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貴財団より助成金を受領して行った中国人研究者・技術者招聘について報告いたします。

添付資料：研究報告書

受給者氏名：裏出 良博

所属機関名：(財) 大阪バイオサイエンス研究所

所属部署：分子行動生物学部門 職名：研究部長

〒 565-0874

所在地：大阪府吹田市古江台6-2-4

電話：06-6872-4851 内線：320

1．助成金額 750,000 円

2．研究テーマ アデノシン受容体遺伝子欠損マウスを用いたカフェイン覚醒調節の解析

3．成果の概要（100字程度）

野生型マウス、アデノシンA1およびアデノシンA2A受容体遺伝子欠損マウスへのカフェイン投与実験により、カフェインの覚醒効果は、脳内の第2の睡眠物質であるアデノシンのA2A受容体が関与することを証明した。

4．被招聘者

氏名：Xu Xin-Hong 職名：講師

所属機関：中国 復旦大学上海医学院 部署：薬理学教室

5．滞在日程概要（日付、主な活動・工程等）

5月 マウスの睡眠覚醒を測定する方法のトレーニング。カフェインを3つの投与量で投与した後、脳波と筋電図を記録することで、A1R KOマウスと野生型マウスのカフェインによる覚醒作用を比較し、A1アデノシン受容体の貢献度を調べる。

6月 カフェインを3つの投与量で投与した後、脳波と筋電図を記録することで、A2AR KOマウスと野生型マウスのカフェインによる覚醒作用を比較し、A2Aアデノシン受容体の貢献度を調べる。

7月、8月 自然な状態でA1R、A2ARのKOマウスの睡眠覚醒サイクルを測定し、A1RとA2ARの遺伝子欠損が及ぼす効果を明らかにする。

9月、10月 断眠による睡眠リバウンドに対するA1R、A2ARの貢献度を調べる。

11月 化学発光免疫ブロッティング法を用いて、A1R KOマウス、A2AR KOマウスと野生型マウスにカフェインを投与した後にDARPP-32のリン酸化を測定する。そして、カフェインの情報伝達におけるアデノシン受容体サブタイプの重要性を明らかにする。

12月 結果解析および論文・報告書作成

---------日中医学協会助成事業--------

Adenosine A_{2A} receptor mediates arousal effect of caffeine in mice

研 究 者氏名　Xing-Hong Xu
中国所属機関　複旦大学医学部薬理
日本研究機関　大阪バイオサイエンス研究所
指 導 責任者　部長　裏出　良博
共同研究者名　Wei-Min Qu, 松本 直実, Zhi-Li Huang

Abstract

Caffeine is a component of tea, coffee and cola, and widely consumed in the world. Caffeine induces wakefulness and antagonizes the depressant effects of adenosine with similar affinities for A_1 receptors (A_1Rs) and A_{2A} receptors (A_{2A}Rs) at low doses in human and rodents, both of these 2 receptors have been proposed to be involved in sleep-wake regulation. However, what receptor mediated the arousal effect of caffeine remains controversial. Here we report that caffeine at dose of 15 mg kg^{-1} increased wakefulness after an intraperitoneal injection at 9 a. m. in both A_1R wild-type and its knockout mice. In contrast, caffeine increased wakefulness only in A_{2A}R wild-type mice, but not at all in A_{2A}R knockout mice. These results clearly indicate that caffeine-induced wakefulness is mediated by adenosine A_{2A}R.

Keywords: Adenosine A_1 receptor; caffeine; knockout mice; sleep-wake regulation; DARPP-32 phosphorylation.

Introduction

Adenosine is an inhibitory neuromodulator involved in sleep-wake regulation[1]. There are 4 subtypes of adenosine receptors, A_1, A_{2A}, A_{2B}, and A_3, expressed in the central nervous system[2]. Several lines of evidence indicate that both A_1 receptor (A_1R) and A_{2A} receptor (A_{2A}R) subtype are involved in mediating the sleep-inducing effect of adenosine[3-7]. Caffeine has the effect opposite to that of adenosine on sleep; i.e., it promotes wakefulness. In doses consumed by humans, it binds to A_1R and A_{2A}R with similar affinities as a receptor antagonist[3]. As the pharmacological tools used to determine the receptor involved show limited selectivity and/or incomplete blockade, we decided to use A_1R and A_{2A}R knockout (KO) mice to elucidate which receptor is involved in caffeine-induced wakefulness. Here, we administered caffeine to A_1R, A_{2A}R KO and wild-type (WT) mice and found that blockade of A_{2A}R of caffeine attributed to its arousal effect, with an increase in DARPP-32 (dopamine- and cyclic AMP-regulated phosphoprotein of relative molecular mass 32,000) at Thr 75.

Material and Methods

Mice and Chemicals. A_{2A}R and A_1R KO mice were generated by Chen's[4,5] and Fredholm's[6] laboratories, respectively. Male A_1R KO, A_{2A}R KO and WT mice of the inbred C57BL/6 strain, weighing 23-27 g (11-13 weeks old), were maintained at Oriental Bioservice Ltd (Kyoto, Japan) and used for the experiments. Caffeine (Sigma) was dissolved in PBS and intraperitoneally injected in mice at 9 a.m.

