phenyl-4-oxazolyl)ethyl]phenyl]propyl]-carbamic acid ethyl ester (GW6741) (Tocris Bioscience, Bristol, UK) are selective antagonists of CB1, CB2, and PPARα receptors, respectively. OL135 was diluted in the same vehicle as ST4070 and URB597. Chloral hydrate and pregabalin were diluted in sterile saline solution (0.9% NaCl). SR141716A and SR144528 were dissolved in 2% Tween 80, 2% ethanol, and sterile water. GW6471 was dissolved in a 1:1:8 mixture of ethanol/Tween/aqueous solution (pH 4.5). All compounds were administered in a volume of 5 and 10 ml/kg for rats and mice, respectively.

Animals. Sprague-Dawley male rats (Harlan, Udine, Italy) or CD1 male mice (Charles River Italia, Calco, Italy), weighing 320 to 350 and 35 to 40 g, respectively, were housed in standard transparent plastic cages in groups of three (rats) or four (mice) under a standard 12-h light/dark cycle (lights on at 7:00 AM), with food and water available ad libitum. Mechanical nociceptive threshold and vincristine-induced neuropathy were tested in rats, whereas behavioral responses to streptozotocin and CCI-induced neuropathies, as well as FAAH activity and endocannabinoid levels, were analyzed in mice. All experiments were conducted in accordance with the guidelines for care and use of experimental animals of the European Communities Directive (86/609 EEC, 27.01.1992, No. 116) and approved by the company veterinary doctor and the Italian Ministry of Health. Experiments were performed according to a randomized schedule, and the observer was blind to the treatment received by the animals.

Mechanical Nociceptive Threshold. The mechanical nociceptive threshold, expressed in grams, was measured in rats by applying increasing pressure to the left and right hind paws, using a Randall-Selitto algometer (Ugo Basile, Comerio, Italy). The parameter used to quantify the nociceptive threshold was defined as the pressure (grams) at which the rat withdrew its paw. Rats were habituated to testing procedures and handling by the investigator during the week before the experiment. Vehicle or ST4070 (10, 30, and 100 mg/kg) was administered orally to rats (n = 13 per group). The animals were subjected to the paw withdrawal test at 0, 60, 120, and 240 min after treatment.

Vincristine-Induced Neuropathic Pain. Neuropathy was induced in rats by intraperitoneal administration of 0.15 mg/kg vincristine three times a week for 2 weeks. Rats were divided into two groups: vehicle (n = 12) and vincristine (n = 24). After 2 weeks, rats were distributed into three groups of 12 animals each: 1) vehicle, 2) vincristine + vehicle, and 3) vincristine + ST4070 (50 mg/kg by oral administration).
The Randall-Selitto test was as described above. Paw withdrawal thresholds were measured 0, 60, 120, and 240 min after treatment.

**STZ-Induced Diabetic Neuropathic Pain.** Diabetic neuropathy was induced in CD1 mice by acute intraperitoneal administration of STZ (200 mg/kg) after an overnight fasting. Control mice received vehicle by the same route. Blood glucose levels were measured 14 days after STZ administration in blood samples obtained by tail prick. Blood glucose levels were measured using a Glucometer Elite device (Bayer AG, Wuppertal, Germany), and mice with glucose levels above 250 mg/dl were considered diabetic. Mechanical allodynia was tested by an automatic von Frey apparatus (Ugo Basile Dynamic Plantar Aesthesiometer). In brief, mice were placed on a metal mesh table and adapted to the new environment for 30 min. A mechanical stimulus was delivered to the plantar surface of both hind paws from below the floor of the test chamber by an automated testing device. A steel rod (0.5 mm in diameter) was pushed against the hind paw with ascending force, from 0 to 5 g over a 10-s period. When the animal withdrew its hind paw, the mechanical stimulus was automatically stopped, and the force at which the withdrawal occurred was recorded. Withdrawal responses were taken from five consecutive trials with at least 10-s intervals between trials. Mechanical allodynia was tested 16 days after STZ treatment. Vehicle was administrated to diabetic (n = 18) and vehicle-treated mice (n = 18), whereas ST4070 (10, 30 and 100 mg/kg) was orally administrated to diabetic animals (n = 19 per dose). ST4070 and vehicle were administrated 1 h before testing. At the end of the experiment, the animals were sacrificed, and the brains were removed and stored at −80°C until assays of FAAH activity and brain levels of AEA, 2-AG, and PEA were performed.

