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BRIEF COMMUNICATION

Modulating P1 Adenosine Receptors in Disease Progression of SOD1^{G93A} Mutant Mice

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal progressing neurodegenerative disease; to date, despite the intense research effort, only two therapeutic options, with very limited effects, are available. The purinergic system has been indicated as a possible new therapeutic target for ALS, but the results are often contradictory and generally confused. The present study was designed to determine whether P1 adenosine receptor ligands affected disease progression in a transgenic model of ALS. SOD1^{G93A} mice were chronically treated, from presymptomatic stage, with a selective adenosine A_{2A} receptor agonist (CGS21680), antagonist (KW6002) or the A_1 receptor antagonist DPCPX. Body weight, motor performance and survival time were evaluated. The results showed that neither the stimulation nor the blockade of adenosine A_{2A} receptors modified the progressive loss of motor skills or survival of mSOD1^{G93A} mice. Conversely, blockade of adenosine A_1 receptors from the presymptomatic stage significantly attenuated motor disease progression and induced a non-significant increase of median survival in ALS mice. Our data confirm that the modulation of adenosine receptors can elicit very different (and even opposite) effects during the progression of ALS course, thus strengthens the importance of further studies to elucidated their real therapeutic potential in this pathology.

Keywords Adenosine A_{2A} receptors (A_{2A}Rs) · Adenosine A_1 receptors (A_1Rs) · Amyotrophic lateral sclerosis (ALS) · SOD1^{G93A} mice

Introduction

Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disease which compromises motor functions and progresses in severity until death. ALS is actually considered a multi-factorial disease, which partially explains why, in spite of the intense basic research efforts, it still represents an unmet medical need. Indeed, despite the many molecules tested in phase II and phase III clinical trials, Riluzole and Edaravone are the only drugs approved, and they only offer minimal advantages for patients.

A potential beneficial effect of caffeine (the most widely used psychostimulant in the world) in preventing ALS was suggested by Beghi and coworkers, who found a reduced risk of developing ALS in patients taking caffeine [1]. Such an association was however not always confirmed by subsequent studies [2, 3].

Caffeine is a non-selective antagonist of the metabotropic adenosine receptor family comprising the more prominent A_1 (A_1Rs) and A_{2A} receptors (A_{2A}Rs), the subtypes A_{2B} and A_3Rs, which typically show lower expression levels and are in most cases only stimulated by high, pathological adenosine concentrations. Considering that A_{2A}R antagonists are viewed as potential neuroprotective drugs, and that the effects of chronic caffeine administration are mainly ascribed to A_{2A}R blockade, in a previous work we tested caffeine as a potential therapeutic approach in mSOD1^{G93A} ALS mice [4]. Contrarily to our hypothesis, however, we found that the drug dramatically reduced mice survival. We

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then argued that the detrimental effects of caffeine were due to $A_{2A}R$ blockade, and hypothesized that the stimulation of these receptors might rather be protective. In line with this view, Yanpallewar et al. [5] demonstrated that the selective $A_{2A}R$ agonist CGS21680 slowed the onset of motor neuron degeneration and muscle weakness when administered to mutant $SOD1^{G93A}$ ALS mice starting from presymptomatic phases. On the other hand, however, partial genetic ablation and pharmacological inhibition of $A_{2A}Rs$ with the selective antagonist KW6002 (Istradefylline) has been reported to delay disease progression when administered to symptomatic mice of the same strain [6]. Ng and coworkers also demonstrated that $A_{2A}R$-mediated adenosine signaling induces motor neuron cell death "in vitro".

These contrasting findings reveal that the role exerted by $A_{2A}Rs$ in this pathology is very complex; furthermore, the fact that the effects exerted by A_1 and A_{2A} receptors at the neuromuscular junction of $SOD1^{G93A}$ mice change during the course of the disease [7] adds further complications.

