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Adenosine A₃ receptors regulate heart rate, motor activity and body temperature

Jiangning Yang^{1,*}, Yingqing Wang^{1,2,*}, Pablo Garcia-Roves¹, Marie Björnholm^{1,3}, and Bertil B. Fredholm^{1,†}

¹Department of Physiology & Pharmacology, Karolinska Institutet, S-171 77 Stockholm

²College of Pharmacy, Jilin University, 130021, China

³Department of Molecular Medicine and Surgery, Karolinska Institutet, S-171 77 Stockholm

Abstract

Aim—We wanted to examine the phenotype of mice that lack the adenosine A₃ receptor (A₃R).

Methods—We examined the heart rate, body temperature and locomotion continuously by telemetry over several days. In addition the effect of the adenosine analogue R - N⁶-phenylisopropyl-adenosine (R-PIA) was examined. In addition, we examined heat production and food intake.

Results—We found that the marked diurnal variation in activity, heart rate and body temperature, with markedly higher values at night than during day time, was reduced in the A₃R knockout mice. Surprisingly, the reduction in heart rate, activity and body temperature seen after injection of R-PIA in wild type mice was virtually eliminated in the A₃R knock-out mice. The marked reduction in activity was associated with a decreased heat production, as expected. However, the A₃R knock-out mice, surprisingly, had a higher food intake but no difference in body weight compared to wild type mice.

Conclusions—The mice lacking adenosine A₃ receptors exhibit a surprisingly clear phenotype with changes in e.g. diurnal rhythm and temperature regulation. Whether these effects are due to a physiological role of A₃ receptors in these processes or if they represent a role in development remains to be elucidated.

Keywords

Sex differences; heat production; body weight; diurnal; knock-out

INTRODUCTION

Of the four adenosine receptors, the A₃ receptor was the one most recently identified by cloning (Zhou *et al.* 1992). This receptor is often described as being “low affinity” (e.g. Bozarov *et al.* 2009) but there is evidence that at comparable receptor densities adenosine is as good an agonist at the A₃ receptor as it is on A₁ and A_{2A} receptors (Fredholm *et al.* 2001). Interestingly, inosine is a partial agonist at the A₃ receptor and this adenosine metabolite appears to mediate some responses via A₃ receptors (Jin *et al.* 1997; Gomez &

*These authors contributed equally

†To whom correspondence should be sent. Bertil B. Fredholm, Department of Physiology & Pharmacology, Karolinska Institutet, S-171 77 Stockholm, Bertil.fredholm@ki.se

Conflict of Interest.

There is no conflict of interest.

Sitkovsky 2003). The late discovery and the fact that there are major species differences in structure and pharmacology hampered the elucidation of its functional role(s) by pharmacological methods. The development of a knock-out mouse (Salvatore *et al.* 2000) has therefore been very useful to delineate physiological and pathophysiological situations where A₃ receptors are important (Yaar *et al.* 2005). In many ways the study of genetically modified mice has stimulated the interest in adenosine A₃ receptor pharmacology (Borea *et al.* 2009).

Already the first study on the knock-out mice emphasized that A₃ receptors are important in modulating responses of inflammatory cells (Salvatore *et al.* 2000). Activation of A₃ receptors enhances responses of mast cells (Jin *et al.* 1997; Salvatore *et al.* 2000; Zhong *et al.* 2003), which can further lead to increased vascular permeability (Tilley *et al.* 2000), inflammatory pain (Wu *et al.* 2002) as well as increased bronchoconstriction (Tilley *et al.* 2003; Hua *et al.* 2008). On the other hand, there can be an A₃ receptor-mediated reduction of the release of TNF α (Salvatore *et al.* 2000), and methotrexate, by increasing adenosine levels, appears to utilize this mechanism to reduce TNF α responses during inflammation (Montesinos *et al.* 2003). However, the role of A₃ receptors is less important than A_{2A} receptors in several circumstances (Kreckler *et al.* 2006; Montesinos *et al.* 2006). Adenosine A₃ receptors stimulate the migration of eosinophils (Young *et al.* 2004) and neutrophils (Chen *et al.* 2006b; Inoue *et al.* 2008; van der Hoeven *et al.* 2008).

There are also vascular effects. Although basal blood pressure is not altered in A₃R (-/-) mice the blood pressure fall after adenosine was enhanced (Zhao *et al.* 2000), and the coronary flow after adenosine A_{2A} receptor stimulation is higher in the A₃R knock-out mouse (Talukder *et al.* 2002). There are also A₃ receptors that mediate the ability of adenosine to, via changes to the glycocalyx, increase the vascular permeability (Platts & Duling 2004).

