Rapid Ammonia Gas Transport Accounts for Futile Transmembrane Cycling under NH_3/NH_4^+ Toxicity in Plant Roots[1][C][W]

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Futile transmembrane NH_3/NH_4^+ cycling in plant root cells, characterized by extremely rapid fluxes and high efflux to influx ratios, has been successfully linked to NH_3/NH_4^+ toxicity. Surprisingly, the fundamental question of which species of the conjugate pair (NH_3 or NH_4^+) participates in such fluxes is unresolved. Using flux analyses with the short-lived radioisotope ^{13}N and electrophysiological, respiratory, and histochemical measurements, we show that futile cycling in roots of barley (*Hordeum vulgare*) seedlings is predominately of the gaseous NH_3 species, rather than the NH_4^+ ion. Influx of $^{13}NH_3/^{13}NH_4^+$, which exceeded 200 μmol g^{-1} h^{-1}, was not commensurate with membrane depolarization or increases in root respiration, suggesting electroneutral NH_3 transport. Influx followed Michaelis-Menten kinetics for NH_3 (but not NH_4^+), as a function of external concentration (K_m = 152 μM, V_{max} = 205 μmol g^{-1} h^{-1}). Efflux of $^{13}NH_3/^{13}NH_4^+$ responded with a nearly identical K_m. Pharmacological characterization of influx and efflux suggests mediation by aquaporins. Our study fundamentally revises the futile-cycling model by demonstrating that NH_3 is the major permeating species across both plasmalemma and tonoplast of root cells under toxicity conditions.

Ammonia/ammonium (NH_3/NH_4^+) toxicity in higher plants has resulted in crop reduction and forest decline (Pearson and Stewart, 1993; Vitousek et al., 1997; Britto and Kronzucker, 2002), biodiversity loss (Stevens et al., 2004; Bobbink et al., 2010), and species extirpation (de Graaf et al., 1998; McClean et al., 2011). These major ecological and economic problems have been aggravated by an accelerated global nitrogen (N) cycle caused primarily by the industrialized production and use of N fertilizers (Galloway et al., 2008; Gruber and Galloway, 2008). With increasing global population and demands on agricultural production, there is no sign of this trend easing: anthropogenic N fixation has reached 210 teragrams $year^{-1}$, an approximately 12% increase from 2005 and an approximately 1,300% rise from 150 years ago (Galloway et al., 2008; Fowler et al., 2013).

Although considerable knowledge of the causes and mechanisms of NH_3/NH_4^+ toxicity has accrued in recent years, our understanding of the key processes remains rudimentary (Gerendas et al., 1997; Britto and Kronzucker, 2002). A major hypothesis is that of futile transmembrane NH_4^+ cycling, which proposes a pathological inability of root cells to restrict the primary entry of NH_4^+ at high external concentrations ($[NH_4^+]_{ext}$); many downstream toxicological events are contingent upon this entry (Britto et al., 2001b). In this model, a rapid, thermodynamically passive influx of NH_4^+ is coupled to an active efflux of NH_4^+ that is nearly as rapid, constraining normal cellular function and energetics and resulting in plant growth decline and mortality. This phenomenon is thought to occur in NH_4^+-sensitive species such as barley (*Hordeum vulgare*) and, to a lesser extent, in tolerant species such as rice (*Oryza sativa*), which can be susceptible at higher thresholds (Balkos et al., 2010; Chen et al., 2013).

Most soils are typically acidic, especially when $[NH_4^+]$ is high (i.e. in the millimolar range; Van Breemen et al., 1982; Bobbink et al., 1998; Britto and Kronzucker, 2002), and given the pKa of 9.25 for the conjugate pair NH_3/NH_4^+, $[NH_3]$ is generally low (Izaurralde et al., 1990; Weise et al., 2013). Consequently, the fluxes of NH_3 have largely been considered negligible (Britto et al., 2001a; Britto and Kronzucker, 2002; Loqué and von Wirén, 2004), in contrast to NH_4^+ fluxes, which are well characterized physiologically (Lee and Ayling, 1993; Wang et al., 1993a, 1993b; Kronzucker et al., 1996) and at the molecular level (Rawat et al., 1999; von Wirén et al., 2000; Ludewig et al., 2007), at least at lower concentrations. However, the transport of NH_3 across membranes has received new attention in the light of evidence that some members of the aquaporin (AQP) family of transporters, a diverse and ubiquitous class of major intrinsic proteins (Maurel et al., 2008; Hove and Bhave, 2011), can mediate NH_3 fluxes in single-cell systems (Jahn et al., 2004; Holm et al., 2005; Loqué et al., 2005; Saparov et al., 2007). However, a convincing demonstration that AQPs transport NH_3 in planta is currently

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lacking. Given the unusually high capacity of AQP-mediated fluxes relative to those of ion channels and other transporters (Kozono et al., 2002), it is possible that sizable NH_3 fluxes can be conducted through AQPs, even at very low external NH_3 concentration ($[NH_3]_{ext}$).

Here, we have critically reexamined the hypothesis that futile cycling is composed of cationic NH_4^+ fluxes across the plasmalemma, of which an active efflux mechanism accounts for energetic demands directly contributing to toxicity (Britto et al., 2001b). We present evidence for the following alternative scenario: 1) futile cycling consists mainly of the passive electroneutral flux of the conjugate base NH_3; 2) such fluxes rapidly span both major membrane systems in root cells (i.e. plasmalemma and tonoplast); 3) AQPs mediate such fluxes; and 4) a thermodynamic equilibrium of NH_3 is established throughout the cell, resulting in hyperaccumulation of NH_4^+ in the acidic vacuole. This evidence comes primarily from positron emission tracing with the short-lived radioisotope ^{13}N, used to characterize the component fluxes of futile cycling at the cellular level in the model species barley. We have coupled this with $^{42}K^+$ radiotracing, to provide comparison with a well-understood cationic flux, as well as electrophysiological, respiratory, pharmacological, and histochemical analyses.