Electroencephalogram (EEG) and Electromyogram (EMG) Recordings. Under pentobarbital anesthesia (50 mg kg^{-1}, i.p.), mice were implanted with EEG and EMG electrodes for polysomnographic recordings as previously described[7,8]. Baseline recordings were taken in each animal for 24 h, beginning at 8 a.m., which served as the control for the same animal. Vehicles were injected at 9 a.m. in the baseline day. On the next day, Caffeine (15 mg kg^{-1}) was injected in mice at 9 a.m. The vigilance states were automatically classified off-line by 10-s epochs into 3 stages of wake, rapid eye movement

(REM) and non-REM sleep by SLEEPSIGN, according to the standard criteria [8,9].

Determination of phosphoThr75-DARPP-32. Male WT and KO mice for A_1R and $A_{2A}R$ were i.p. injected with vehicle or caffeine and killed 15 min later. The heads of the animals were immediately immersed with liquid nitrogen for six seconds. The dorsal parts of the striata rapidly (20 s) were dissected out on an ice-cold surface, sonicated in 750ml of 1% sodium dodecylsulphate and boiled for 10 min. Equal amounts of protein were separated and quantified for phospho-DARPP-32 bands as decribed[10].

Statistical Analysis. See each figure legend. In all cases, $P < 0.05$ was taken as the level of significance.

Results and Discussion

To explore adenosine receptors involved in arousal effect induced by caffeine, firstly, we determined the effect of caffeine on sleep architecture in A_1R knockout (KO) mice during the light period, the majority of time is spent sleeping.

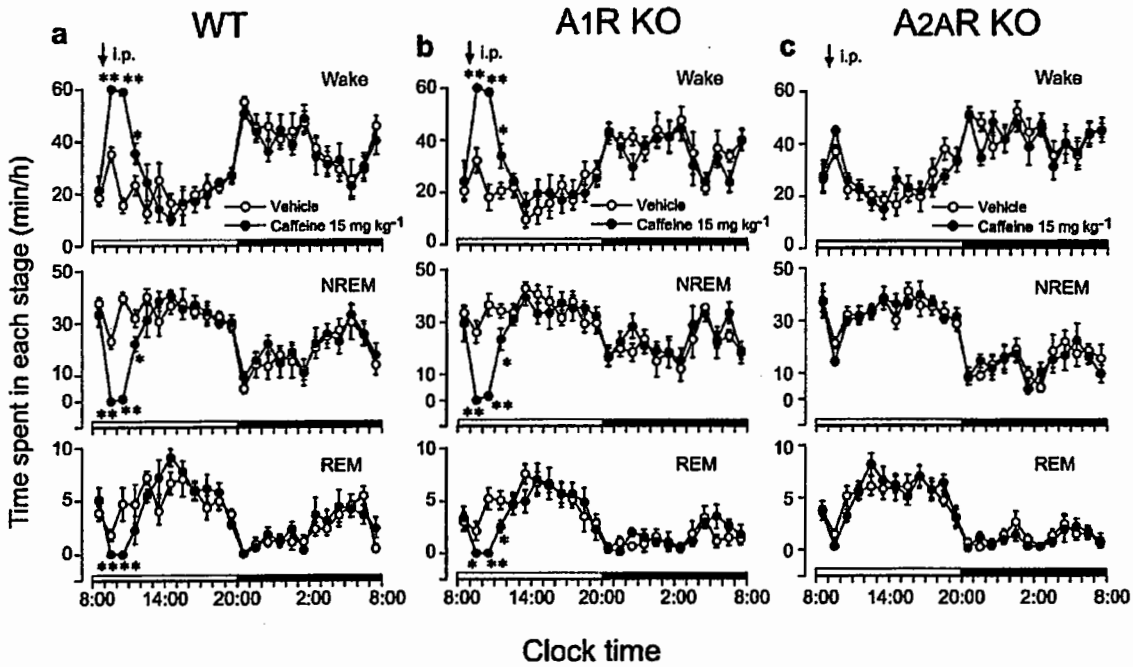

Figure 1 Time-course changes in caffeine 15 mg kg^{-1} treatment group in WT (**a**), A_1R KO (**b**) and $A_{2A}R$ KO (**c**) mice. Each circle represents the hourly mean ± s.e.m. of wakefulness, NREM or REM sleep. Open and closed circles stand for the baseline and experimental day profiles, respectively. The arrows indicate the injection time at 9 a.m. The long horizontal open and filled bars on the χ-axes indicate the 12 hr light and dark periods, respectively. Asterisk, $P < 0.05$; double asterisk, $P < 0.01$ by the paired *t*-test.

