PERSPECTIVE

P2X7 receptor antagonism in amyotrophic lateral sclerosis

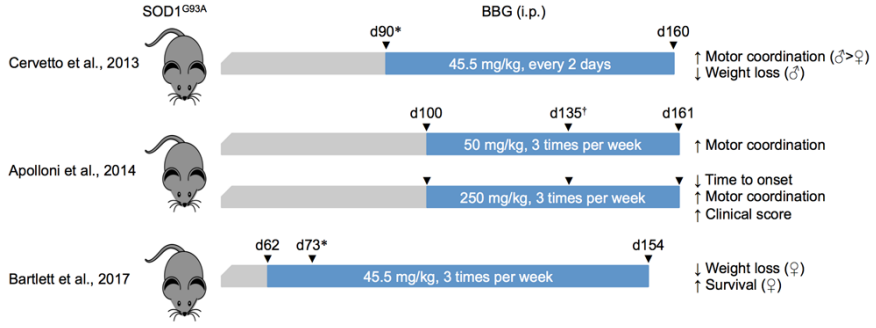
Amyotrophic lateral sclerosis (ALS) is a rapidly progressive disorder characterized by the degeneration and subsequent loss of upper and lower motor neurons, resulting in reduced muscle function and paralysis (Mathis et al., 2017). Current therapies are limited to the oral administration of riluzole, which improves survival by only 3 months (Mathis et al., 2017). Thus, new therapies are urgently required to treat this neurodegenerative disorder. Over the past decade, the intercellular signaling pathway involving extracellular adenosine triphosphate (ATP) activation of the P2X7 receptor channel has emerged as a potential therapeutic target in ALS (Volonte et al., 2016). To this end, we evaluated recently whether the P2X7 antagonist, Brilliant Blue G (BBG), could alter disease progression in a murine model of ALS (Bartlett et al., 2017). In this model, mice overexpress a human variant of mutant superoxide dismutase 1 ($SOD1^{G93A}$) and typically develop clinical signs of ALS from day 70 or later depending on the laboratory, and the transgene copy number, gender and genetic background of the mice (Heiman-Patterson et al., 2005). Mutations in the gene encoding SOD1 (including $SOD1^{G93A}$) are associated with 15–20% of familial ALS cases in humans, with human $SOD1^{G93A}$ transgenic mice being one of the most commonly used animal models of this disease (Heiman-Patterson et al., 2005).

In our study (Bartlett et al., 2017), intraperitoneal (i.p.) injection of BBG (45.5 mg/kg) into $SOD1^{G93A}$ mice 3 times per week from 62 days of age (late pre-onset) delayed weight loss and prolonged survival of female, but not male, mice. BBG treatment also delayed weight loss in both genders combined. BBG treatment had no effect on clinical score or motor coordination in either gender, nor did it prevent motor neuron loss, microgliosis, or affect the amount of lumber SOD1 or P2X7 protein at end-stage. Therefore, results from this study demonstrate that P2X7 antagonism with BBG has some therapeutic benefit in ALS, at least in female $SOD1^{G93A}$ mice.