RESULTS

To gauge the relative contributions of NH_3 and NH_4^+ transport, concentration-dependent root NH_3/NH_4^+ influxes and their associated plasma membrane depolarization (change in membrane potential [$\Delta\Delta\Psi_m$]) were compared with those of the macronutrient ion potassium (K^+; Fig. 1, A–C). In these experiments, plants were grown under nontoxic conditions, using a complete nutrient medium with K^+ and NH_4^+ both provided at 0.1 mM (pH 6.25). Direct influx measurements with $^{42}K^+$ and $^{13}NH_3/^{13}NH_4^+$, determined between 0.1 and 10 mM for each (with the other held constant at 0.1 mM), show influx of the two ions to have vastly different rates and isotherm shapes. For instance, steady-state NH_3/NH_4^+ influx (i.e. measured at the growth concentrations of 0.1 mM K^+ and NH_4^+) was 11.5-fold higher than that of K^+ (13.12 ± 0.85 versus 1.26 ± 0.06 μmol g^{-1} h^{-1}, respectively). As each substrate's concentration independently rose to 10 mM, its influx increased 4- to 5-fold, peaking with a NH_3/NH_4^+ influx 13-fold higher than that of K^+ (Fig. 1A). Interestingly, however, the rise in NH_3/NH_4^+ influx was not commensurate with membrane depolarization, indicating that most of the influx observed was not electrogenic and supporting the idea that NH_3, not NH_4^+, is the main transported N species. The significance of this result was underscored when compared with changes in $\Delta\Delta\Psi_m$ observed with K^+: increases in external K^+ concentration resulted in up to 4-fold greater depolarization than seen with comparable changes in NH_3/NH_4^+ (Fig. 1B), despite NH_3/NH_4^+ influx being more than 10 times higher than K^+ influx. Only at 10 mM were depolarizations of similar magnitude observed (approximately 60 mV). Figure 1C further illustrates this disproportion, by showing $\Delta\Delta\Psi_m$ as a function of influx for each substrate. The 9-fold steeper slope with

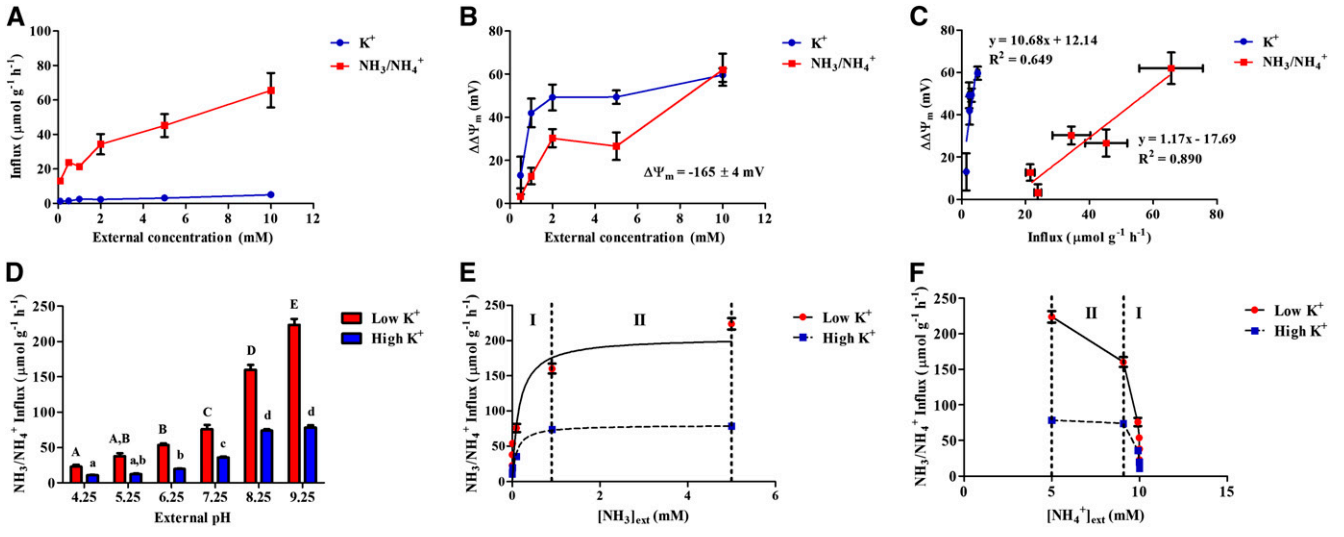

Figure 1. NH_3 (not NH_4^+) is the main permeating species in barley roots. A and B, Concentration dependence of influx (A) and $\Delta\Delta\Psi_m$ (B) of NH_3/NH_4^+ (red) and K^+ (blue) in plants grown at 0.05 mM $(NH_4)_2SO_4$ and K_2SO_4. C, Data from A and B replotted to show relationship between $\Delta\Delta\Psi_m$ and influx. D, Root NH_3/NH_4^+ influx as a function of external pH. E and F, Data from D replotted to show dependence of NH_3/NH_4^+ influx on NH_3 (E) or NH_4^+ (F) concentrations, which were predicted from solution pH, according to the Henderson-Hasselbalch equation (pKa of NH_3/NH_4^+ = 9.25). Area I represents pH 4.25 to 8.25; area II represents pH 8.25 to 9.25. Plants were grown at 5 mM $(NH_4)_2SO_4$ and either low (0.01 mM, red) or high (2.5 mM, blue) K_2SO_4. For all sections, error bars represent ± SE of the mean ($n \geq 3$). Letters in D denote significantly different means ($P < 0.05$) as determined by a one-way ANOVA with Tukey's posthoc test. [See online article for color version of this figure.]