Elimination of A₃ receptors appears to increase resistance to ischemic damage in the heart (Cerniway *et al.* 2001; Guo *et al.* 2001), and the negative effect of pressure overloading (Lu *et al.* 2008) but does not alter early preconditioning (Guo *et al.* 2001; Eckle *et al.* 2007). Paradoxically activation of A₃ receptors also has been reported to confer resistance to myocardial ischemic damage (Harrison *et al.* 2002; Ge *et al.* 2006), and this occurs via activation of sarcolemmal k_{ATP} channels (Wan *et al.* 2008). Ischemic kidney damage is similarly reduced in mice lacking A₃ receptors (Lee *et al.* 2003) whereas ischemic brain damage is increased (Chen *et al.* 2006a), as is skeletal muscle ischemia (Zheng *et al.* 2007). Tissue damage following sepsis is reduced by endogenous A₃ receptor activation (Lee *et al.* 2006). Although the expression of A₃ receptor is restricted and occurs late during development the over expression leads to embryonic lethality (Zhao *et al.* 2002). It was recently reported that the hepatic glucose mobilization after ischemia/reperfusion was virtually completely eliminated by an antagonist of adenosine A₃ receptors (Cortés *et al.* 2009).

The behavioural consequences of an A₃ receptor deletion are complex: Basal locomotor activity is increased (Fedorova *et al.* 2003; Björklund *et al.* 2008a), the activity is reduced in tests assumed to predicted changes in mood (Fedorova *et al.* 2003), and the behavioural activation by caffeine and amphetamine was reduced as was the natural increase in activity when the animals enter the dark night time (Björklund *et al.* 2008a). The expression of A₃ receptors in brain is so limited that a role has been questioned (Rivkees *et al.* 2000), but it is clear that microglial cells and astrocytes possess functional A₃ receptors (Hammarberg *et al.* 2003; Björklund *et al.* 2008c). The presence in nerve cells is supported by the finding that apparently selective A₃ receptor activation increases serotonin transport in synaptosomes

(Zhu *et al.* 2007) and by effects on long term potentiation (LTP, Costenla *et al.* 2001), which are eliminated in A₃R knock-out mice (Maggi *et al.* 2009).

Thus, there are several indications that A₃ receptors are involved in several physiological and pathophysiological reactions. However, there is little data where unrestrained mice have been followed for longer periods of time with respect to basic physiological parameters. Here we report initial findings using such methodology following up on earlier studies where mice lacking A₁ and/or A_{2A} receptors have been examined (Yang *et al.* 2007, 2009a-b). We confirm that mice lacking A₃ receptors show clear alterations in activity (Björklund *et al.* 2008a), but we also demonstrate an unexpected role in temperature regulation and metabolism.

MATERIALS AND METHODS

Animals

The study was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US NIH, and was approved by the Animal Ethics Committee of Northern Stockholm. We used adenosine A₃ receptor deficient (A₃R^{-/-}) mice generated by Merck Research Laboratories (with permission from Drs Marlene Jacobson and Stephen Tilley) and wild type control mice (C57Bl/6). To minimize genetic drift there was frequent interbreeding to heterozygotes. The genotyping of A₃R^{-/-} mice was performed via using PCR. The mice were backcrossed repeatedly against C57Bl6 (more than 10 generations) to ensure that they were practically congenic.

The telemetry system

As previously described (Yang 2009), we used a telemetry system (Data Sciences, St. Paul, MN) consisting of implantable transmitters (TA10ETA-F20), telemetry receivers (DSI PhysioTel Receivers-RPC-1 Model), and eight universal adapters (UA 10 PC). Anaesthesia was induced and maintained by isoflurane. The transmitter was implanted into the peritoneal cavity and the leads were sutured in a lead II position. Mice were allowed to recover at least 7 days before the recordings of each individual mouse, kept in its own cage, were started. A computer program (Data quest A.R.T gold acquisition) sampled calibrated values of heart rate and body temperature as well as uncalibrated activity counts. After 5 days of baseline registration some of the mice received the adenosine analogue R(-)N6-(2-phenylisopropyl) adenosine (R-PIA, 50 µg/kg, Sigma, i.p.).