Under baseline conditions, the amounts and circadian profiles of sleep and wakefulness were identical between A_1R KO and wild-type (WT) mice (Fig. 1a, b), consistent with previous report[11]. When caffeine was intraperitoneally (i.p.) given to WT mice at a dose of 15 mg kg^{-1}, which would correspond to an intake of approximately about 3-4 cups of coffee in human[3], it significant increased wakefulness during the first, second and third hour after injection by 1.7, 3.8 and 1.5 fold (Fig. 1a), respectively, when the wakefulness was compared with that of the baseline day. This enhancement of wakefulness was concomitant with decreases in NREM and REM sleep. Caffeine (15 mg kg^{-1}) decreased the NREM sleep by 100%, 97% and 31%, and reduced REM sleep by 100%, 100% and 52%, respectively, during the first, second and third hour after injection. Like WT mice, A_1R KO mice displayed similar changes in sleep-wake stages after

administration of caffeine (Fig. 1b). These data clearly indicated that adenosine A_1R is not involved in arousal effect of caffeine.

To investigate the role of $A_{2A}R$ in the arousal effect of caffeine, we employed $A_{2A}R$ KO mice[4]. When caffeine (15 mg kg^{-1}) was injected into the $A_{2A}R$ KO mice, the KO mice did not exhibit any significant change in time spent in wake, NREM and REM sleep after caffeine administration (Fig. 1c). These results clearly indicate that adenosine $A_{2A}R$ plays a crucial role in caffeine-induced wakefulness.

Since the stimulatory effects of caffeine has been reported to be associated with an increase in the state of phosphorylation of DARPP-32 (dopamine- and cyclic AMP-regulated phosphoprotein of relative molecular mass 32,000) at Thr 75 (Ref [10]). We quantified the level of the phospho-DARPP-32 after treatment with caffeine in A_1R, $A_{2A}R$ KO mice and their WT mice to further clarify importance of adenosine receptor subtype in the signal transduction pathway of caffeine. Administration of caffeine (7.5 mg kg^{-1}) produced an increase in Thr 75 phosphorylation in A_1R KO, A_1R and $A_{2A}R$ WT mice by 1.7-, 1.5- and 1.7-fold, respectively, but not in $A_{2A}R$ KO mice, as compared with corresponding vehicle control (Fig. 2), suggesting that caffeine-induced increase in Thr 75 phosphorylation depends on the action on $A_{2A}R$. However, the roles of phosphorylation of Thr 75 of DARPP-32 in sleep-wake regulation need further investigation. We previously reported that activation of $A_{2A}R$ induces sleep in rats [12,13]. In striatopallidal neurons, activation of $A_{2A}R$ stimulates the cAMP/ protein kinase A (PKA) pathway [14] and blockade of $A_{2A}R$ results in the decease in PKA activity. It was recently demonstrated that phospho-Thr 75-DARPP-32 is an effective inhibitor of PKA [15], therefore, the caffeine-induced increase in the state of phosphorylation of Thr 75 would further lower PKA activity, thereby providing a positive feedback amplification mechanism for shutting down $A_{2A}R$-stimulated PKA signaling pathway.

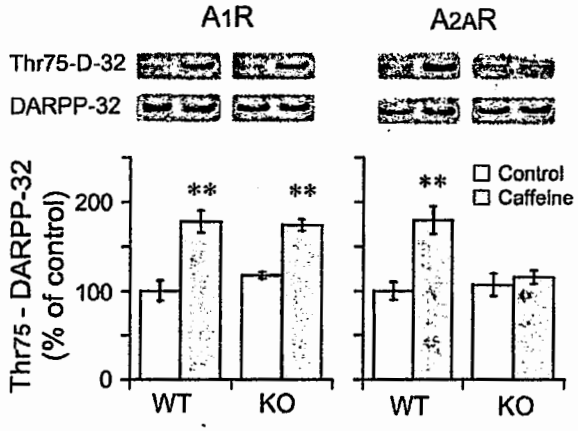

Figure 2 Effect of caffeine on DARPP-32 phosphorylation at thr 75 in WT and KO mice for A_1R and $A_{2A}R$. Mice were treated i.p. with caffeine (7.5 mg kg^{-1}) and decapitated 15 min after injection. The striatal levels of phospho-Thr 75-DARPP-32 were determined as described in Methods. Upper panels show representative image of immunoblot; lower panels show summary of data expressed as means ± s.e.m. (n = 6 - 8). The amount of phosphorylated DARPP-32 is expressed as a percentage of that determined after vehicle administration. Double asterisk, $P < 0.01$ versus vehicle-injected WT mice, Student's t-test.

In the striatum, $A_{2A}R$ are highly expressed postsynaptically by a large population of medium-sized spiny neurons [16,17] and the cells expressing $A_{2A}R$ represent about 50% of all DARPP-32-containing neurons [10]. These cells play a critical role in the functioning of the basal ganglia, a group of nuclei involved in the control of voluntary movements, as well as in motivational, emotional and cognitive aspects of motor behavior.

In conclusion, low doses of caffeine exhibited a striking wake-promoting effect in A_1R KO mice, but not in $A_{2A}R$ KO mice, clearly indicating that the wake-promoting effect of caffeine requires functioning $A_{2A}R$.

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