K^+ relative to NH_3/NH_4^+ illustrates the much greater electrical response elicited by K^+ transport.

To further test the capacity of NH_3 transport in planta, we monitored NH_3/NH_4^+ influx as a function of each conjugate species' external concentration independently, by adjusting solution pH and thus the $[NH_3]$ to $[NH_4^+]$ ratio (pKa = 9.25; Fig. 1, D–F). Seedlings were grown under high (10 mM) external NH_3/NH_4^+ concentration ($[NH_3/NH_4^+]_{ext}$) in a full-nutrient medium (pH 6.25), then placed in growth solution with pH ranging between 4.25 and 9.25 for 10 min, prior to influx measurement with $^{13}NH_3/^{13}NH_4^+$. Influx showed significant stimulations with rising pH over the entire range (Fig. 1D) and followed clear Michaelis-Menten kinetics with rising $[NH_3]_{ext}$ (derived using the Henderson-Hasselbalch equation; Fig. 1E). By contrast, influx as a function of rising $[NH_4^+]_{ext}$ showed a declining pattern, particularly above 9 mM (Fig. 1F). These effects were observed under both low (0.02 mM) and high (5 mM) external K^+ concentrations, which were applied in context of the known regulation of NH_3/NH_4^+ fluxes by K^+ (Szczerba et al., 2008; Balkos et al., 2010; ten Hoopen et al., 2010). Under low K^+, where NH_3/NH_4^+ toxicity is most severe (Britto and Kronzucker, 2002; Balkos et al., 2010), total influx plateaued at approximately 200 μmol g^{-1} h^{-1}, the highest transmembrane flux of NH_3/NH_4^+ hitherto reported in any plant system. Under high K^+, where relief from toxicity is observed (Britto and Kronzucker, 2002), $[NH_3]_{ext}$-dependent influx was significantly lower, as apparent in the decrease in V_{max} (from 204.8 ± 14.5 to 80.0 ± 4.5 μmol g^{-1} h^{-1} for low- and high-K^+ plants, respectively). By contrast, no significant differences in K_m were observed between K^+ conditions (0.15 ± 0.05 versus 0.09 ± 0.03 mM for low- and high-K^+ plants, respectively). The energetic consequences of increases in NH_3/NH_4^+ influx with pH were also tested using root respiration measurements. We found that, despite the much higher influx observed when pH was changed from 6.25 to 9.25, steady-state root O_2 consumption decreased by approximately 55% within 5 min of this change in low-K^+, high-NH_3/NH_4^+ plants (Fig. 2). By contrast, no such changes were observed when nitrate (NO_3^-) was the sole N source.

As with influx, efflux of $^{13}NH_3/^{13}NH_4^+$ from prelabeled roots was strongly stimulated by alkaline solution pH (and thus, higher external $[NH_3]$ to $[NH_4^+]$ ratios; Supplemental Fig. S1A). In plants grown on high NH_3/NH_4^+ and low K^+, sudden (at 8 min; Supplemental Fig. S1A, see arrow) upward shifts in external pH immediately and significantly stimulated $^{13}NH_3/^{13}NH_4^+$ efflux, with greater stimulations observed at higher pH values. When tracer release was plotted as a function of the concomitant NH_3/NH_4^+ influx (measured at the identical pH shift), we observed a strong linear relationship between efflux and influx (Supplemental Fig. S1B). Moreover, tracer release as a function of $[NH_3]_{ext}$ (which we suggest may be equivalent to cytosolic $[NH_3]$; see below) resulted in Michaelis-Menten kinetics similar to those seen with influx, having K_m values ranging from 0.10 to 0.36 mM $[NH_3]$ (Supplemental Fig. S1B, inset).

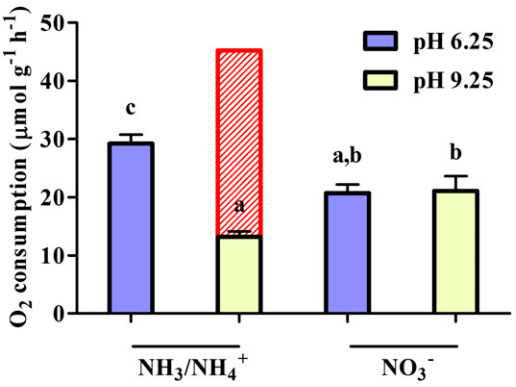

Figure 2. Effect of 5-min exposure to elevated pH (pH 9.25) on root respiration in barley plants grown with 0.01 mM K_2SO_4 and 5 mM of either $(NH_4)_2SO_4$ or $Ca(NO_3)_2$. Red bar represents O_2 consumption, predicted if NH_3/NH_4^+ influx at pH 9.25 is comprised entirely of cationic NH_4^+ fluxes (see Fig. 1F). Letters denote significantly different means ($P < 0.05$) as determined by one-way ANOVA with Tukey's posthoc test. [See online article for color version of this figure.]

We sought to further characterize $^{13}NH_3/^{13}NH_4^+$ efflux in intact roots under toxicity conditions (low K^+, high NH_3/NH_4^+) to identify the compartmental origin of tracer release (Britto and Kronzucker, 2003; Coskun et al., 2010). In roots prelabeled with tracer, $^{13}NH_3/^{13}NH_4^+$ efflux was immediately suppressed by sudden (at 8 min; Fig. 3A, see arrow) exposure to 4°C or upon withdrawal of external NH_3/NH_4^+, while an external pH shift to 9.25 (from 6.25) resulted in an immediate and sizable efflux stimulation. Importantly, these findings demonstrate that efflux analysis under toxic conditions captures physiological (i.e. transmembrane) events, not artifacts of apoplastic exchange (Coskun et al., 2010, 2013). Thus, compartmental analysis by tracer efflux could be applied (Lee and Clarkson, 1986; Kronzucker et al., 1997; Britto and Kronzucker, 2003), revealing efflux to influx ratios of approximately 80% and extremely rapid rates of both unidirectional fluxes characteristic of futile cycling (Britto et al., 2001b).