During the course of our pre-clinical trial (Bartlett et al., 2017), two similar studies were published (Cervetto et al., 2013; Apolloni et al., 2014). These studies also reported some therapeutic benefit with BBG treatment in $SOD1^{G93A}$ mice, but with notable differences to our study (**Figure 1**). In the first of these studies (Cervetto et al., 2013), injection of BBG (45.5 mg/kg i.p.) every 2 days from day 90 (onset) improved motor coordination in mice of either gender, with greater improvement in male than female mice. BBG treatment also delayed weight loss in male and both genders combined, but not in female mice. BBG treatment did not alter survival. In the second of these studies (Apolloni et al., 2014), injection of BBG (50 mg/kg i.p.) 3 times per week from day 100 (late pre-onset) improved motor coordination, but did not alter disease onset or survival, with no differences observed between genders. Injection of BBG (50 mg/kg i.p.) 3 times per week from day 135 (onset) had no effect on motor coordination, disease onset or survival (Apolloni et al., 2014). $SOD1^{G93A}$ mice were also injected with a higher dose of BBG (250 mg/kg i.p.) 3 times per week from either day 40 (asymptomatic), day 70 (pre-onset) or day 100 (late pre-onset) (Apolloni et al., 2014). BBG treatment from day 40 or 70 did not alter any observed disease parameter, but BBG treatment from day 100 delayed disease onset, and improved clinical score and motor coordination, but did not delay weight loss or prolong survival. BBG treatment from day 100 also increased motor neuron survival, and reduced microgliosis and pro-inflammatory markers (nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and interleukin-1β) at end-stage. As with treatment using BBG at 50 mg/kg, no differences were observed between genders with this compound at 250 mg/kg.

The effect of BBG treatment on the survival of female $SOD1^{G93A}$ mice in our study (Bartlett et al., 2017) paralleled the prolonged survival observed in female, but not male, heterozygous and homozygous P2X7 knockout ($P2X7^{KO}$)/$SOD1^{G93A}$ mice compared to P2X7 wild-type ($P2X7^{WT}$)/$SOD1^{G93A}$ mice (Apolloni et al., 2013). In contrast, genetic ablation of P2X7 anticipated clinical onset, worsened disease progression and increased motor neuron loss, microgliosis, astrogliosis and pro-inflammatory markers (NADPH oxidase and inducible nitric oxide synthase) towards or at end-stage (Apolloni et al., 2013). Consistent with this, BBG treatment in our study caused an increase in pro-inflammatory marker serum monocyte chemoattractant protein-1 in end-stage $SOD1^{G93A}$ mice (Bartlett et al., 2017), although group sizes were insufficiently powered to detect a significant difference. Together these two studies demonstrate a dual role for P2X7 activation in this model of ALS (at least in female $SOD1^{G93A}$ mice) by promoting disease progression but limiting aspects of neuroinflammation. Moreover, it may be of value to compare the effects of BBG treatment in $P2X7^{WT}$/$SOD1^{G93A}$ and $P2X7^{KO}$/$SOD1^{G93A}$ mice (which to the best of our knowledge has not been reported) to confirm P2X7 antagonism by BBG in $SOD1^{G93A}$ mice expressing functional P2X7.

Collectively the four studies discussed above (Apolloni et al., 2013, 2014; Cervetto et al., 2013; Bartlett et al., 2017) reveal a complex role for P2X7 in the $SOD1^{G93A}$ murine model of ALS and by extension ALS in humans. The various and contrasting gender differences observed with P2X7 antagonism or genetic ablation in these studies remain unexplained, but it is well known that disease progression in this model of ALS is gender dependent (Heiman-Patterson et al., 2005). It is noteworthy, that disease progression accelerates in ovariectomized $SOD1^{G93A}$ mice and that this effect can be reversed by administration of 17β-oestradiol (Choi et al., 2008), a compound which can also inhibit P2X7 activation (Cario-Toumaniantz et al., 1998). Whilst, these



Mutant human superoxide dismutase 1 ($SOD1^{G93A}$) transgenic mice were injected intraperitoneally (i.p.) with BBG from each day (d) as indicated (blue bars) or with respective saline-based diluent (not shown) to end-point. Mice were assessed for weight loss, clinical score, motor coordination and survival as described in the respective studies. Disease onset was defined as: first signs of tremor and/or hind limb motor deficits*; or 10% decline in rotarod performance†. Arrows represent improved (↑) or delayed (↓) clinical parameters. In two studies, differences in some clinical parameters were observed only in male (♂) or female (♀) mice.