With the purpose of increasing our knowledge of the field, we tested, under the same experimental conditions used in the caffeine study, the effects of selective ligands of adenosine A_{2A} and A_1 receptors on the disease severity and survival of ALS mice.

Materials and Methods

We used $SOD1^{G93A}$ mutant and wild-type (WT) transgenic mice bred by 'The Jackson Laboratory' under the strain designations B6SJL-TgN ($SOD1^{G93A}$) 1 Gur/J and B6SJL-TgN (SOD1) 2 Gur/J, respectively. Only male mice were used in this study.

The animals were kept under standardized temperature, humidity and lighting condition, with free access to water and food (standard pellets). Animal care and use followed the directives of the Council of the European Communities (86/609/EEC) and adequate measures were taken to minimize pain or discomfort. When mice showed substantial motor impairment pellets soaked in normal water, were supplied inside the cage. The experimental protocol was approved by the Italian Ministry of Health.

Drugs used were KW6002 (Istradefylline), 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride (CGS21680) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX).

Stock solutions of CGS21680 and DPCPX were made in dimethyl sulfoxide (DMSO). At the maximal concentration applied to the preparation the DMSO concentration was 0.5%.

On the basis of our own experience on the motor depressant effects of CGS21680 in mice [8, 9] and taking into account the range of "protective doses" reported by Chou et al. [10] in a mouse model of Huntington's disease, 2.5 mg/kg/day of CGS21680 were administered intraperitoneally (i.p.) five times per week from 70 days of age until death.

The A_1 antagonist DPCPX was administered at 0.75 mg/kg i.p. five times per week from 70 days of age. The dose was selected on the basis of our preliminary experiments, showing that it is devoid of any significant effects on locomotor activity (Potenza et al. unpublished results).

KW6002 (or Istradefylline) was synthesized as previously described [11]. A stock solution of KW6002 (0.25 mg/ml, 0.5% methylcellulose) was daily diluted in drinking water and administered per os. Mice were given ad libitum access to normal water (0.03% methylcellulose, Veh) or water mixed with drug. The water intake/mouse was calculated three times a weeks. On average, each mouse drank 5 ml per day yielding a daily KW6002 dose of 3 mg/kg.

As measures of disease progression body weight, motor function and survival time were evaluated.

Body weight measurements were recorded for each animal three times a week from 9 a.m. to 12 a.m. to avoid diurnal variations. Motor function was tested using an accelerated rotarod device (Columbus Instruments, Columbus, OH). At 9 weeks of age mice were trained for 2 days to become acquainted with the rotarod. The testing began at 70 days of age using a ramped accelerating program: the animals were positioned on the rotating bar, time was started and the rod was accelerated at a constant rate from 8 to 32 rpm for a maximum of 180 s. Mice were given three consecutive trials (10 min of interval), and for each animal the longest latency to fall was recorded as a measure of the motor function competence. Rotarod testing was performed once a week until the animals reached the pre-established minimum performance (5 s).

Disease onset was defined as the time (days) at which a 10% reduction in rotarod performance of the $mSOD1^{G93A}$ mice was observed. The survival time was considered as the actual age of death or the time (defined as end stage) when mice were sacrificed because of the loss of the ability to right themselves within 30 s after having been placed on their sides, according to established guidelines for drug testing in ALS mouse models [12].

Disease onset was statistically analyzed by log-rank test and Fisher's exact probability test. Kaplan–Meier survival analysis and Logrank (Mantel–Cox) were used for survival comparisons.

Data of body weight and rotarod performance were analyzed with Holm–Sidak *t* test for multiple comparisons. Mice died unexpectedly before reaching the very symptomatic stage, presumably due to injury caused by the intraperitoneal injection, were excluded from the statistic survival analysis; however, their clinical data until the day before their death were retained.

Results

Effects of $A_{2A}R$ Stimulation or Blockade

As expected for this mutant mice strain, $mSOD1^{G93A}$ mice treated with vehicle reached maximum weight at 100 days of age; from this time the weight started to decrease gradually until the end stage.