Further evidence for the intracellular origin of effluxed tracer was seen in a silver (Ag^+)-induced stimulation of $^{13}NH_3/^{13}NH_4^+$ release (Fig. 3B). We have previously shown that sudden exposure to Ag^+ causes extensive damage to both major membrane systems (plasmalemma and tonoplast) in barley roots (Coskun et al., 2012). By contrast, we observed no effect of mercury (Hg^{2+}) application on tracer release (Fig. 3C), suggesting a lack of membrane disintegrity occurring. Importantly, this qualifies the use of Hg^{2+} as a potential inhibitor of AQPs (see below). With respect to the Ag^+-induced stimulation in tracer efflux, this effect allowed for quantification of released substrate (in terms of μmol g^{-1} root fresh weight) via integration of the ^{13}N loss and estimated intracellular specific activity, as shown previously (Coskun et al., 2012). The chemical quantity of NH_3/NH_4^+ released during Ag^+ application (64 ± 3 μmol g^{-1}) was very similar to that of total root tissue NH_3/NH_4^+ content under control conditions, as

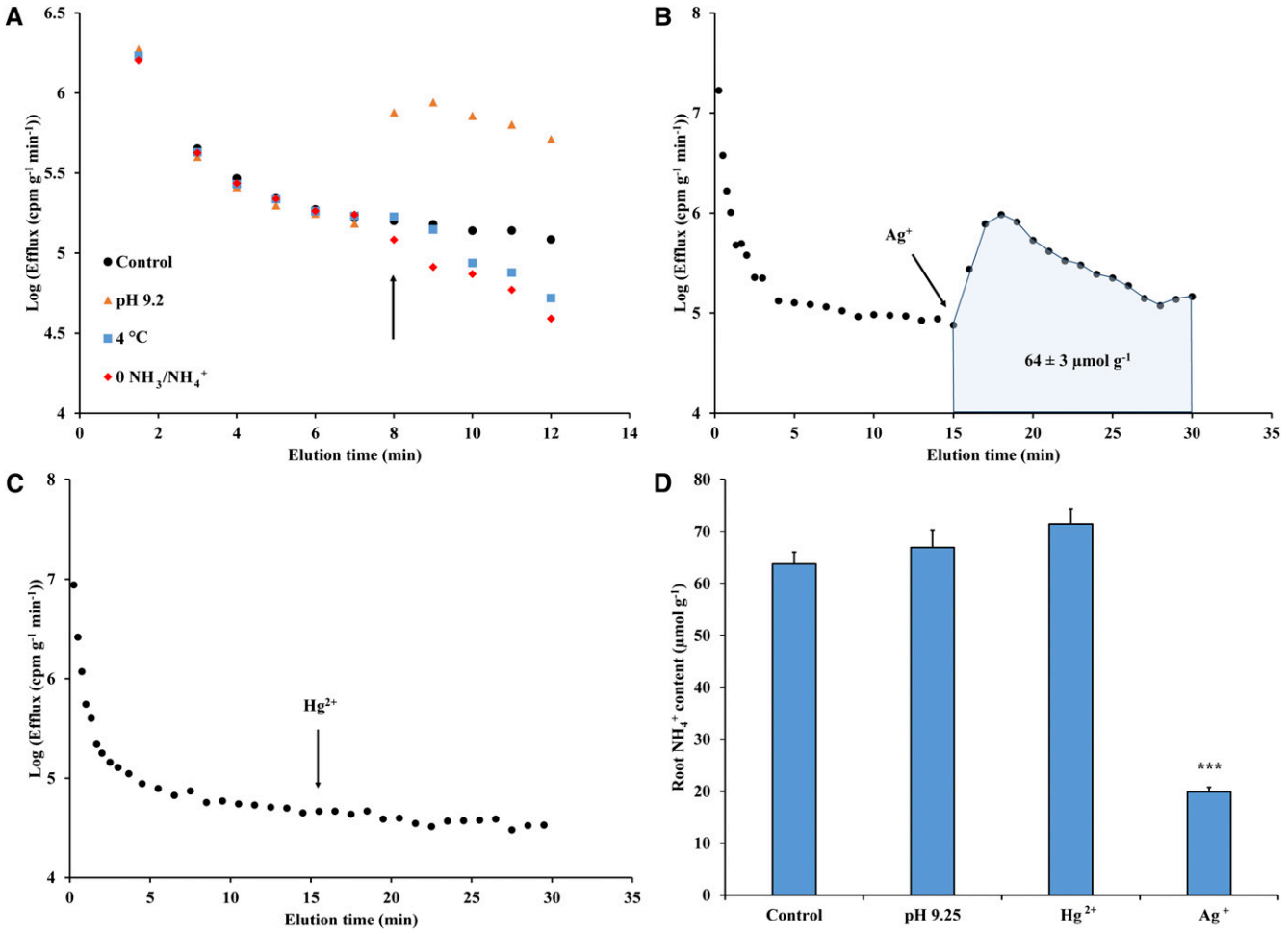

Figure 3. Characterization of NH_3/NH_4^+ efflux from roots of intact barley seedlings. A, Effect of sudden (at 8 min; see arrow) exposure to either pH 9.25, 4°C, or N withdrawal from the external medium. Each point represents the mean of three to seven replicates (SE of the mean <15% of the mean). B, Effect of sudden (at 15 min; see arrow) exposure to 500 μM Ag^+. Integration range of tracer release due to Ag^+ and results of integration are given in shaded area. Each point represents the mean of four replicates (SE of the mean <15% of the mean). C, Lack of effect of 500 μM Hg^{2+} application (at 15.5 min; see arrow) on root $^{13}NH_3/^{13}NH_4^+$ efflux. Each point represents the mean of three replicates (SE of the mean <15% of the mean). D, Root NH_4^+ content measured using OPA and its effect due to 15-min exposure to pH 9.25, 500 μM Hg^{2+}, or 500 μM Ag^+. Each bar represents mean ± SE of the mean ($n \geq 6$). Asterisks denote significant difference from control ($P \leq 0.001$) as determined by one-way ANOVA with Dunnett's posthoc test. In all sections, plants were grown at 5 mM $(NH_4)_2SO_4$ and 0.01 mM K_2SO_4. [See online article for color version of this figure.]