**Chronic Constriction Injury-Induced Neuropathic Pain.** CCI of sciatic nerve was used as a model of neuropathic pain, following the method described previously for rats (Bennett and Xie, 1988). CCI was performed under anesthesia with chloral hydrate (500 mg/kg i.p.), and the middle third of the right sciatic nerve was exposed through a 1.5-cm longitudinal skin incision. Three ligatures (5-0 chromic gut; Ethicon, Cornelia, GA) were tied loosely around the sciatic nerve proximal to the sciatic trifurcation. The wound was automatically stopped, and the force at which the withdrawal of the hind paw occurred was recorded. Withdrawal responses were taken from five consecutive trials with at least 10-s intervals between trials. Mechanical allodynia was tested 16 days after CCI treatment. Vehicle was administrated to diabetic (n = 18) and vehicle-treated mice (n = 18), whereas ST4070 (10, 30 and 100 mg/kg) was orally administrated to diabetic animals (n = 19 per dose). ST4070 and vehicle were administrated 1 h before testing. At the end of the experiment, the animals were sacrificed, and the brains were removed and stored at −80°C until assays of FAAH activity and brain levels of AEA, 2-AG, and PEA were performed.

**Data Analysis.** Statistical differences between groups were evaluated by using one-way ANOVA. Post hoc comparisons were carried out by using Dunn’s method.

**Results**

**Mechanical Nociceptive Threshold.** ST4070 at oral doses of 10 and 30 mg/kg increased dose-dependently the mechanical nociceptive threshold, measured in the Randall-Selitto test after 60 and 120 min, without any further significant increase at the highest dose of 100 mg/kg (Fig. 1). Vincristine-Induced Neuropathic Pain. The neuropathy induced by vincristine was counteracted by ST4070. The oral administration (50 mg/kg) of the FAAH inhibitor significantly increased the mechanical withdrawal threshold in vincristine-treated rats, with the analgesic effect still being significant 240 min after drug administration (Fig. 2).
Diabetic Hyperglycemia was observed in 72 of 75 mice. Three animals were therefore excluded from the analysis. ST4070 increased significantly mechanical withdrawal threshold in a dose-dependent manner (Fig. 3).

CCI-Induced Neuropathic Pain. Figure 4 shows that CCI induced a significant decrease in mechanical nociceptive threshold in vehicle mice by ~50% in the ipsilateral hindpaw compared with the contralateral hindpaw (Fig. 4A). Similarly to URB597, OL135, and pregabalin, ST4070 counteracted this deafferentation of CCI animals (data not shown).

The main result of the present study is that the oral administration of ST4070 exerts powerful analgesic effects in several animal models of neuropathic pain, supporting this new selective, reversible FAAH inhibitor as a promising candidate for fighting this chronic pathological condition. In addition, the observation that ST4070 is effective in enhancing mechanical nociceptive threshold in naive animals demonstrates that this FAAH inhibitor modulates the endocannabinoid’s tone. The study of the effects on FAAH enzymatic activity and substrates further reveals that ST4070 can provide analgesia via the selective increase of endogenous PEA and AEA, but not 2-AG, brain levels. Neuropathic pain involves a problematic clinical and pharmacological management, with a significant individual and social impact. Growing evidence from experimental and clinical studies has pointed to cannabinoids as potential analgesic agents (Walker and Hohmann, 2005; Ashton and Milligan, 2008), even if the use of cannabinoid agonists is limited by their abuse potential and prolonging the duration of action of endogenously released AEA, the pharmacological inhibition of FAAH may represent an interesting different strategy. The hypothesis
that FAAH inhibition can boost the analgesic effect of endocannabinoids is supported by the finding that FAAH knockout mice (Cravatt et al., 2001) display hypoalgesic phenotype, as well as by the analgesic effects of the FAAH inhibitors OL135 and URB597 in various rodent models of nociception (Lichtman et al., 2004b; Chang et al., 2006; Jhaveri et al., 2006; Palmer et al., 2008; Kinsey et al., 2009). The antinociceptive potential of FAAH inhibitors has also been recently underlined in two rodent models of osteoarthritis (Schuelert et al., 2011), thus corroborating the idea that endocannabinoid enhancers may exert anti-inflammatory effects by reducing levels of proinflammatory cytokines such as interleukin-1β and tumor necrosis factor α (Naidu et al., 2010). However, as for the analgesic effects of FAAH inhibition on neuropathic pain, the results described are not always consistent. Jayamanne and coworkers (2006) reported that URB597 reduces allodynia and hyperalgesia in a model of inflammatory pain, whereas it did not affect allodynia in neuropathic rats, so our data with ST4070 in different neuropathic animal models seem to be particularly relevant.

