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Mice Deficient in L-12/15 Lipoxygenase Show Increased Vulnerability to 3-Nitropropionic Acid Neurotoxicity

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Abstract

Considerable evidence supports a contributory role for leukocyte-type 12/15 Lipoxygenase (L-12/15 LO) in mediating hippocampal and cortical neuronal injury in models of Alzheimer's disease and stroke. Whether L-12/15 LO contributes to neuronal injury in a model of Huntington's disease (HD) has yet to be determined. HD is characterized by marked striatal neuronal loss, which can be mimicked in humans and animals by inhibition of mitochondrial complex II using 3-Nitropropionic acid (3-NP). Herein, we compared histological and behavioral outcomes between mice that were wild-type or null for L-12/15 LO following systemic injection of 3NP. We found that mice deficient in L-12/15 LO had a higher incidence of striatal lesions coincident with an increase in morbidity as compared to their wild-type littermate controls. This could not be explained by differential metabolism of 3-NP as striatal succinate dehydrogenase activity was inhibited to the same extent in both genotypes. The present results show that deleting L-12/15 LO is detrimental to the striatum in the setting of chronic, systemic 3-NP exposure and are consistent with the overall conclusion that region-specific effects may determine the ultimate outcome of L-12/15 LO activation in the setting of brain injury.

Keywords

Huntington's disease; oxidative stress; 3-nitropropionic acid; striatal injury; L-12/15- lipoxygenase

1. Introduction

Lipoxygenases (LOs) catalyze the oxidation of free and esterified phospholipid fatty acids generating bioactive lipid mediators and reactive oxygen species (ROS) [13, 42]. The leukocyte-type 12/15 LO (L-12/15 LO) is expressed throughout brain parenchyma in both neurons and glial cells of the cerebral cortex, basal ganglia, and hippocampus [33]. Physiologically, L-12/15 LO metabolites modulate several neuronal ion channel conductances [9, 29, 35, 47]. Pathophysiologically, L-12/15 LO activation is linked to

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neuronal cell death in animal models of both cerebral ischemia and Alzheimer's disease [13, 22, 37, 46, 51] and in various *in vitro* models of oxidative stress [22, 26, 27, 32, 38, 54].

Although the exact mechanism(s) by which L-12/15 LO facilitates neuronal injury remain(s) to be fully elucidated, it is known to oxidatively modify lipoproteins and phospholipids [24, 44, 53] and to damage mitochondria [45]. Mitochondrial dysfunction is considered to be a common mediator of many acute and chronic neurological diseases, though it most closely and directly links to the pathophysiology of Huntington's disease (HD), an adult-onset autosomal-dominant inherited neurodegenerative disease caused by a mutation in the short arm of human chromosome 4p16.3 [14, 28, 30]. Whether L-12/15 LO activation contributes to striatal damage in HD models has yet to be explored. 3-nitropropionic acid (3-NP) —a phyto/fungal toxin — irreversibly inhibits the electron transport enzyme succinate dehydrogenase (SDH) and produces striatal lesions and cognitive and motor dysfunction in rodents and primates that closely resemble that of human HD [5, 7, 34]. Thus, it was used herein to compare histological and behavioral outcomes of mice wild-type or null for *alox15,* the gene that encodes for the protein L-12/15 LO.

2. Materials and methods

2.1 Animal husbandry

Wild-type and mutant mice for study (male, 15–18 wks) were derived from F1 heterozygous (+/−) breeding units obtained by crossing *Alox15* −/− (JAX, # 002778) male mice with wild-type C57BL/6J (+/+) female mice (JAX, #000664). F2 and F3 generations were used. Genotyping was performed via PCR analysis of tail genomic DNA samples: WT primers (266 bp) 5′-CGT GGT TGA AGA CTC TCA AGG -3′ (forward), 5′-CGA AAT CGC TGG TCT ACA GG -3′ (reverse); mutant primers (280 bp) 5′-CTT GGG TGG AGA GGC TAT TC -3′ (forward), 5′-AGG TGA GAT TAC AGG AGA TC -3′ (reverse). Mice were housed three to five per cage on a 12 hr light/dark schedule in an AALAC-accredited Animal Care facility. Mouse chow and water were provided *ad libitum.* Housing/breeding strategies were employed to control for genetic and environmental influences [49, 50].