measured by chemical (orthophthaldehyde [OPA]) analysis (63.8 ± 2.3 μmol g^{-1}; Fig. 3D). We should note that, although efflux still proceeded after termination of the Ag^+ treatment (Fig. 3B), the apparent premature curtailment of the treatment resulted in an underestimate of no more than approximately 1 μmol g^{-1}, which was considered negligible. Tissue analysis (determined by OPA assay) revealed that approximately 70% of root NH_3/NH_4^+ was lost during Ag^+ exposure, demonstrating that the majority of cellular (i.e. both cytoplasmic and vacuolar) NH_3/NH_4^+ was released (Fig. 3D). By contrast, pH 9.25 and Hg^{2+} resulted in no change in tissue NH_3/NH_4^+ content (Fig. 3D), despite the significant effects on both influx and efflux of the former (see above).

Lastly, to gain mechanistic insight into NH_3/NH_4^+ influx, the possible involvement of different types of membrane transporters in NH_3/NH_4^+ influx was tested by means of pharmacological profiling, in low-K^+, high-NH_3/NH_4^+ plants (Fig. 4). Hg^{2+}, a well-known blocker of AQP activity, was applied with significant effect (36% inhibition at pH 6.25), while further support for AQP involvement was observed with treatments known to induce intracellular acidosis, which can cause closure of AQPs via protonation of conserved His residues on the cytoplasmic side (Tournaire-Roux et al., 2003; Törnroth-Horsefield et al., 2006; Ehlert et al., 2009). Hydrogen peroxide (H_2O_2) and propionic acid (PA) were two such effective treatments (30% and 54% inhibition relative to control, respectively). N_2 treatment, however, was not as effective, despite its efficacy in other systems (Tournaire-Roux et al., 2003). Note that these acidifying treatments were only effective at lower external pH

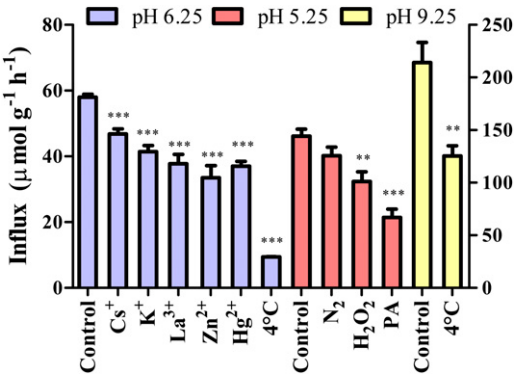

Figure 4. Pharmacological profile of NH_3/NH_4^+ influx into roots of barley seedlings at varying external pH. Ionic inhibitors were applied as chloride salts, except for K^+ (applied as K_2SO_4). Influx at pH 9.25 corresponds to *y* axis on the right. Each bar represents mean ± SE of the mean ($n \geq 5$). Asterisks denote significantly different means (**, $P \leq 0.01$; ***, $P \leq 0.001$) from respective control, as determined by one-way ANOVA with Dunnett's posthoc test (at pH 6.25 and 5.25) or Student's *t* test (at pH 9.25). Plants were grown as in Figure 3. [See online article for color version of this figure.]

(pH 5.25). Also, Hg^{2+} could not be tested at high pH (pH 9.25) due to hydroxide precipitation (Schuster, 1991). Other significant inhibitors of NH_3/NH_4^+ influx at pH 6.25 included $Cs^+ < K^+ < La^{3+} < Zn^{2+} \ll 4°C$. The highest influx, seen at pH 9.25, was also suppressible at 4°C by approximately 44%.

DISCUSSION

This study critically reexamines the nature of futile transmembrane NH_3/NH_4^+ cycling in barley roots, a phenomenon with ties to NH_4^+ toxicity in a wide range of higher plants (Feng et al., 1994; Britto et al., 2001b, 2002; Chen et al., 2013). We have addressed the fundamental question of which species of the conjugate pair (NH_3 or NH_4^+) is transported in the futile cycle to thus enable insight into mechanisms of transport, compartmentation, and toxicity of NH_3/NH_4^+.

The lack of agreement between $\Delta\Delta\Psi_m$ and changes in NH_3/NH_4^+ influx, in contrast to K^+, suggests that, above a small baseline cationic NH_4^+ flux no higher than that of K^+ (<5 μmol g^{-1} h^{-1}; Fig. 1A), electroneutral NH_3 transport accounts for the observed rapid rates of ^{13}N transport in intact barley roots (Fig. 1, A–C). While previous tracer studies have also demonstrated that NH_3/NH_4^+ fluxes exceed those of K^+ at equimolar concentrations (Scherer et al., 1984; Vale et al., 1988; Wang et al., 1996), none have provided parallel membrane potential measurements. A comparison between fluxes and $\Delta\Delta\Psi_m$, however, is of great utility in gauging the relative apportionment of NH_3 and NH_4^+ fluxes, as we show here.