Like other FAAH inhibitors (McKinney and Cravatt, 2005; Fezza et al., 2008), the novel enol carbamate ST4070 is highly selective toward FAAH (Gattinoni et al., 2010) and prevents AEA and PEA, but not 2-AG, substrate degradation. The fact that ST4070 increased PEA content at doses lower than those necessary to increase AEA levels suggests that PEA might play a key role in the analgesic activity of ST4070. PEA belongs to the family of cannabimimetic fatty acids derivatives able to indirectly modulate and/or potentiate the biological effects of endocannabinoids. In particular, PEA can potentiate the effects of AEA stimulation on transient receptor potential vanilloid receptor type 1 channels (De Petrocellis et al., 2001), whose rapid desensitization contributes to increase refractariety to nociceptive stimulation.
and analgesic effects. Because PEA competes with FAAH-induced AEA hydrolysis and down-regulates FAAH expression, its action has been described in terms of potentiation ("entourage effect") of the responses mediated by AEA (Lambert and Di Marzo, 1999; De Petrocellis et al., 2001). PEA is endowed with peripheral and central anti-inflammatory and antinoceptive properties, as shown in the rodent formalin model of inflammatory pain, in spinal cord injury, as well as in carrageenan-induced paw edema and hyperalgesia (Calignano et al., 2001; Costa et al., 2002, 2008; Lo Verme et al., 2005; D'Agostino et al., 2007, 2009; Genovese et al., 2008; Endocannabinoid Research Group et al., 2010). The relevance of PEA level increases for the relief of mechanical allodynia and inflammation has been emphasized by the minocycline-induced inhibition of activated microglia in the spinal nerve ligation model (Guasti et al., 2009). Moreover, PEA administration has been recently shown to be effective against mast cell degranulation underlying chronic granulomatous inflammation (Endocannabinoid Research Group et al., 2010; De Filippis et al., 2011) and against inflammation and motor disability in a virus model of multiple sclerosis (Loria et al., 2008). In line with these data, the anti-inflammatory and analgesic effects of PEA may involve the down-regulation of substance P-induced mast cell degranulation via the so-called ALIA ("autacoid local inflammation antag- onism") effect (Aloe et al., 1993). Another possible mechanism involves the ability of PEA to stimulate receptors such as the nuclear peroxisome PPAR-α, the transient receptor potential vanilloid receptor type 1, or a yet-uncharacterized CB2-like receptor (Lo Verme et al., 2005; Costa et al., 2008). However, although CB2 receptor blockade has been shown to inhibit some PEA-mediated analgesic effects (Calignano et al., 2001), the action of PEA seemed insensitive to CB2 antagonism in the carrageenan-induced acute paw inflammation, in which PEA administration reduced edema by decreasing cyclooxygenase activity and nitric-oxide production (Costa et al., 2002).

