The molecular mechanism of ‘ryegrass staggers’ a neurological disorder of K⁺ channels.

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Running Title Page

a) Running title: “BK β4 subunit role in toxin-induced ataxia”

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Abstract

“Ryegrass staggers” is a neurological condition of unknown mechanism that impairs motor function in livestock. It is caused by infection of perennial ryegrass pastures by an endophytic fungus that produces neurotoxins, predominantly the indole-diterpenoid compound lolitrem B. Animals grazing on such pastures develop uncontrollable tremors and become uncoordinated in their movement. Lolitrem B and the structurally related tremor inducer, paxilline, both act as potent large conductance calcium-activated potassium (BK) channel inhibitors. Using patch-clamping we show that their different apparent affinities correlate with their toxicity in vivo. To investigate whether the motor function deficits produced by lolitrem B and paxilline are due to inhibition of BK ion channels, their ability to induce tremor and ataxia in mice deficient in this ion channel (Kcnma1−/−) were examined. Our results show that mice lacking Kcnma1 are unaffected by these neurotoxins. Furthermore, doses of these substances known to be lethal to wild-type mice had no effect on Kcnma1−/− mice. These studies reveal the BK channel as the molecular target for the major components of the motor impairments induced by ryegrass neurotoxins. Unexpectedly, when the response to lolitrem B was examined in mice lacking the β4 BK channel accessory subunit (Kcnmb4−/−), only low level ataxia was observed. Our studies therefore reveal a new role for the accessory BK β4 subunit in motor control. The β4 subunit could therefore be considered as a potential target for treatment of ataxic conditions in animals and in humans.
Introduction

A neurological disease of farm animals, involving ataxia and tremors, was first described in New Zealand more than a century ago (NZ Country Journal, 1880). This condition was subsequently recognized in farm animals in temperate regions throughout the world and named ‘ryegrass staggers’ (Cheeke, 1995) (Supplemental Movie). This disease occurs when animals ingest ryegrass infected with the endophytic fungus, Neotyphodium lolii (Fletcher and Harvey, 1981), which produces neurotoxic indole-diterpenoids, predominantly lolitrem B (Gallagher et al., 1981). The impaired motor coordination produced by lolitrem B results in major animal production and management problems as well as significant livestock losses. In 2002 a major outbreak of ryegrass staggers in Australia resulted in the death of tens of thousands of sheep and hundreds of cattle (Reed et al., 2005), and this disease has been estimated to cost New Zealand $100M per annum in lost animal production. The molecular target for lolitrem B in producing ryegrass staggers is unknown. Although endophytes cause animal disease, their presence in ryegrass pastures is advantageous because in addition to tremorgens they produce compounds that prevent insect attack (Lane et al., 2000). These beneficial compounds improve pasture survival particularly under New Zealand conditions. Novel endophyte-grass combinations have been obtained through selective breeding to maximize the beneficial effects of the endophytes (insect anti-feedant compounds) while minimizing those detrimental to mammals (indole-diterpenoids). It is not feasible to resow extensive areas of New Zealand and Australia, therefore these pastoral improvements have limited application. A better understanding of the mechanism of ryegrass staggers and the development of treatments is therefore of considerable interest to farmers.
Since ryegrass staggers causes motor phenotypes suggestive of hyperexcitation of the central nervous system, we proposed potassium channel inhibition as a potential mechanism of lolitrem B effects (Dalziel et al., 2005). Several reports have implicated large conductance calcium-activated potassium (BK) channels in the control of movement in flies (Atkinson et al., 2000; Elkins et al., 1986), mice (Sausbier et al., 2004), and humans (Du et al., 2005). The BK channel α subunit (Slo), encoded by the Kcnma1 gene, is functionally diverse and widely expressed among tissues. These channels are particularly prevalent in neural tissue where they play an important role in controlling cellular excitability (Gribkoff et al., 2001a). BK channel α subunits assemble as homo-tetramers to form the channel pore and can combine with one of four accessory β subunits encoded by Kcnmb genes. In brief, the β1 subunit is expressed most highly in smooth muscle, β2 in the ovary, β3 in the testis, and β4 in neural tissue (Brenner et al., 2000a; Jiang et al., 1999). Functional and pharmacological diversity is conferred by the various tissue-specific subunit combinations. For example, BK channels in cells expressing both α and β4 channels have a complex concentration-dependence to activation by calcium, slowed gating kinetics, and are essentially insensitive to the peptide inhibitors charybdotoxin and iberiotoxin (Behrens et al., 2000; Brenner et al., 2000a; Meera et al., 2000; Weiger et al., 2000).