Because such rapid NH_3 fluxes in planta are simply without precedent, additional investigation was called for. Further evidence in support of NH_3 uptake was seen in the Michaelis-Menten character of the $[NH_3]_{ext}$-dependent influx isotherms (Fig. 1E). By contrast, NH_4^+ influx was seen to decline with rising $[NH_4^+]_{ext}$ (Fig. 1F), ruling out a sizeable contribution from that N species. We should note, however, that because these isotherms were obtained using changes in external pH, there may be pH-specific and/or NH_4^+-specific effects on transport. Such effects require examination, although they are inherently difficult to ascertain because pH and $[NH_3]$ to $[NH_4^+]$ ratios are inextricably linked. It should also be noted that in a study on rice, Wang et al. (1993b) observed a decline in $^{13}NH_3/^{13}NH_4^+$ influx with rising pH at 10 mM $[NH_3/NH_4^+]_{ext}$. However, the fluxes in their study were much lower than in this study and also were determined in an NH_4^+-tolerant species. Further investigation is necessary to determine whether this is a part of the strategy by which a plant may achieve tolerance to this N source.

We also provide evidence for NH_3 (but not NH_4^+) efflux under toxic (low-K^+, high-NH_3/NH_4^+) conditions. Firstly, the trans-inhibition and -stimulation of efflux in response to changes in NH_3 provision (by substrate withdrawal and pH 9.2, respectively; Fig. 3A) suggests that NH_3 efflux is highly dependent on NH_3 influx (see also the linear dependence of the fluxes; Supplemental Fig. S1B), which is consistent with observations that efflux to influx ratios increase with rising influx (Wang et al., 1993a; Britto et al., 2002; Britto and Kronzucker, 2006). Such trans-inhibition and -stimulation of efflux have previously been shown in barley (Britto and Kronzucker, 2003) and in the mammalian literature, specifically for amino acids (White and Christensen, 1982; Sweiry et al., 1991). In the latter case, trans-stimulation of efflux has been attributed to a large counterflow through a single transporter mediating bidirectional fluxes and, as such, could in large part explain the futile NH_3 cycling in this paper. Further evidence that the ^{13}N efflux trace represents $^{13}NH_3$ and not $^{13}NH_4^+$ is found in its saturating response to $[NH_3]_{ext}$ (Supplemental Fig. S1B, inset), which resembles that of influx (Fig. 1E). K_m values for efflux, which were comparable to those for influx (ranging between 0.10–0.36 mM NH_3), suggests a similar, if not identical, mechanism of NH_3 transport for the two fluxes. It is not clear why the efflux step should respond so readily to changes in external NH_3, when substrate binding to an efflux transporter must take place intracellularly. Intriguingly, it may be that NH_3 transport responds to $[NH_3]_{ext}$ in a manner that leads to a rapid equalization between NH_3 pools on either side of the plasma membrane, and thus NH_3 efflux kinetics are in fact directly responding to cytosolic $[NH_3]$ and only indirectly to $[NH_3]_{ext}$. NH_3 may shuttle rapidly among multiple cellular compartments, establishing similar equilibrium concentrations in each, where membrane permeabilities permit (see below).

The Michaelis-Menten analyses under low-K^+, high-NH_3/NH_4^+ conditions (see above; Fig. 1E) revealed a V_{max} of about 200 μmol g^{-1} h^{-1} for NH_3 influx, the highest bona fide transmembrane flux hitherto reported in any plant system. Such rapid fluxes are orders of magnitude

higher than typical fluxes of mineral (ionic) nutrients (Britto and Kronzucker, 2006). Although fluxes of sodium (Na^+) under toxic (saline) conditions have been reported to reach or exceed such values (Lazof and Cheeseman, 1986; Essah et al., 2003; Malagoli et al., 2008), the validity of these fluxes have recently come into question, particularly with respect to their unrealistic energetic requirements (Britto and Kronzucker, 2009; Kronzucker and Britto, 2011); moreover, such fluxes are generally reported at much higher external substrate concentrations (typically, 100 mM or higher). On the other hand, such energetic limitations do not apply to the passive electroneutral fluxes of NH_3. In fact, root O_2 consumption was found to decline under such conditions (i.e. pH 9.25; Fig. 2), a result that further discounts NH_4^+-specific futile cycling, which is predicted to involve a thermodynamically active efflux (Britto et al., 2001b). Figure 2 highlights, in red, the theoretical increase in O_2 consumption necessary to power an active efflux mechanism of NH_4^+ when fluxes are as high as 200 μmol g^{-1} h^{-1} based on current models of ion transport and O_2 consumption (Poorter et al., 1991; Kurimoto et al., 2004; Britto and Kronzucker, 2009). This large energy deficit is consistent with the idea that futile cycling is primarily of the conjugate base, NH_3. However, unlike with the previously proposed NH_4^+ cycling, the term futile here does not refer to an energy-dissipating process (e.g. Amthor, 2000), but more generally to the lack of apparent functional utility in the NH_3 cycle. The fact that the pH shift did not affect root O_2 consumption in NO_3^--grown plants shows an N source specificity of this effect that will require further investigation to explain.