Figure 1 P2X7 antagonism with Brilliant Blue G (BBG) can reduce amyotrophic lateral sclerosis (ALS) progression in mice.

observations do not completely reconcile the gender differences observed between the pre-clinical trials with BBG in SOD1^{G93A} mice (Cervetto et al., 2013; Apolloni et al., 2014; Bartlett et al., 2017), they do suggest possible interactions between P2X7 and 17β-oestradiol, which may influence treatment design and disease outcomes in ALS.

Gender differences aside, consideration of the pre-clinical trials with BBG (Cervetto et al., 2013; Apolloni et al., 2014; Bartlett et al., 2017) along with the P2X7 ablation study (Apolloni et al., 2013) indicate that P2X7 antagonism may be of most therapeutic benefit when treatment commences during late pre-onset or early onset. However in the absence of suitable biomarkers to predict disease onset and given that the average time to diagnosis is 10–12 months in humans (Mathis et al., 2017), the potential application of P2X7 antagonism in ALS is currently limited. Thus, given the current knowledge about ALS in humans, future pre-clinical studies of P2X7 antagonism in the SOD1^{G93A} murine model of ALS would be best served with drug treatments commencing at disease onset to translate any therapeutic benefits observed in mice to people with ALS. Moreover, pre-clinical studies of P2X7 antagonism are required in other transgenic mouse models of ALS, such as TAR DNA-binding protein 43 kDa (TDP-43) or C9Orf72 (Mathis et al., 2017), to determine if this approach has therapeutic benefits in ALS associated with molecular events other than mutant SOD1.

A limitation of pre-clinical trials with BBG in SOD1^{G93A} mice (and other murine models of disease) is that this compound is not specific for P2X7. BBG can impair murine P2X7 with a half maximal inhibitory concentration of 2 μM (Bartlett et al., 2013), but this compound can also inhibit other channels including murine neuronal voltage-gated sodium channels at similar concentrations to murine P2X7 (Jo and Bean, 2011). Despite this caveat, BBG has been a well-recognized P2X7 antagonist since the year 2000, and has been the most widely used P2X7 antagonist *in vivo* to date since its first use almost a decade ago in rodents models of multiple sclerosis, Alzheimer's disease, Huntington's disease or spinal cord injury (Bhattacharya and Biber, 2016). Nonetheless, future studies exploring P2X7 antagonism in ALS should consider using specific P2X7 antagonists, many of which are inhibitory at concentrations lower than that of BBG (Bhattacharya and Biber, 2016).

An emerging limitation of pre-clinical trials with BBG in SOD1^{G93A} mice is whether this compound can cross an intact blood-brain barrier. In the absence of blood-brain barrier damage, BBG demonstrates no significant occupancy of rat brain P2X7, whilst the brain to plasma ratio of BBG may be as low as 0.0065, which is well below a target ratio of 0.2 or more (Bhattacharya and Biber, 2016). Consistent with these findings, in SOD1^{G93A} mice treated with BBG 3 times per week for up to 90 days (Bartlett et al., 2017), we observed no blue coloration of the brains or spinal cords either macroscopically or microscopically, despite extensive blue coloration of the skin macroscopically, and of the livers and spleens microscopically (Bartlett R, Sluyter R, Yerbury JJ, unpublished). Thus, pre-clinical studies with P2X7 antagonists that can cross the blood-brain barrier (Bhattacharya and Biber, 2016) may be required to determine if P2X7 antagonism is of therapeutic benefit in ALS.

Our recent study (Bartlett et al., 2017) combined with studies by others (Cervetto et al., 2013; Apolloni et al., 2014) show that P2X7 antagonism has therapeutic benefits in SOD1^{G93A} mice, but the observed gender differences between these studies remain unexplained. Nevertheless these studies suggest that P2X7 may play a role in ALS progression, which is supported by other observations in rodents and humans with ALS (Volonte et al., 2016). However, much work is required to ascertain a potentially complex role of this receptor in this multifaceted, currently incurable neurodegenerative disorder, and to determine if P2X7 represents a realistic therapeutic target in people with ALS.

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