Paxilline, a tremorgenic compound structurally related to the ryegrass fungal toxins (Munday-Finch et al., 1997), inhibits BK currents (Knaus et al., 1994), and both paxilline and lolitrem B have been shown to be high affinity BK inhibitors in heterologously expressed channels (Dalziel et al., 2005; Gribkoff et al., 1996; Sanchez
and McManus, 1996; Strøbæk et al., 1996). Reports that non-tremorgenic derivatives of paxilline also inhibit BK channels argue against them being the molecular target for the motor function deficits that occur \textit{in vivo} (Knaus et al., 1994). However, the more recent finding that BK channel knock-out mice have an ataxic phenotype (Meredith et al., 2004; Sausbier et al., 2004) suggests that BK channel inhibitors could impair motor function via this mechanism. In the present study we used a mouse model to test the hypothesis that BK channels selectively mediate the pharmacological effect of fungal toxins to produce ryegrass staggers. We used the fungal toxins lolitrem B and paxilline as pharmacological tools and compared their effects on wild-type and BK channel $\alpha$ subunit deficient mice ($Kcnma1^{-/-}$) and correlated this with their effects on $hSlo$ $\alpha$ subunit channels expressed in human embryonic kidney cells as revealed by patch-clamping. We also carried out experiments using $\beta$ subunit deficient mice and heterologously expressed $hSlo$ with $\beta1$ or $\beta4$ subunits to investigate possible subunit-specific differences in neurotoxicity.

\section*{Methods}

\subsection*{Ethical approval}

Animal manipulations were approved by the AgResearch Ruakura Animal Ethics Committee established under the Animal Protection (Code of Ethical Conduct) Regulations Act, 1987 (New Zealand). Animal protocols performed at Stanford University complied with the US National Institutes of Health guidelines and were reviewed by the Stanford University Animal Care and Use Committee. Five to eight animals were used per experiment. At the conclusion of the experiment mice were killed by carbon dioxide inhalation.
Isolation of mycotoxins

Lolitrem B was extracted from ryegrass seed infected with *Neotyphodium lolii* and purified by chemical methods as described previously (Miles et al., 1994). The resulting crystalline solid was shown to be at least 97% pure by NMR spectroscopy. Paxilline was isolated from *Penicillium paxilli* cultures as previously described (Munday-Finch et al., 1996). The resulting white powder was shown by NMR spectroscopy to be at least 98% pure. Elemental analysis indicated that paxilline was in the form of an acetonitrile solvate (paxilline.CH₃CN). Chemical structures have been published previously for paxilline (Springer et al., 1975) and lolitrem B (Gallagher et al., 1984).

Mice

BK channel-null mice (*Kcnma1−/−*) had an FVB background (Meredith et al., 2004) and their wild-type littermates were used as controls. β1 and β4 subunit-null (*Kcnmb1−/−* and *Kcnmb4−/−*) mice had a mixed C57BL/6J background and C57BL/6J wild-type mice were used as strain controls (Brenner et al., 2005; Brenner et al., 2000b). Male and female mice aged between 3 and 24 months were used and treatment groups were matched for age and sex.

Mouse tremor assay

The intensity of tremor induced by the toxins was measured using a previously described mouse bioassay method (Gallagher and Hawkes, 1986). This bioassay has been used extensively in our laboratories during structure-activity studies of
tremorgenic toxins (Miles et al., 1994; Miles et al., 1992; Munday-Finch et al., 1997). Toxins were administered by intraperitoneal injection as a solution in 9:1 (v/v) DMSO (dimethyl sulfoxide)-water (50 µl). Animals were closely observed after dosing and the tremors assessed at 15 minute intervals for the first hour and then at hourly intervals for the next 4-6 hours and then at least 3 times daily until the tremors subsided. The severity of tremor was scored using a tactile and visual rating scale from 0 to 5 (Gallagher and Hawkes, 1986). To ensure results for the tremor assay were reproducible, unbiased, and had low variability between investigators, results were scored separately by two investigators for the same treatment group then compared. Results showed data to be consistent and reproducible (Supplemental Figure 1). For ethical reasons, toxin doses given to wild-type mice were selected to obtain a moderate tremor response to avoid undue stress to the animal, and a score of 3 on the tremor scale was the maximum desired response.