2.2 3-nitropropionic acid (3-NP) Dosing

3-NP (Sigma) was dissolved in 0.9% saline at 25 mg/mL, adjusted to pH 7.4 using 5N NaOH, filter sterilized, and stored at 4°C for up to one week. Mice —who had been acclimated to handling for 5 days prior — received a total systemic dose of 780 mg/kg i.p. 3-NP (b.i.d 8–12 h intervals) given over nine days using an escalating dosing protocol as follows: 20 mg/kg × 2d, 30 mg/kg × 2d, 50 mg/kg × 1d, and 60 mg/kg × 4d. Mice completing the study were sacrificed ~12 hr after the final injection.

2.3 Behavioral Scoring

The severity scale for 3-NP-induced motor disorders was performed as described [15]. Hindlimb clasping, general locomotor activity, hindlimb dystonia, truncal dystonia and postural adjustment reflexes were assessed twice per day just prior to each injection by an observer blinded to genotype. Three scales were assigned corresponding to no abnormality, moderate or severe deficits. Any mouse attaining a cumulative behavioral score ≥ 9 or

sustaining a weight loss ≥ 20% was immediately sacrificed. Five separate experiments were performed over 6 months.

2.4 Histology

Frozen brain sections (20 μm) cut serially through the rostro-caudal extent of each brain (+1.54 to −0.22 relative to bregma) were stained with 0.5% thionin essentially as described [12]. Images (1200 pixels) were captured by scanning (Epson Perfection 3170). The lesion area — denoted by pale thionin staining — as well as the total striatal area was measured using NIH ImageJ at three levels from bregma (+1.10, +0.86 and +0.50) by three individuals blind to the mouse's genotype. For each level, the percent striatal damage was calculated as follows: (L/T) × 100, where L and T represent the lesioned area and total striatal area, respectively. Data are expressed as the mean lesion volume + SEM of all three levels derived from the mean calculated for all individuals.

2.5 Succinate dehydrogenase (SDH) assay

SDH activity was determined in crude brain mitochondrial preparations two hr following injection with either saline or 3-NP (200 mg/kg, i.p.) exactly as described [41].

2.6 Statistical analysis

Statistical analyses were performed using GraphPad Prism, Version 6.03 as described in each figure legend. Percent data were transformed (arcsin square root) prior to analysis. Weight data were analyzed via two-way repeated measures ANOVA. Behavioral data were analyzed by two-way repeated measures ANOVA following log transformation of the raw data [Y = log (Y+1)].

3. Results

Alox15 $^{+/+}$ and *Alox15* $^{-/-}$ were used to examine how loss of function of L-12/15 LO would affect 3-NP-induced motor system impairment and striatal toxicity. After the first four injections of 3-NP, mice did not display any visible neurological deficit symptoms (Figure 1). Motor symptoms increased progressively thereafter in both genotypes similarly with increased days of dosing with 3-NP (Fig. 1A, $p < 0.0001$ for day and $p = 0.827$ for genotype) as did the expected reduction in gross body weight (Fig. 1B, $p < 0.0001$ for day and $p = 0.469$ for genotype). Despite this, not all mice, regardless of genotype, developed histologically-identifiable lesions (Fig. 2). However, the incidence of lesions between genotypes did differ (Fig. 2B). Only 19% of *Alox*15$^{+/+}$ mice showed 3-NP induced striatal neurodegeneration, whereas the incidence of lesions in *Alox*15 $^{-/-}$ mice more than doubled to 43% (Fig. 2B, p=0.031). This increase was dominated by a high proportion of smaller lesions with a mean size of 20 ± 4% of the total striatal area (Fig. 2B, $p = 0.006$). Coincident with this was a genotype-dependent increase in morbundity with 44.4% of the *Alox15* $^{-/-}$ attaining a score of ≥ 9 compared to just 24.2% of *Alox15* $^{+/+}$ littermate controls (Fig. 3A, $p = 0.039$). However, the rate at which the mice in each genotypic group were removed — graphed as % survival — did not differ (Fig. 3B, p=0.086). To rule out the possibility that this underlying sensitivity to 3-NP was due to genotypic differences striatal SDH inhibition, we quantified striatal SDH activity after an acute systemic injection of 3-NP (200mg/kg,

i.p.). SDH activity in *Alox15*$^{+/+}$ and *Alox15*$^{-/-}$ was equally reduced (64.4% vs 66.4%, respectively) (Fig. 4).