In the light of the above, the preferential ST4070-induced potentiation of PEA activity seems to support the existence of several targets (e.g., PPAR-α) underlying the antinoceptive effects of the drug observed in CCI, vincristine-induced, and STZ-induced neuropathic pain. In addition, although both AEA and 2-AG are subjected to multiple metabolic pathways (Simon and Cravatt, 2010), our findings corroborate the concept that FAAH is the major regulator of the endogenous tone of AEA and PEA, whereas the degradation of 2-AG is controlled mainly by different enzymes such as monoacylglycerol lipase (Petrosino and Di Marzo, 2010). Hence, considering the ST4070-induced enhancement of PEA and AEA brain levels and the fact that PEA does not bind CB1 or CB2 receptor sites while it seems to binds CB2-like receptors located on mast cells (Facci et al., 1995; Calignano et al., 1998), we investigated whether separate CB1, CB2, or PPAR-α receptor blockade could prevent the antiallodynic effects of ST4070 administration. In agreement with ST4070 enzymatic activity as well as with the antiallodynic potential of increased AEA and PEA levels, our data provide evidence that the antinoceptive activity of ST4070 may be ascribed to a concurrent action on both CB receptor- and PPAR-α receptor-mediated signaling. Moreover, because sciatic nerve ligation produces mast cell activation and degranulation (Zuo et al., 2003), there is the possibility that conditions of neuropathic pain triggered by peripheral nerve damage may benefit from the dual preventive action on AEA and PEA degradation ("entourage effect") conferred by the ST4070 treatment.

It is noteworthy that FAAH is overexpressed in multiple sclerosis (Benito et al., 2007) and its inhibition has also been associated with a significant neuroprotective action, as demonstrated by the URB597-induced decrease of hippocampal neuron hyperactivity or excitotoxic-induced neuronal loss (Karanian et al., 2005; Coomber et al., 2008). In this view, considering the multiple underlying factors (e.g., nerve trauma, metabolic, or chemical intoxication) of neurodegeneration and its association to neuropathies, the design of novel FAAH inhibitors possessing neuroprotective activity can represent an additional strategy for neuropathic pain management. FAAH inhibitors are not cataleptogenic and are effective in treating kainic acid-induced epileptic seizure and brain damage (Karanian et al., 2007). Considering that anticonvulsants (such as pregabalin) are established pharmacological options for neuropathic pain treatment (Backonja and Glanzman, 2003) the antiepileptic properties shown by FAAH inhibitors add value to the current development of this class of drugs. Indeed, in spite of different treatment options (e.g., anticonvulsants, antidepressants, opioids, and several topical medications), the persistent nature and the intrinsic refractoriness to pharmacotherapy that characterize neuropathic pain strongly limit the attainable benefits. Hence, because current clinical treatments are far from being satisfactory, there is great need to develop more effective agents for neuropathic pain relief.

In the present study, we provide evidence that acute oral ST4070 administration is effective in reducing mechanical

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**Fig. 5.** Effects of ST4070 (ST) administration (30 mg/kg p.o.) alone or combined with SR141716A (3 mg/kg), SR144528 (3 mg/kg), or GW6471 (1 mg/kg) on neuropathic pain induced by CCI of sciatic nerve. Time-course effects of ST4070 administration are shown from 0.5 to 2 h (D4). ***p < 0.001 versus vehicle. **p < 0.001 versus ST4070. Points are mean ± S.E.M. (n = 10). Statistical differences between groups were assessed by two-way ANOVA for repeated measures: treatment, F<sub>1,45</sub> = 59.23, p < 0.001; time, F<sub>2,150</sub> = 27.39, p < 0.001; treatment × time interaction, F<sub>4,150</sub> = 9.03, p < 0.001. Post hoc comparisons were carried out by using the Fisher test.
hypersensitivity and allodynia associated with vincristine-induced neuropathies, diabetes, and nerve injury. Furthermore, we demonstrated that ST4070 administration is more efficient in relieving CCI-induced allodynia compared with the other FAAH inhibitors tested, most notably pregabalin, a reference compound of therapeutic value for human neuropathic pain. In fact, ST4070 displayed a longer-lasting action than that observed after pregabalin, OLI35, or URB597 administration. However, given the critical importance of sex hormones in different chronic pain syndromes (Alosi and Bonifazi, 2006), future studies should address the efficacy of ST4070 in neuropathic pain management in female rats and mice.

Finally, the antiallodynic effects of ST4070 were offset by CB1, CB2, and PPAR-α receptor blockade, thus supporting the notion that this novel FAAH inhibitor can act via multiple endocannabinoid and noncannabinoid mechanisms of action.

This study highlights the clinical potential and the therapeutic efficacy of ST4070 in neuropathic pain management, by way of concurrent PEA- and AEA-mediated signaling pathways.

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Authorship Contributions

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References


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