Motor function and coordination

The rotarod test was conducted using a Rotamex 4 rotarod (Columbus Instruments, Ohio). The rotational speed was increased from 13 to 79 rpm over 12 minutes. The mean duration to fall (in seconds) of two trials was recorded and averaged for each mouse. The mice were first trained to use the rotarod for 4 trials per day over a 3 day period.

hSlo expression in HEK cells

The human large conductance calcium-activated potassium channel, hSlo (accession number U11058) in pcDNA5 was stably expressed in the Flp-In™-293 cell line
Cells were grown in DMEM (Dulbecco’s Modified Eagle Medium) and 2.5 mM HEPES (N-[2-hydroxyethyl]-piperazine-N’-[2-ethanesulfonic acid]), supplemented with minimal essential amino acids and 10% foetal bovine serum at 37°C. Cells were plated onto coverslips coated with 0.01% poly-L-lysine.

**hSlo + β subunit expression in HEK cells**

Where hSlo + β subunits are coexpressed, the enhanced green fluorescent protein (eGFP) mammalian vector constructs containing mouse β1 or human β4 subunit isoforms (Brenner et al., 2000a) were transiently expressed either by cotransfection with hSlo (pcDNA) into HEK 293 cells (4 μg DNA/well, of 24 wells) or transfected into stably transfected hSlo-Flip-in 293 cells. Transfected cells were identified by their fluorescence under UV light.

**Electrophysiological measurements**

Macroscopic currents were recorded from inside-out membrane patches from cells expressing hSlo with or without accessory β subunits. Cells were bathed at room temperature in an intracellular solution that contained 140 mM KMeSO₃, 2 mM KCl, 20 mM HEPES, 5 mM HEDTA (N-(2-hydroxyethyl)ethylene-diaminetriacetic acid) and 3.65 mM CaCl₂ to give 10 µM free calcium, pH 7.2. Pipettes were pulled from borosilicate glass using a 5-stage microelectrode puller (Flaming/Brown, Sutter Instruments) to give resistances of 3-5 MΩ when filled with extracellular solution that contained 140 mM KMeSO₃, 2 mM KCl, 20 mM HEPES and 2 mM MgCl₂, pH 7.2. Macroscopic currents were recorded from voltage-clamped cells at a holding potential of -80 mV using an EPC-10 amplifier (HEKA, Germany) and PC based Pulse 8.53 data acquisition system.
collection software (HEKA, Germany). Data were filtered at 5 kHz and sampled at 20 µs intervals. Fast transients were cancelled using capacitance compensation and leak subtraction was used. Data were analyzed using Pulse Tools 8.5 (HEKA, Germany) and Sigma Plot 9.0 software. Dose-response data were fitted with a logistic 4 parameter sigmoidal function to estimate the concentration that gave half-maximal inhibition.

Toxins were applied directly to a bath of 2.5 ml volume, and where possible, recovery was measured at the completion of the experiment by perfusing the bath at a rate of 4 ml/min. Where inhibition could not be reversed, the toxin was applied at a concentration that completely inhibited channel activity.

Statistical analyses

Results where \( n \geq 3 \) are expressed as means. Error bars in each figure show ± standard error of means. Statistical comparisons were made using GenStat version 10.2 (VSN International Limited, Hemel Hempstead, UK). One way analysis of variance was used to compare treatments. Where the variance was not homogenous across treatment groups, the data were log transformed. *, \( P < 0.05 \), **, \( P < 0.01 \), ***, \( P < 0.001 \).

Results

BK channel inhibitors impair motor function

Motor function deficits induced by two indole-diterpenoid BK channel inhibitors were examined. Tremors were assessed using a visual rating scale which evaluates both the degree of spontaneous tremor and that which is induced by activity. The dose rates of lolitrem B (4 mg/kg) and paxilline (8 mg/kg) were chosen to give a similar peak tremor response and reflect their different potencies. The tremors induced by lolitrem B
peaked at 4 hours post-dosing and slowly diminished over 96 hours (Fig 1A). In contrast, the tremors induced by paxilline had a faster onset and shorter duration, peaking at 15-30 minutes and recovering over 10 hours (Fig 1A). Mice dosed vehicle (DMSO-water) showed no tremor response (Fig 1C). Tremors induced by these compounds in wild-type mice have been previously reported (Gallagher and Hawkes, 1986; Miles et al., 1992; Munday-Finch et al., 1997) and are consistent with our findings. These results show significant differences in the time-course of tremors induced by lolitrem B and paxilline which is independent of dose rates administered and consistent with previous studies (Gallagher and Hawkes, 1986; Miles et al., 1992).