The statistical analysis performed from the start of treatment up to the 101th day of life, when all the animals are still alive and the body weight is minimally affected by the progression of the disease, shows that chronic treatment with CGS21680 induces a significant loss of weight when compared with Veh-treated animals (Fig. 1a; *$p < 0.01$ Holm–Sidak *t* test for multiple comparisons). Although in our study we didn't measure food intake, this effect could be related to the modulation of feeding behaviour exerted by A_{2A} receptor activation in the striatum [13, 14]. Furthermore, the pharmacological stimulation of A_{2A} receptors was recently proposed as a potential anti-obesity approach in light of its ability to induce lipolysis in brown adipocytes with an increase in energy expenditure [15, 16].

Conversely, no significant weight changes were observed in KW6002-treated as compared with control $mSOD1^{G93A}$ mice (Fig. 1b).

Neither the A_{2A} agonist nor the antagonist affected the onset and the progressive loss of motor skills of $mSOD1^{G93A}$ mice: values for rotarod performance of drug-treated $mSOD1^{G93A}$ mice (CGS21680 2.5 mg/kg n = 12; KW6002 3 mg/kg n = 10) and vehicle-treated $mSOD1^{G93A}$ (Veh (CGS) n = 10; Veh (KW) n = 8) were indistinguishable ($p > 0.05$ vs. ALS-vehicle group; Holm–Sidak *t* test for multiple comparisons).

The median survival of Veh and treated mice was also superimposable in the four groups (median survival = 128, 127 and 126 days for both Veh groups, CGS21680 and KW60021 respectively).