Whole Body Energy Homeostasis

Food intake, oxygen consumption, and locomotor activity were measured using a Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH). Mice were housed individually with *ad libitum* access to standard chow and water. Mice were acclimatized to the metabolic cages for 48 h prior to a 24-h period of automated recordings every 25 min. Oxygen consumption (VO₂) was determined by an open-circuit Oxyman. Oxygen consumption was calculated as the difference between the input oxygen flow and the output oxygen flow. The system will also calculate carbon dioxide production (VCO₂) and the respiratory exchange ratio (RER) as the ratio between VCO₂ and VO₂. The rate of heat production is calculated by first assessing the calorific value (CV) of the food being metabolized. The CV is based on the observed RER in a manner tabulated by Lusk (1928). The equations are: Heat = CV × VO₂ - CV = 3.815 + 1.232 × RER and from this heat production was calculated. Ambulatory locomotor activity was measured by consecutive beam breaks in adjacent beams under the 24 h period.

Statistical analysis

Student's t-test or ANOVA were employed for evaluation of differences between groups, using the procedures in GraphPad Prism.

RESULTS

Heart rate

Basal heart rate differed between male and female mice, as reported earlier (Yang *et al.* 2007). In male mice there was little difference between $A_3R^{+/+}$ and $A_3R^{-/-}$ mice (Fig 1). By contrast, there was a major difference between genotypes in the females. Whereas female $A_3R^{+/+}$ mice showed the expected large diurnal variation, with higher rates at night, especially in the beginning, the heart rate curve was essentially flat in the $A_3R^{-/-}$ mice (Fig 1). Another major surprise was that the well known cardio depressant effect of the adenosine analogue R-PIA, which is commonly ascribed to an action on cardiac A_1 receptors (Johansson *et al.* 2001; Yang *et al.* 2007), was virtually eliminated in the $A_3R^{-/-}$ mice (Fig 2). This was true for male as well as female mice.

Body temperature

The registration of body temperature generated results that were very similar to those for heart rate, as reported earlier in other genotypes (Yang *et al.* 2007). Temperature was somewhat higher in females, than in males, and the diurnal variation normally seen in female mice was absent in the mice lacking A_3R receptors (Fig 3). Furthermore, the substantial decrease in body temperature induced by R-PIA was essentially eliminated in the $A_3R^{-/-}$ mice of both sexes (Fig 4).

Locomotor activity

As expected the results from measurements of locomotion gave similar results. As reported earlier the increased over all activity seen in female mice when the light is about to turn off was essentially eliminated in the $A_3R^{-/-}$ mice (Fig 5) (Björklund *et al.* 2008b). This was not seen to the same extent in the male mice studied with this technique (but see below). When animals were given an injection of R-PIA they initially were stimulated, but in the wild type animals this was followed by a long fall in activity both in males and females (Fig 6). In the $A_3R^{-/-}$ mice, by contrast, there was no apparent depression of activity.

Locomotor activity could also be determined in the clams system. As seen in Fig 7 when this way of recording locomotion was used there appeared to be a clear-cut reduction in night time activity not only in females, but also in males (Fig 7, A, B, E), however in males it was not statistically significant if calculated as groups means. By contrast there were no significant reduction in day time activity (Fig 7D).

Heat production

From measurements of oxygen consumption heat production can be calculated. As expected, given the decreased physical activity, the $A_3R^{-/-}$ mice exhibited a lower heat production, especially at night (Fig 8). During the light period, $A_3R^{-/-}$ mice produce 0.363 ± 0.021 kcal heat in one hour, which is significantly, $p < 0.001$, less than the WT mice (0.422 ± 0.024 kcal/hr); in the dark, both genotypes had a higher heat production, but $A_3R^{-/-}$ mice (0.420 ± 0.021 kcal/hr) still produced significantly, $p < 0.0001$, less than WT mice (0.513 ± 0.024 kcal/hr).

Food intake and weight gain

Given the lower activity and the lower heat production we expected to see a lower food intake as well. However, to our surprise, $A_3R^{-/-}$ mice actually consumed more food than the corresponding wild types (Figure 9). During light time, on average, each male WT mouse consumed 0.073 ± 0.001 gram (g) in one hour, and the $A_3R^{-/-}$ mouse consumed almost 30 % more (0.109 ± 0.015 g/hr); during the dark time, the male WT mouse consumed about twice as much (0.171 ± 0.020 g/hr), and the $A_3R^{-/-}$ mouse still consumed more (0.203 ± 0.021 g/hr). Essentially similar data were obtained in females. The increased food intake in the face of lower heat production suggests a major increase in body weight. However, this was not observed. Instead the weight gain with age in $A_3R^{+/+}$ and $A_3R^{-/-}$ mice, could be virtually superimposed, but with different curves for males and females (Figure 10).