Since livestock affected by ryegrass staggers show severe incoordination, in addition to tremor, we used a method to assess coordination and ataxia in mice dosed with tremorgens. This was achieved using an accelerating rotarod to measure the ability of mice to balance and walk on a moving rod. Results show that both lolitrem B and paxilline severely affect motor control and that their durations of action were similar to that observed for tremor (Fig 1B). The peak effect for lolitrem B on rotarod performance was 2 hours post-dosing, compared to 4 hours for the tremor effect. The peak effect for paxilline was observed at 15-30 min post-dosing, similar to that observed for the tremor effect. Control animals dosed with vehicle showed no impairment of motor control (Fig 1D). Both tremor intensity and incoordination were shown to be dose-dependent for lolitrem B (Fig 1C, 1D). The results show that 1 mg/kg lolitrem B was sufficient to produce mild tremors (Fig 1C) but insufficient to impair rotarod performance (Fig 1D). A higher dose of lolitrem B (2 mg/kg) was required to significantly impair rotarod performance ($P < 0.01$).
**hSlo channel inhibition**

To investigate the molecular basis of the kinetic differences between lolitrem B and paxilline *in vivo*, we used the patch-clamp technique to examine inhibition of currents from human BK channels stably expressed in HEK cells (Fig. 2A). To determine the concentration required to inhibit *hSlo* potassium currents by half (IC$_{50}$) we constructed inhibition curves by applying increasing concentrations of each compound to inside-out membrane patches. Currents were elicited by +150 mV voltage pulses in 10 μM free calcium. Figure 2A shows inhibition of the initial response to increasing concentrations of paxilline or lolitrem B. Figure 2B shows the mean inhibition curve and Table 1 gives the IC$_{50}$ values. BK channels were found to have a 5-fold higher apparent affinity for lolitrem B than for paxilline (Fig. 2B, Table 1), which could explain the higher potency of lolitrem B in disrupting motor function. Channel inhibition by paxilline in patch-clamp experiments fully recovered following 30 min of wash-out (Fig 2C), but that induced by lolitrem B did not (Fig 2D), as reported previously (Dalziel et al., 2005). This result is consistent with the slower recovery of mice treated with lolitrem B compared to paxilline and suggests that the toxins may exert their pharmacological effects via BK channels.

**Kcnma1$^{-/-}$ mice are insensitive to toxin effects**

To determine whether the motor impairment induced by lolitrem B and paxilline is specifically due to BK channel inhibition and if there are any physical responses initiated by these toxins interacting with other targets, we examined the effect of both compounds on mice with a targeted deletion in the gene encoding the pore-forming...
alpha subunit of the BK channel (Kcnma1−/−). The phenotypes of Kcnma1−/− mice include mild baseline tremor measuring 1.5 ± 0.1 on the tremor scale (Fig. 3A) and moderate ataxia (Fig. 3B), indicated by poor rotarod ability (Meredith et al., 2004). Earlier studies have shown that motor learning is not impaired and performance improves with training at a rate similar to wild-type mice (Meredith et al., 2004; Sausbier et al., 2004). Lolitrem B had no effect on Kcnma1−/− motor function. Both tremor (Fig. 3A) and coordination (Fig. 3B) remained unchanged after administration of the toxin. Paxilline was similarly without effect (Fig. 3E & 3F). By comparison, both toxins produced moderate tremor and impaired rotarod ability in FVB wild-type littermate controls (Fig. 3A & 3B). DMSO was without effect either in Kcnma1−/− mice or in controls. These data suggest the neurotoxic effects of paxilline and lolitrem B are mediated through BK channels. To investigate whether the sensitivity of Kcnma1−/− mice to the toxins is merely reduced in the absence of BK channel regulation, higher toxin concentrations were administered. In wild-type mice, a dose of 8 mg/kg lolitrem B induces convulsive episodes (Gallagher and Hawkes, 1986) but a dose of 12 mg/kg given to Kcnma1−/− mice had no additional effect on baseline tremor, ataxia or behaviour (Fig. 3C, D). This finding indicates that these toxins impair motor function by specifically inhibiting BK channels.