These results have important consequences for the compartmentation and toxicity of NH_3/NH_4^+. Compartmental analyses with $^{13}NH_3/^{13}NH_4^+$ in roots and shoots of plants grown under toxic (high-NH_3/NH_4^+) conditions generally yield extremely high "pool sizes" of many hundred millimolar (Britto et al., 2001b, 2002), leading to the speculation that the entire cell, not simply the cytosol, acts as a single compartment of tracer origin (Britto and Kronzucker, 2003; Balkos et al., 2010). This study provides the first evidence in support of this "whole-cell" hypothesis. Because a high-capacity, thermodynamically passive NH_3 transport can account for futile cycling, it is feasible that NH_3 rapidly equilibrates across intracellular membranes and among cellular compartments, particularly the vacuole and cytosol (Fig. 5). How such rapid unidirectional fluxes can persist given the apparent lack of an NH_3 concentration gradient across cellular compartments is an interesting question and can be most simply explained by passive diffusion through high-capacity membrane channels such as AQPs (see below). The NH_3 equilibration across cellular compartments can explain why tracer accumulation (measured as counts retained in tissue or released with Ag^+ application; Fig. 3B) closely agreed with that of chemical (OPA) analyses measuring tissue NH_3/NH_4^+ content (Fig. 3D). Thus, the NH_4^+ content within each compartment may be ultimately determined by NH_3 permeation and compartmental pH, as illustrated in Figure 5. In this revised model of futile cellular N cycling, the 0.5 to 1.5 mM range of cytosolic [NH_4^+] agrees well with measured values from studies using methods such as ion-selective microelectrodes and NMR (Lee and Ratcliffe, 1991; Wells and Miller, 2000). Importantly, the model reveals that a hyperaccumulation of NH_4^+ in the vacuole would ultimately exist (Fig. 5), due to vacuolar acid trapping, and could explain the frequently observed suppressions in cationic nutrients, notably K^+ (but also Ca^{2+} and Mg^{2+}; Barker et al., 1967; Van Beusichem et al., 1988; Lang and Kaiser, 1994; Kronzucker et al., 2003), which may ultimately be the major cause of NH_3/NH_4^+ toxicity in higher plants.

It is likely that the rapid NH_3 cycling reported here is mediated by AQPs (Fig. 4), which have high transport capacity and are known to conduct NH_3 fluxes (Jahn et al., 2004; Holm et al., 2005; Loqué et al., 2005; Saparov et al., 2007; Hove and Bhave, 2011). Kozono et al. (2002) estimated the rate of water transport through AQP1 to be 3×10^9 molecules per subunit per second, roughly 30-fold higher than K^+ transport via the potassium crystallographically-sited activation (KcsA) channel, which is among the fastest ion channels (Morais-Cabral et al., 2001). AQP involvement is suggested by the pharmacological profiling of NH_3 influx in our study, particularly in the effect of Hg^{2+}, a classic AQP inhibitor (Fig. 4). Importantly, unlike with Ag^+

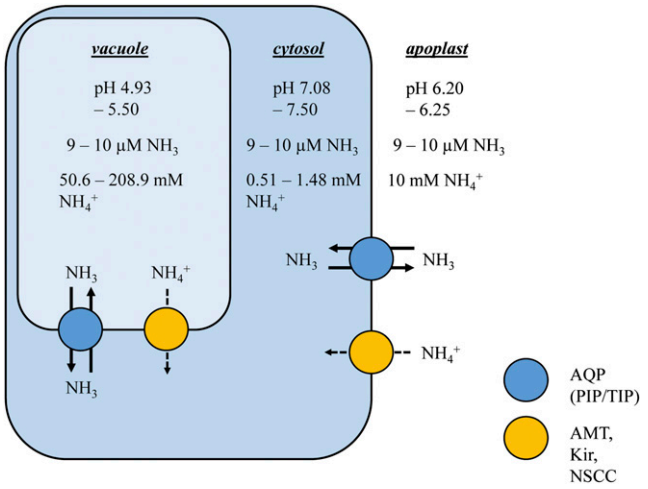

Figure 5. Revised model of futile transmembrane NH_3/NH_4^+ cycling in root cells of higher plants. Uncharged NH_3 rapidly equilibrates across both major membrane systems (plasmalemma and tonoplast) and is likely mediated by AQPs specific to each system. A relatively minor channel/carrier-mediated flux of NH_4^+ may also occur across both membrane systems. NH_4^+ concentrations are a function of NH_3 equilibration and compartment pH (Roberts et al., 1982; Walker et al., 1996; Kosegarten et al., 1997; Kosegarten et al., 1999). PIP, Plasmalemma-intrinsic protein; TIP, tonoplast-intrinsic protein; AMT, ammonium transporter; Kir, K^+ inward rectifier; NSCC, nonselective cation channel. [See online article for color version of this figure.]

(Fig. 3B), Hg^{2+} showed no sign of causing membrane damage in our system (as manifest in lack of efflux stimulation or tissue content losses; Fig. 3, C and D; compare with Coskun et al., 2012). The strong suppressions of influx by H_2O_2 and PA also support AQP involvement (Fig. 4; Tournaire-Roux et al., 2003; Törnroth-Horsefield et al., 2006; Ehlert et al., 2009). It is worth highlighting here, however, that pharmacological profiling, like any method, is not without its caveats. The lack of specificity of several blockers/chemical treatments (Coskun et al., 2013), as well as the need to employ relatively high concentrations at times, can potentially have secondary effects. This by no means invalidates the use of pharmacology, but highlights the importance of a diversity of experimental approaches. Future experiments with AQP antisense/knockout lines (Martre et al., 2002; Javot et al., 2003), particularly for AQPs already shown to mediate NH_3 fluxes (Jahn et al., 2004; Holm et al., 2005; Loqué et al., 2005), could help further elucidate their involvement in futile NH_3 cycling. In addition, such mutant analyses could provide a critical test of the "whole-cell distribution" hypothesis for NH_3 presented above (Fig. 5).

We end by highlighting the suppression of V_{max} for NH_3 influx by high-plant K^+ status, while K_m remains unaffected (Fig. 1E). It appears that K^+ status has no effect on the substrate affinity of NH_3 transporters but regulates NH_3 influx by other means. One such mechanism might involve the modulation of AQP activity, which may well be expected, because, in plants, K^+ acquisition is the chief means of establishing osmotic balance and cell turgor (Britto and Kronzucker, 2008; Grzebisz et al., 2013). The effects of K^+ in the short-term suppression of NH_3/NH_4^+ influx and efflux (Szczerba et al., 2008; Balkos et al., 2010; see also Fig. 4) also suggest a post-translational regulation of NH_3 transporters by K^+. Such a mechanism may explain the agriculturally important alleviation of NH_3/NH_4^+ toxicity in higher plants by K^+ (Barker et al., 1967; Szczerba et al., 2008; Balkos et al., 2010) and thus pave the way for future studies.