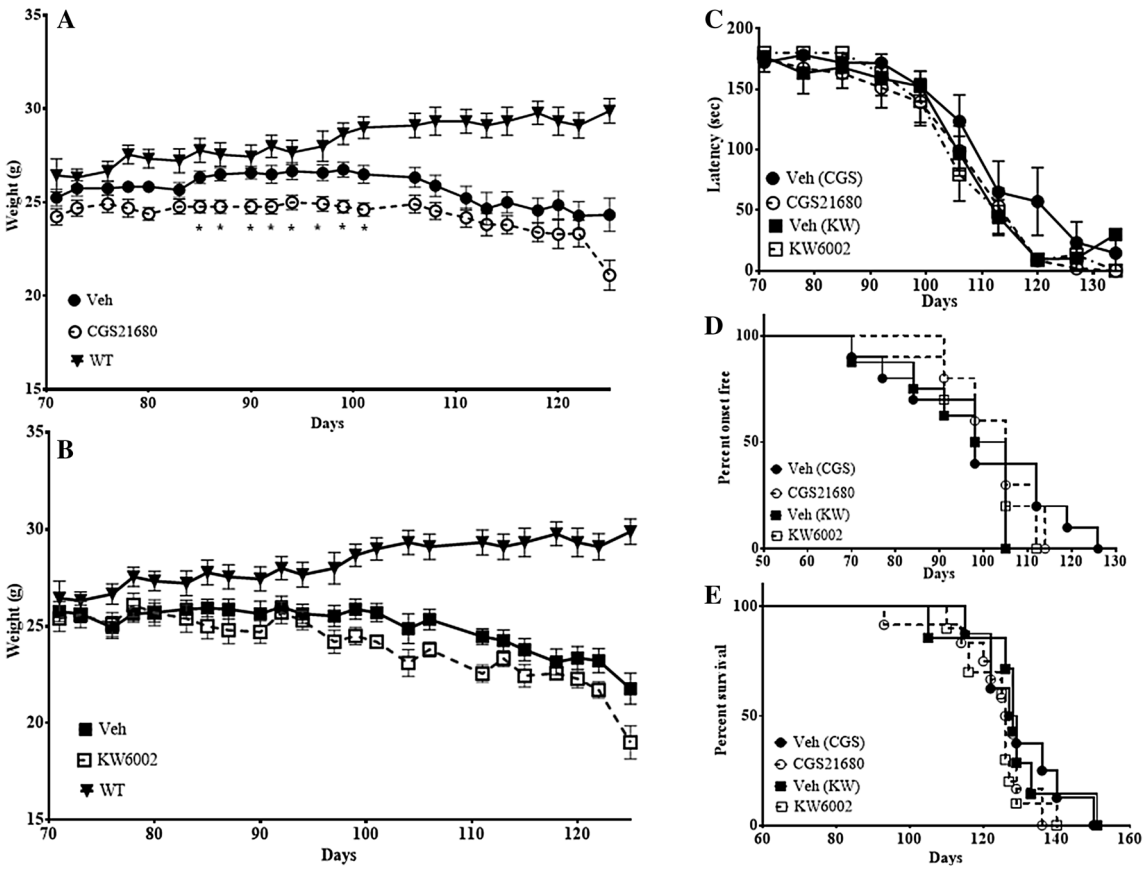

Fig. 1 Effect of A_{2A} ligand administration on body weight, rotarod test, motor onset and survival. Chronic administration of the $A_{2A}R$ agonist CGS21680 (**a**) (2.5 mg/kg i.p.) significantly worsened the pathological decrease of body weight in $mSOD1^{G93A}$ mice (**a**; *$p < 0.01$ Holm–Sidak *t* test for multiple comparisons). The $A_{2A}R$ antagonist KW6002 (3 mg/kg) showed no significant effect on body weight of $mSOD1^{G93A}$ mice (**b**). Chronic administration of both $A_{2A}R$ ligands (the agonist CGS21680 2.5 mg/kg or the antagonist KW6002 3 mg/kg) did not affect the progressive loss of motor skills (**c**), the disease onset (**d**) or survival (**e**) of $mSOD1^{G93A}$ mice. Kaplan–Meier survival analysis and Logrank (Mantel–Cox) were used for onset and survival comparison

Effects of A_1R Blockade

A_1R desensitization has been consistently reported to occur in cells, brain slices or intact animals after prolonged exposure to receptor agonists (for a review see [17]). For this reason, given the chronic nature of the treatment to be done, in our study we decided to only test the effects of a selective A_1R antagonist (DPCPX).

As shown in Fig. 2c, although there was no statistically significant difference in the overall disease onset, after 4 weeks of treatment (98 days of age), the number of disease-free animals treated with DPCPX (0.75 mg/kg n = 10) was significantly greater than that of vehicle treated animals (n = 12) (Fisher's exact probability test p = 0.02). Accordingly, DPCPX-treated mice showed a better motor performance throughout the trial, reaching a statistically significant difference in the 16th week of life (Fig. 2b; *p < 0.01 Holm–Sidak *t* test for multiple comparisons).

Furthermore, the A_1R antagonist induced a tendency (not significant) to increase the median survival of $mSOD1^{G93A}$ mice (Fig. 2d; median survival = 128 and 136 days for Veh and DPCPX respectively). No effects were observed on the weight loss induced by ALS progression (Fig. 2a).

Discussion

Adenosine signaling plays important roles in many physiological and pathological conditions through the activation of G protein-coupled receptors (A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R) expressed in a variety of cells; in the central nervous system, in particular, adenosine behaves as a neuromodulator acting through its high affinity A_1 and A_{2A} receptors.

In ALS patients, a significant increase of $A_{2A}Rs$ in lymphocytes, and a positive correlation between $A_{2A}R$ density and ALS Functional Rating Scale was demonstrated [18]. This finding, together with a number of experimental evidence collected in many models of neurodegenerative diseases [19–22], encouraged the idea of $A_{2A}R$ as a possible therapeutic target for ALS. Despite the intense research on this topic, however, the picture is still far from clear.