Kcnmb4−/− mice have reduced sensitivity to toxin-induced ataxia

BK-specific accessory beta subunits confer heterogeneity in physiological function and pharmacology to BK channels. We therefore investigated whether BK channel complexes containing beta subunits were involved in toxin-induced motor deficits. We were particularly interested in examining the role of β4 (encoded by the Kcnmb4 gene)
because these subunits are co-localised with hSlo and highly expressed throughout the brain, where they modify the gating kinetics of the channel and can alter its pharmacology (Brenner et al., 2005; Brenner et al., 2000a; Ha et al., 2004; Weiger et al., 2000). Conversely, since the β1 subunit (encoded by the Kcnmb1 gene) is exclusively expressed in smooth muscle, but also modulates BK channel function, we used Kcnmb1−/− mice (Brenner et al., 2000b; Meera et al., 1996; Tseng-Crank et al., 1996) as a control, expecting that they would respond to the neurotoxins in a manner similar to wild-type mice. Although Kcnmb4 knock-out mice (Kcnmb4−/−) are prone to seizures (Brenner et al., 2005), their locomotor and tremor phenotype is similar to wild-type mice, as are those of Kcnmb1−/− mice (Brenner et al., 2000b). The tremor effect of lolitrem B and paxilline in Kcnmb1−/− and Kcnmb4−/− was similar to that in strain controls (C57BL/6J wild-type mice) (Fig. 4A & 5A). Furthermore, the tremor response in Kcnmb4−/− was dose-dependent for lolitrem B as observed in wild-type mice (Supplemental Figure 2). These results show that the β4 subunit is not involved in the tremor effects of these toxins. As expected, lolitrem B (Fig. 4B) and paxilline (Fig. 5B) reduced the motor coordination and balance of Kcnmb1−/− mice in an analogous fashion to that observed in wild-type mice. In contrast, the coordination of Kcnmb4−/− mice dosed with lolitrem B (Fig. 4B) and paxilline (Fig. 5B) was much less affected compared to C57BL/6J wild-type mice (P < 0.001). A comparison of Kcnmb4−/− mice dosed toxin and vehicle showed a small difference in ataxia (lolitrem B; P = 0.049, paxilline: P = 0.02). These results show that the severe toxin-induced ataxia seen in the mouse model of ryegrass staggers requires the presence of Kcnmb4 subunits. It is possible that administration of higher doses of the toxins to Kcnmb4−/− mice might further impair their rotarod performance. However, this experiment was not feasible due to the unacceptable tremor
response this would induce. During this study it was necessary to use different wild-type mouse controls since the knock-out mice were produced on different background strains. It is interesting to note that the performance of the different strains was variable, in particular for the rotarod task (Fig 1A&B; Fig 4A&B, Fig 3E&F; Fig 5A&B). Strain difference effects have been observed previously (Brooks et al., 2004).

Inhibition of BK channels containing beta subunits

To understand how the Kcnmb4 subunit modulates BK channel activity in response to the toxins, we investigated the sensitivity of channels containing hSlo + β4 compared to hSlo alone and hSlo + β1 controls. Current responses to increasing concentrations of each compound were recorded (Figs 4C & 5C) and inhibition curves constructed (Figs 4D & 5D) for lolitrem B and paxilline. These results showed that the presence of the β4 subunit increased the apparent affinity for lolitrem B two-fold, but that the effects of paxilline were unchanged (Table 1). Thus BK channels in Kcnmb4−/− mice would have reduced sensitivity to lolitrem B, which may partially explain why ataxia is relatively low in these mice.

Discussion

The tremor effects of indole-diterpenoids have previously been considered to be unrelated to their inhibition of BK channels since certain non-tremorgenic indole-diterpenoids are reported to inhibit BK channels (Knaus et al., 1994). However, we have found that doses of paxilline and lolitrem B that induce tremors in wild-type controls have no effect on mice lacking BK channels indicating that BK channels are required for their tremorgenic and ataxic effects. This finding is further strengthened by
the observation in *Kcnma1*−/− mice that lolitrem B does not induce tremors at levels known to induce severe tremors and convulsions in wild-type mice. Furthermore, the lack of any other visible behavioural changes in these mice shows a specificity of action for lolitrem B as a BK channel inhibitor. It is unclear why non-tremorgenic indole-diterpenoids affect BK channels without inducing tremors but preliminary experiments suggest that this is due to a reduced potency and reversibility of action of these compounds on BK channels. It is interesting to note that the tremors induced by the toxins in wild-type mice are significantly greater than the baseline phenotype of mice lacking BK channels. This may be attributed to a phenomenon described as ‘homeostatic remodelling’ which has been used to describe instances where expression of other potassium channels is increased during development and this functionally compensates for the absence of BK channels (Nerbonne et al., 2008; Rieg et al., 2007).