DISCUSSION

The present study has revealed several surprisingly strong phenotypes for the $A_3R^{-/-}$ mouse, and there are a number of novel observations. The first major finding was that much of the effect of the purportedly A_1 selective agonist R-PIA on heart rate, body temperature and activity was lost in the $A_3R^{-/-}$ mouse. R-PIA was one of the first adenosine analogues used in the characterizations of adenosine receptors and was used in the initial definition of A_1 receptors (Vapaatalo *et al.* 1975; van Calker *et al.* 1979; Londos *et al.* 1980). When it was shown that much (if not all) the cardiodepression and temperature lowering effect of R-PIA was lost in $A_1R^{-/-}$ mice (Johansson *et al.* 2001; Yang *et al.* 2007) the case appeared settled. However, it has been known that R-PIA is also quite potent as a ligand at A_3 receptors (Klotz *et al.* 1998). Our finding indicates that both A_1 and A_3 receptors are important. They also suggest that some of the responses hitherto solely ascribed to A_1 receptors may actually also have a component dependent on A_3 receptors. It is important to note that there is no change in the expression of A_1 receptors in brains of A_3R knock-out mice (Björklund *et al.* 2008b).

The second major finding is the disruption of the normal diurnal rhythm seen in especially female mice lacking the A_3 receptor. We have earlier noted that the arousal induced by pharmacological agents such as caffeine and amphetamine is also reduced in these mice (Björklund *et al.* 2008b). This suggests that the A_3 receptor is not specifically involved in regulation of diurnal rhythm, but rather that they play some role in arousal, irrespective of how it is induced. In the experiments with telemetry there appeared to be a very clear sex difference, but this was less clear-cut in the experiments using the CLAMS system. We have no good explanation for this apparent sex difference, but we note that the reduction in the effect of stimulatory drugs is seen in both sexes (Björklund *et al.* 2008b).

The third major finding relates to the surprising fact that the lower heat production was accompanied by increased food consumption. The decreased heat production was expected from the lower activity of the animals and we have no good reason to doubt this measurement. The food consumption is directly assessed by the system and again we have no good reason to doubt that measurement. A high food intake in the presence of decreased energy consumption/activity is obviously a recipe for considerable weight gain. However, we found no evidence for this in the A_3R knock-out mice. It could be that the CLAMS system we used provides a completely artificial situation and the activity and food intake etc in the “normal” situation of the mice is quite different. We have little reason to assume that this is the case as for example activity measurements with telemetry gave at least somewhat similar results. An alternative possibility is that the absorption of nutrients is compromised in A_3R knock-out mice and future work will try to test this possibility. We will also test if the body composition is altered even if the body weight is not.

Given that the A₃ receptor was unknown for so long and that adenosine physiology and pharmacology were studied so long (without finding evidence for A₃ receptors) the long list of phenotypes of the mice with a targeted A₃ receptor gene deletion is surprising. In particular, quite a lot of the data indicate an altered basal physiology. Physiological adenosine levels are low (Zetterström *et al.* 1982; Lönnroth *et al.* 1989; Ballarín *et al.* 1991), and such concentrations are expected only to activate adenosine receptors where they are quite abundant (Fredholm *et al.* 2005). A₃ receptors are generally not very abundant, and we are therefore left with a conundrum. This is amplified by the fact that often when a physiologically important mechanism is ablated genetically there are compensations. We certainly do not know the solution to this, but believe that one should consider the possibility that A₃ receptors play an important role in development and that some of the phenotypes we observe are, in fact, consequences of such early developmental effects. We think it is possibly relevant in this context that, as indicated in the Introduction, A₃ receptors do play a role in the migration of eosinophils and neutrophils (Young *et al.* 2004; Chen *et al.* 2006b; Inoue *et al.* 2008; van der Hoeven *et al.* 2008) and possibly other cells, and in the regulation of the extracellular matrix (Platts & Duling 2004). Irrespective of whether A₃ receptors regulate physiology or regulate the development of the physiological systems, it is clear that much work is needed to further elucidate their roles.

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Figure 1.

Registrations of heart rate (HR) in male [wild type (WT), $n=9$; A_3R knockout ($A_3R^{-/-}$), $n=13$; shown in [panel A](#) and female (WT, $n=13$; $A_3R^{-/-}$, $n=13$; shown in [panel B](#)) mice. The dark period (shadowed) is from 7 PM to 7 AM. Data are shown as a 5-day average and at 10 min intervals; data are presented as mean values $\pm$ SE. The indicated p- value refers to the result of a 2-way ANOVA.