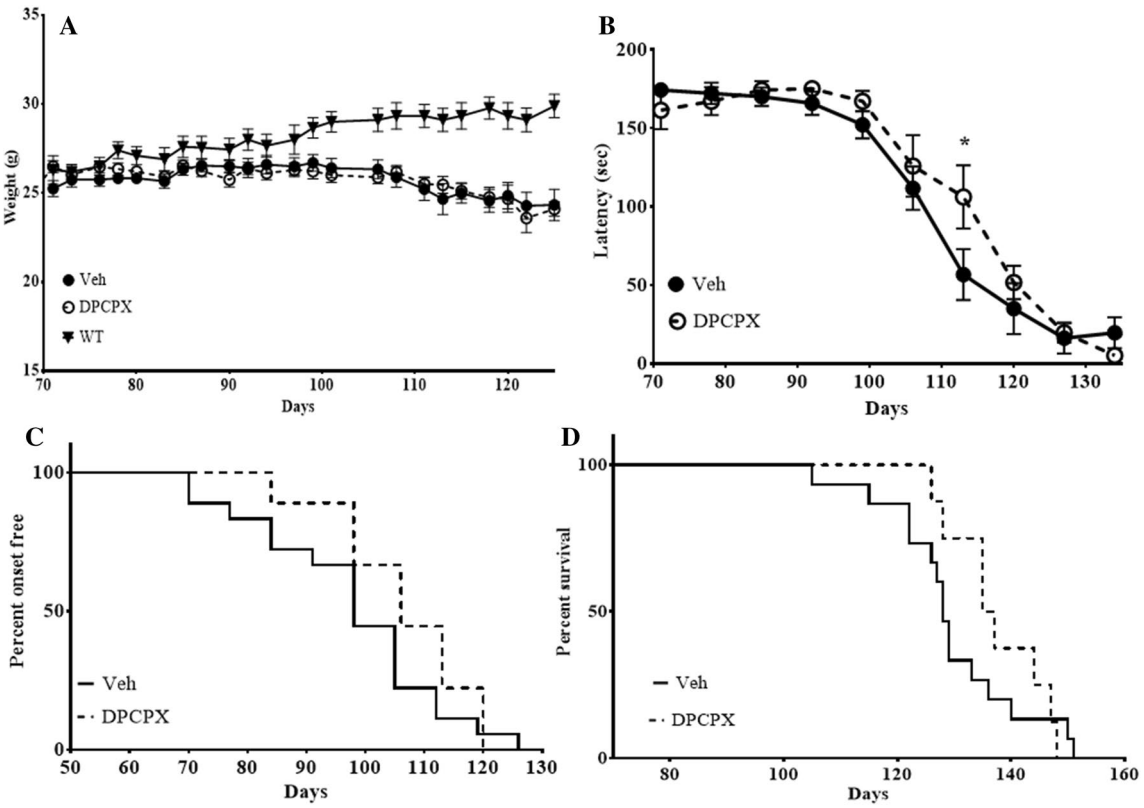

Fig. 2 Effect of A_1 antagonist administration on body weight, rotarod test, motor onset and survival. Body weight loss of $mSOD1^{G93A}$ mice was not changed by treatment with DPCPX (0.75 mg/kg, administered i.p. from 70 days of age until death) (**a**). Mice treated with DPCPX showed a better motor performance (**b**) throughout trial reaching a statistically significant difference in the 16th week of life (*p < 0.01 Multiple *t* test). The A_1R antagonist induced a tendency (not significant) to increase median disease onset (**c**) and survival (**d**) of DPCPX treated $mSOD1^{G93A}$ mice. Kaplan–Meier survival analysis and Logrank (Mantel–Cox) were used for onset and survival comparison

As a matter of fact, both a selective $A_{2A}R$ agonist [5] and a selective $A_{2A}R$ antagonist [6] were shown to delay disease onset or progression in SOD1 mice. These contrasting findings, reported in the same mice model of ALS, reveal that the role exerted by $A_{2A}Rs$ in this disease is very complex.