The differences between paxilline and lolitrem B observed *in vivo* can also be explained using *in vitro* experiments on BK channels. Using the mouse model, the tremor effect of lolitrem B was greater than that of paxilline. This correlated with a 5-fold higher apparent affinity of lolitrem B for BK channels in comparison to paxilline. The time course of action of paxilline and lolitrem B was also markedly different *in vivo*, with paxilline-induced tremors lasting only 10 hours, compared to 96 hours for lolitrem B. This is consistent with *in vitro* experiments showing a lack of reversibility of channel inhibition for lolitrem B while a full recovery was observed for paxilline. We therefore conclude that the toxins impair motor function by specifically inhibiting BK channels and that it is unlikely that any other receptors are involved. This discovery means that a
prevention or cure for ryegrass staggers is now feasible and further work is planned to assess the effectiveness of BK channel activators for this use.

The use of both tremor and rotarod methods have allowed the simultaneous assessment of two different aspects of motor function, tremor and ataxia. This is the first time that these two effects have been separated in the study of ryegrass staggers and have allowed mechanistic information on each component to be determined. The control data showed that in mice lacking accessory $\beta_1$ or $\beta_4$ subunits, there is no baseline tremor or ataxia, unlike the baseline tremor phenotype when the $\alpha$ BK pore-forming subunit is absent.

The analogous tremor response to paxilline and lolitrem B seen in wild-type, $\textit{Kcnmb1}^{-/-}$ and $\textit{Kcnmb4}^{-/-}$ mice in comparison to the nil effect seen in $\textit{Kcnma1}^{-/-}$ mice indicates that the BK $\alpha$ subunit is essential for tremor generation. Given the high expression level of the $\alpha$ subunit in skeletal muscle (Jiang et al., 1999), a direct effect of the toxins on BK channels in this tissue might be possible and our data do not rule this out. The low level of toxin-induced ataxia observed in $\textit{Kcnmb4}^{-/-}$ mice, in comparison to wild-type or $\textit{Kcnmb1}^{-/-}$ indicates that the $\beta_4$ subunit is important for toxin-induced ataxia. The molecular basis for this effect is unclear. Given the overlapping expression of the $\alpha$ subunit ($\textit{Slo}$) and $\beta_4$ in the cerebellum (Behrens et al., 2000; Brenner et al., 2000a; Weiger et al., 2000), the ataxic effect may be due to inhibition of BK channels in motor control pathways. Ataxia and tremor could involve inhibition of BK channels in different brain regions, particularly if splice variants differ in their pharmacology. The two-fold higher apparent affinity of lolitrem B for BK channels containing a $\beta_4$ subunit may partially explain the reduced ataxia in mice lacking this subunit. However, this relatively small pharmacological difference is unlikely to fully account for the very low
level of lolitrem B-induced ataxia in the absence of this subunit. Since 
\( hSlo + \beta 4 \) and 
\( hSlo \) BK channels do not differ in their apparent affinity for paxilline, subunit differences in pharmacology cannot account for reduced ataxia by this compound in mice lacking the \( \beta 4 \) subunit. In the absence of the \( \beta 4 \) subunit other ion channels may partially compensate for the functional role of 
\( hSlo + \beta 4 \) BK channels in motor coordination, which would explain why toxin-induced ataxia is greatly reduced in 
\( Kcnmb4^{+/} \) mice.

The data in this study show that the main causative fungal toxin in ryegrass staggers, lolitrem B, acts via BK channels to induce an analogous neurological disorder in a mouse model. It also provides the first evidence that the BK \( \beta 4 \) subunit has a modulatory role in motor coordination. BK channels are currently being pursued as pharmacological targets in neural and smooth muscle dysfunction (Du et al., 2005; Gribkoff et al., 2001b), and our study of BK channels in motor function adds to a relatively new set of complex physiological systems where BK channels are potent regulators of excitability (Brenner et al., 2005; Filosa et al., 2006; Meredith et al., 2006).

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References


Footnotes

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Legends for Figures

Figure 1. Lolitrem B and paxilline impair motor function.