Figure 2.

Effects of R(-)N6-(2-phenylisopropyl) adenosine (R-PIA, 50 µg/kg) on heart rate in $A_3R^{-/-}$ mice. R-PIA was administered into male [wild type (WT), $n=5$; A_3 adenosine receptor knockout ($A_3R^{-/-}$), $n=9$] and female (WT, $n=10$; $A_3R^{-/-}$, $n=5$) via intra *peritoneum*. The heart rate was measured at 10-min intervals until 240 min after injection. Data are presented as means $\pm$ SE (*compared with WT, $P < 0.05$). Abbreviations: bpm, Beats/min.

Figure 3.

Registrations of body temperature for consecutive 10-min periods in male [wild type (WT), n=9; A₃R knockout (A₃^{-/-}), n=13]; shown in [panel A](#) and female (WT, n=13; A₃^{-/-}, n=13) mice; shown in [panel B](#). The dark period (shadowed) is from 7 PM to 7 AM. Data are shown as a 5-day average; data are presented as mean values $\pm$ SE (* p < 0.05 compared with WT mice). The significance value refers to results of a 2-way ANOVA.

Figure 4.

Effects of R(-)N6-(2-phenylisopropyl) adenosine (R-PIA, 50 µg/kg i.p.) on body temperature in $A_3^{-/-}$ mice. R-PIA was administered into male [wild type (WT), n=5; A_3 adenosine receptor knockout ($A_3^{-/-}$), n=9] and female (WT, n=10; $A_3^{-/-}$, n=5). The body temperature was measured at 10-min intervals until 240 min after injection. Data are presented as means $\pm$ SE (*compared with WT, $P < 0.05$).

Figure 5.

Baseline registrations of activity are recorded for consecutive 30-min periods in male [wild type (WT), $n=9$; A_3R knockout ($A_3^{-/-}$), $n=13$; shown in [panel A](#)] and female (WT, $n=13$; $A_3^{-/-}$, $n=13$; shown in [panel B](#)) mice. The dark period is from 7 PM to 7 AM. Data are shown as a 5-day average; data are presented as mean values $\pm$ SE ($*p < 0.05$ compared with WT mice).

Figure 6.

Effects of R(-)N6-(2-phenylisopropyl) adenosine (R-PIA, 50 μ g/kg) on activity in male (Panel A) and $A_3^{-/-}$ mice (Panel B). R-PIA was administered into male [wild type (WT), $n=5$; A_3 adenosine receptor knockout ($A_3^{-/-}$), $n=9$] and female (WT, $n=10$; $A_3^{-/-}$, $n=5$) via intra *peritoneum*. The activity was measured at 30-min intervals until 240 min after injection. Data are presented as means $\pm$ SE (*compared with WT, $P < 0.05$).

Figure 7.

Locomotor activity differences between WT mice and A3^{-/-} mice.

Panel A and Panel B show consecutive locomotor activities in 24 hr. Each point represents number of ambulatory activities during 25min (mean $\pm$ SE). Both in male (WT, n=6; A3^{-/-}, n=7) and female (WT, n=4; A3^{-/-}, n=6), A3^{-/-} mice moved much less than WT mice, especially during the dark time, $p < 0.0001$ (2 way ANOVA). Panel C and D are the activities in light and dark time separately. * $p < 0.05$.

Figure 8.

Heat Production in male WT mice and A3^{-/-} mice (WT, n=6; A3^{-/-}, n=7). The figure shows calculated heat production, at 25 min intervals, over the 24 hr period. Each point represents mean ± SEM. WT mice produced significantly more heat ($p < 0.001$) than A3^{-/-} mice when calculated over the entire period.

Figure 9.

Food intake in male WT and A3^{-/-} mice. Panel A shows accumulated food intake in 24 hr (from noon the first day to noon the second day) of male mice (WT, n=6; A3^{-/-}, n=7).

A3^{-/-} mice consumed much more food during 24hr than WT mice, $p < 0.0001$ (2 way ANOVA). Each point represents accumulated food intake of every 25mins (mean $\pm$ SE).

Figure 10.

Body weight of male and female wild type (WT) and A3 -/- mice with increasing age. Data are mean and S.D. Wild types males were 35, 15 and 17 in the different age groups, wild type females were 40, 10 and 19; A3 -/- males were 20, 8 and 16, and A3 -/- females were 45, 7 and 26 in the age brackets 2-3 months, 3-5 months and 5-9 months.