MATERIALS AND METHODS

Plant Culture

Barley (*Hordeum vulgare* 'Metcalfe') seedlings were grown hydroponically for 4 d (after 3-d germination in sand) in a climate-controlled growth chamber (Coskun et al., 2013). Hydroponic tanks contained aerated, N- and K^+-free, modified Johnson's solution (pH = 6.25) and were frequently exchanged to maintain a nutritional steady state. Depending on the experiment, N was supplied as either NH_4^+ (0.1 or 10 mM, as $(NH_4)_2SO_4$) or NO_3^- (10 mM, as $Ca(NO_3)_2$) and K^+ was supplied at either 0.02, 0.1, or 5 mM as K_2SO_4 (see "Results").

Radiotracer Experiments

^{13}N (half-life = 9.98 min) was used to trace the unidirectional fluxes and cellular compartmentalization of NH_3/NH_4^+ in roots of intact seedlings (Kronzucker et al., 1997; Britto et al., 2001b). For steady-state influx experiments, roots were incubated for 5 min in aerated growth solution spiked with $^{13}NH_3/^{13}NH_4^+$, then desorbed in two sequential steps (for 5 s and 5 min) in nonradioactive growth solution to release tracer from extracellular spaces (Balkos et al., 2010). Treatment conditions were conducted as above, with some modifications. These included concentration-dependent and pH-dependent isotherms, whereby growth solution $[NH_3/NH_4^+]_{ext}$ and pH were adjusted (with NaOH) as specified in all solutions, including a 10-min-preloading solution. Other treatments included 10 mM CsCl, 5 mM K_2SO_4, 10 mM $LaCl_3$, 10 mM $ZnCl_2$, and 500 μM $HgCl_2$ and chilling (4°C) in preloading (10 min) and loading solutions. A subset of experiments involved a 2-h pretreatment at pH 5.25 ± N_2 bubbling (anoxia treatment), 2 mM H_2O_2, or 20 mM PA. For all treatments, after the final desorption, roots were separated from shoots, spun in a low-speed centrifuge for 30 s to remove surface water, weighed, and counted for γ-ray emissions. A small subset of experiments were conducted, as above for the concentration-dependent isotherm, but with ^{42}K (half-life = 12.36 h; Coskun et al., 2013).

For compartmental analysis by tracer efflux, roots were exposed for 1 h in loading solution to maximize intracellular-specific activity of the tracer (Kronzucker et al., 1997), then placed in efflux funnels and eluted of radioactivity with successive 20-mL aliquots of fresh, nonlabeled growth solution for various washout periods. The desorption series was timed as follows, from first to final eluate: 1.5 min (twice), 1 min (nine times), for a total of 12 min. A subset of experiments involved either chemical (pH 7.25, 8.25, and 9.25 or complete NH_3/NH_4^+ withdrawal) or cold (4°C) treatment for the final 5 min of elution (see "Results"). Another subset involved a longer elution protocol (30 min; Coskun et al., 2012) and the sudden (at 15 min) application of 500 μM $AgNO_3$ to disrupt membranes and release tracer accumulated in the cell (Coskun et al., 2012). Following elution, roots were handled as above, and radioactivity in roots, shoots, and efflux eluates were counted.

To quantify the chemical amount of released NH_3/NH_4^+ during Ag^+ application (above), an integration technique was employed as described in detail elsewhere (Coskun et al., 2012). In brief, the summation of radioactivity released (in counts per min) during Ag^+ treatment was divided by the internal specific activity at the time of Ag^+ application (taking into account the exponential rise in specific activity during loading and its decline during elution up to the time of Ag^+ application) and corrected for root fresh weight.

Electrophysiological Measurements

Membrane potential differences in epidermal and cortical root cells from intact barley seedlings were measured as described in detail elsewhere (Schulze et al., 2012). In brief, roots were immersed in growth solution in a plexiglass cuvette mounted onto a light microscope. Root cells were impaled with a glass microelectrode, and potential differences were recorded with the use of an electrometer. Once stable readings were achieved, growth solution was exchanged by use of peristaltic pumps at approximately 7.5 mL min^{-1}. Treatments included growth solution supplemented with rising concentrations of either NH_3/NH_4^+ or K^+ (see "Results"), and $\Delta\Delta\Psi_m$ was recorded.

Respiration Measurements

Root respiration was measured in intact barley seedlings as described in detail elsewhere (Malagoli et al., 2008). In brief, roots of 7-d-old seedlings were immersed in growth solution in a 3-mL Hansatech cuvette/O_2 electrode system, and the decline in dissolved O_2 was recorded over 10 min, after which roots were dried and weighed, as described above.

Tissue Content Measurements

Tissue NH_4^+ content was determined by the OPA method as described in detail elsewhere (Coskun et al., 2012). Briefly, roots of 7-d-old intact seedlings were immersed for 5 min in aerated 10 mM $CaSO_4$ to desorb extracellular NH_4^+, and plant organs were harvested as described above. For treatments, roots were first immersed for 15 min in aerated growth solution supplemented with either 500 μM $AgNO_3$, 500 μM $HgCl_2$, or pH 9.25 (titrated with NaOH) prior to $CaSO_4$ desorption. From there, root and shoot tissue was pulverized under liquid N_2, and NH_4^+ was extracted with 10 mM formic acid (Husted et al., 2000). Purified supernatant was added to OPA reagent (Goyal et al., 1988; Coskun et al., 2012), and the color was left to develop in the dark at room temperature for 30 min. Sample absorbance was measured at 410 nm using a spectrophotometer.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. pH dependence of root $^{13}NH_3/^{13}NH_4^+$ efflux and its relationship to influx.

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