A-B, Wild-type mice (FVB strain) dosed with lolitrem B (blue; 4 mg/kg; n = 8) or paxilline (red; 8 mg/kg; n = 8) showing A, mean tremor score and B, latency to fall. C-D, Wild-type mice (FVB strain) dosed with lolitrem B (1, 2 or 4 mg/kg; n = 8) or DMSO carrier alone (n = 6) showing C, mean peak tremor score and D, peak normalized latency to fall.

Figure 2. Lolitrem B and paxilline inhibit hSlo channels.

Representative recordings from inside-out membrane patches of HEK293 cells expressing hSlo (α subunit). A, Currents are inhibited by paxilline or lolitrem B. B, Concentration-response curves for mean normalized currents inhibited by lolitrem B (blue; n = 18) and paxilline, (red; n = 12). C-D, Current levels after 30 min of washout following complete inhibition by C, paxilline and D, lolitrem B.

Figure 3. Lolitrem B and paxilline do not impair motor function in Kcnma1−/− mice.

A-B, Kcnma1−/− mice (FVB background strain) dosed with lolitrem B (dark blue; 4 mg/kg; n = 8) or DMSO-water (light blue; n = 8) and wild-type littermates dosed with lolitrem B (grey; 4 mg/kg; n = 8) or DMSO-water (black; n = 8) showing A, mean tremor score and B, latency to fall. C, peak mean tremor score of Kcnma1−/− mice dosed with lolitrem B (4, 8 or 12 mg/kg; n = 6) or DMSO control (n = 8). D, peak latency to fall of Kcnma1−/− mice dosed with lolitrem B (4 or 12 mg/kg; n = 6) or DMSO control (n = 8). E-F, Kcnma1−/− mice dosed with paxilline (red; 8 mg/kg; n = 8) or DMSO-water (orange; n = 8) and wild-type littermates (FVB strain) dosed with paxilline (grey;...
8 mg/kg; n = 8) or DMSO-water (black; n = 8) showing E, mean tremor score and F, latency to fall.

**Figure 4.** The influence of the BK β4 subunit on motor function and BK channel function, impaired by lolitrem B.  
A-B, *Kcnmb1−/−* (blue; n = 8), *Kcnmb4−/−* (red; n = 8) (both C57BL/6J background strain) and C57BL/6J wild-type (black dashed; n = 8) mice dosed with lolitrem B (4 mg/kg) showing A, mean tremor score and B, mean latency to fall. DMSO control for *Kcnmb4−/−* (red dashed; n = 8).  
C, Representative recordings from inside-out membrane patches from HEK293 cells coexpressing *hSlo α* and β1 or β4 subunits. Currents are inhibited by lolitrem B.  
D, Concentration-response curves for mean normalized currents from α (n = 8; black), α + β1 (n = 8; blue dashed) or α + β4 (n = 8; red dashed).  
E-F, Summary of changes in motor function induced by lolitrem B (4 mg/kg; n = 8) in the different BK knock-out mice and their background strain controls showing E, mean tremor score and F, normalized time to fall (2 hours post-dose/pre-dose). Significance was tested using least significant difference post-hoc test after ANOVA. Asterisks indicate the significance of each treatment compared to wild-type at 2 hours post-dosing. The time to fall for lolitrem B-treated *Kcnmb4−/−* mice was statistically different from the DMSO control, P = 0.049.

**Figure 5.** The influence of the BK β4 subunit on motor function and BK channel function, impaired by paxilline.  
A-B, *Kcnmb1−/−* (blue; n = 8), *Kcnmb4−/−* (red; n = 8) (both on C57BL/6J background strain) and C57BL/6J wild-type (black dashed; n = 8) mice dosed with paxilline (8
mg/kg) showing A, mean tremor score and B, mean latency to fall. DMSO control for $Kcnmb4^{+/−}$ (red dashed; $n = 8$). C, Representative recordings from inside-out membrane patches from HEK293 cells coexpressing $hSlo$ $α$ and $β1$ or $β4$ subunits. Currents are inhibited by paxilline. D, Concentration-response curves for mean normalized currents from $α$ ($n = 8$; black), $α + β1$ ($n = 8$; blue dashed) or $α + β4$ ($n = 8$; red dashed). E–F, Summary of changes in motor function induced by paxilline (8 mg/kg; $n = 8$) in the different BK knock-out mice and their background strain controls showing E, mean tremor score and F, normalized time to fall (15 min post-dose/pre-dose). Asterisks indicate the significance of each treatment compared to the maximal effect in wild-type (15 min post-dosing). The time to fall for paxilline-treated $Kcnmb4^{+/−}$ mice was significantly different from the DMSO control, $P = 0.02$. 
Table 1. IC\textsubscript{50} concentrations of toxins to inhibit BK channel currents.