Considering that $A_{2A}R$ function remarkably changes with ALS progression [7, 23], to avoid a potential bias linked to disease stage, we started our treatments at the same time (10 week of age) as established in our previous caffeine trial. Under this experimental condition, neither the stimulation nor the blockade of $A_{2A}Rs$ influenced the course of the disease. These findings, although apparently at odds with the above cited studies, can however be explained in light of the recent, comprehensive appraisal of Sebastiao et al. [24]. In their elegant review on the role of adenosine receptors in ALS, the authors indeed propose that there are specific time windows where $A_{2A}R$ stimulation is beneficial and others where such receptors turn detrimental, and their blockade becomes protective instead. In particular, $A_{2A}R$ agonists appear to be beneficial in the presymptomatic/early stages of the disease (i.e. 8 weeks, [5]), while the A_{2A} antagonist KW6002 was shown to be protective when administered starting from more advanced phases (namely 13 weeks [6]). The lack of effects reported in our study (in which treatments were started at 10 weeks), seems to indicate that the time windows hypothesized by Sebastiao et al. not only exist, but are also rather narrow: 10 weeks could represent a phase of the disease in which the agonist is no longer, and the antagonist not yet, effective. This intriguing hypothesis, which is in line with the time-dependence of $A_{2A}R$ effects already reported in other neurodegenerative diseases (see [19, 25, 26] for reviews), deserves further investigation. Anyway, a first consequence of the present findings is that the detrimental effects of caffeine were not due to the simple blockade of $A_{2A}Rs$, as we had hypothesized at the time.

As for our finding of a basically beneficial effect of DPCPX, it is even more complicated, and raises at least two questions: why should an antagonist of A_1Rs be protective? And, why was caffeine found detrimental, considering that under our experimental conditions the $A_{2A}R$ antagonist is ineffective, and the A_1 antagonist even tends to be beneficial? As for the first question, our results can be explained by the increased tonic activation of A_1Rs (which have an inhibitory influence on neuromuscular transmission) reported in symptomatic ALS mice [7].

In order explain the discrepancy between the effects of caffeine and those of DPCPX, it should first be considered that after chronic caffeine treatment, a tolerance to the effects of A_1R blockade occurs [17], thus clearly indicating that the effects of caffeine and DPCPX cannot be superimposable.

Another important point to take into account is that, after chronic (oral) caffeine intake a remarkable increase in extracellular levels of adenosine has been reported in rodents [27]. It is thus conceivable that, while DPCPX is rather beneficial since it removes the aberrant inhibition exerted by A_1Rs in symptomatic phases, caffeine is detrimental because besides becoming unable to block A_1Rs due to the occurrence of tolerance—it further contributes to the activation of A_1Rs by increasing adenosine levels. This hypothesis, of course, needs to be confirmed by specific studies. As much worthy of future investigations is the potential involvement of A_1/A_{2A} receptor heteromers in the effects exerted by both selective and unselective ligands. Even though—to the best of our knowledge—the existence of those heteromers has not been documented so far at the neuromuscular junction, the propensity of A_1 and $A_{2A}Rs$ to heteromerize when they are co-expressed could represent a general phenomenon. Very interestingly, functional A_1/A_{2A} heteromers exist in cortico-striatal glutamatergic terminals, where they modulate glutamate release [28]. This point could be of particular relevance, since A_1/A_{2A} receptor heteromers are considered as a specific target for caffeine [29].

In conclusion, our data confirm that the modulation of adenosine receptors can elicit very different (and even opposite) effects during the progression of ALS, thus adding further complexity to their role (and weakening their therapeutic potential) in this disease. Finally, although the present results show that the effects of caffeine are not reproduced by selective A_{2A} or A_1 receptor blockade, the exact mechanisms of action of this drug in ALS mice remain to be elucidated.

Author Contributions All co-authors have seen and agree with the contents of the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution and approved by the Italian Ministry of Health (Decree 118/2014B).

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