<table>
<thead>
<tr>
<th>BK channel</th>
<th>Lolitrem B</th>
<th>Paxilline</th>
</tr>
</thead>
<tbody>
<tr>
<td>hSlo</td>
<td>4.4 ± 0.3 nM (n=19)</td>
<td>21.6 ± 1.2 nM (n=12)</td>
</tr>
<tr>
<td>hSlo + β1</td>
<td>4.9 ± 0.3 nM (n=8)</td>
<td>23.3 ± 5.0 nM (n=8)</td>
</tr>
<tr>
<td>hSlo + β4</td>
<td>1.8 ± 0.1 nM (n=8)</td>
<td>17.7 ± 2.8 nM (n=8)</td>
</tr>
</tbody>
</table>

IC\textsubscript{50}; concentration that gives half-maximal inhibition. All values are means ± S.E.M., n; number of cells.
Fig. 1

1A  Muscle tremor

1B  Motor Coordination

1C

1D

Normalized latency to fall

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Fig. 2
Fig. 3

3A  Lolitrem B

3B  Lolitrem B

3C  Lolitrem B dose (mg/kg)

3D  Lolitrem B dose (mg/kg)

3E  Paxilline

3F  Paxilline

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Fig. 4

4A. Lolitrem B concentration (nM) vs. Tremor Score over Time (h).

4B. Lolitrem B concentration (nM) vs. Latency to fall (s) over Time (h).

4C. hSlo + β1 and hSlo + β4 current traces with Lol B concentrations.

4D. I/max vs. Lol B concentration (nM).

4E. Tremor score for different mouse types.

4F. Normalized time to fall for different mouse types.

Mouse type:
- Kcnma1-/-
- WT (FVB)
- Kcnmb1-/-
- Kcnmb4-/-
- WT (C57 black)
Fig. 5

**5A**

Paxilline concentration (nM)

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>I/Imax 0.0</th>
<th>I/Imax 0.2</th>
<th>I/Imax 0.4</th>
<th>I/Imax 0.6</th>
<th>I/Imax 0.8</th>
<th>I/Imax 1.0</th>
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<tbody>
<tr>
<td>10</td>
<td>α</td>
<td>β1 -/-</td>
<td>β4 -/-</td>
<td>WT</td>
<td>α</td>
<td>β1 -/-</td>
</tr>
<tr>
<td>100</td>
<td>α</td>
<td>β1 -/-</td>
<td>β4 -/-</td>
<td>WT</td>
<td>α</td>
<td>β1 -/-</td>
</tr>
</tbody>
</table>

**5B**

Latency to fall (s)

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>Latency to fall (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>α</td>
</tr>
<tr>
<td>40</td>
<td>β1 -/-</td>
</tr>
<tr>
<td>100</td>
<td>β4 -/-</td>
</tr>
<tr>
<td>DMSO</td>
<td>WT</td>
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</tbody>
</table>

**5C**

hSlo + β1

- Control
- 10 nM Pax
- 40 nM Pax
- 100 nM Pax

hSlo + β4

- Control
- 10 nM Pax
- 20 nM Pax
- 100 nM Pax

**5D**

Paxilline concentration (nM)

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>I/Imax 0.0</th>
<th>I/Imax 0.2</th>
<th>I/Imax 0.4</th>
<th>I/Imax 0.6</th>
<th>I/Imax 0.8</th>
<th>I/Imax 1.0</th>
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</thead>
<tbody>
<tr>
<td>10</td>
<td>α</td>
<td>β1 -/-</td>
<td>β4 -/-</td>
<td>WT</td>
<td>α</td>
<td>β1 -/-</td>
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<tr>
<td>100</td>
<td>α</td>
<td>β1 -/-</td>
<td>β4 -/-</td>
<td>WT</td>
<td>α</td>
<td>β1 -/-</td>
</tr>
</tbody>
</table>

**5E**

Mouse type

- Kcnma1/-/-
- WT (FVB)
- Kcnmb1/-/-
- Kcnmb4/-/-
- WT (C57 black)

**5F**

Normalized time to fall

Mouse type