Discussion

Herein, we demonstrate that mice deficient in L-12/15 LO are more sensitive to 3-NP-induced toxicity although a substantial individual variability in striatal lesion size in response to 3-NP in both genotypes was observed. This variability is not atypical; several studies demonstrate similar results [5, 6, 8]. Despite this, behavioral outcomes did not differ between genotypes. A caveat to this analysis is that mice null for the *Alox*15 gene demonstrated a 100% increase in behavioral morbidity when compared to their littermate controls, necessitating removal from the study. Additionally, we did not observe any differences in 3-NP-induced weight loss with nearly identical numbers of mice from each genotype [n = 6 (+/+) and 7 (−/−)] being removed for excessive weight loss, a phenomenon that also occurs in HD patients [4]. These findings support the idea that this is a consequence of inhibition of peripheral tissue mitochondrial function [3, 18].

Our findings of enhanced striatal sensitivity are in contrast with what has been previously demonstrated in models of stroke and Alzheimer's disease. Increased L-12/15 LO immunostaining was demonstrated in the peri-infarct area of mice subjected to experimental ischemia [46] as well as in post-mortem brain sections from human stroke patients [53]. The finding that *Alox15* $^{-/-}$ mice or *Alox15* $^{+/+}$ mice treated with a novel selective inhibitor of L-12/15-LO had less cerebral ischemic damage when compared to untreated *Alox15* $^{+/+}$ mice [53] indicated that this increase contributes to pathology. L-12/15 LO immunoreactivity was also increased in post-mortem AD brains when compared with age-matched controls, in a manner that correlated with the extent of lipid peroxidation [37, 52]. Genetic deletion of L-12/15 LO in the Tg2576 model of AD resulted in a significant reduction in the production and deposition of Aβ in the hippocampus that correlated with improvement of cognitive deficits [51], whereas genetic over-expression of L-12/15 LO in this same mouse strain, led to increased brain Aβ levels and worsened memory impairments [11]. Finally, 3xTg mice receiving systemic administration of a selective inhibitor of L-12/15 LO showed significant reductions in Aβ deposition, phosphorylation of tau, and improvements of memory over those receiving placebo [10]. Together, these data have led to the suggestion that inhibition of L-12/15 LO may be a viable therapeutic strategy for the treatment of stroke [53] and AD [21].

Important differences in the function of the enzyme and/or the susceptibility of the different brain areas studied might explain the differing results found in our study. For instance, the stroke and AD studies focus on cortex and hippocampus, respectively, whereas herein the focus in on the striatum. Given that systemic injection of 3-NP causes similar inhibition of hippocampal, cortical and striatal SDH levels (our unpublished observations), a brain area specific mechanism seems plausible. Disparate cellular effects of L-12/15 LO also have been reported. Whereas its enzymatic products might act as pro-inflammatory factors in some settings [37, 51–53], studies also point to its role in suppressing inflammation via production of a class of eicosanoids called lipoxins [19, 39, 40]. Thus,. loss of L-12/15 LO result in unmitigated inflammation following 3-NP treatment [1, 2]. Enhanced inflammatory gene

expression, including increased peripheral expression of interleukin-1β, in L-12/15 LO deficient mice has been reported [23]. We have shown that IL-1β potentiates neuronal cell death under conditions of energy deprivation [16, 17, 20]. Should a similar result occur in brain, it would likely be deleterious. Finally, loss of L-12/15 LO could result in the build-up of arachidonic acid — which itself can be deleterious to brain [31, 48] — or could result in enhanced production of other, perhaps toxic, eicosanoids (e.g., cyclooxygenase [COX] or 5-Lipoxygenase [5-LO] derived metabolites) via substrate diversion [36, 43]. Of note, administration of licofelon, a dual inhibitor of COX/5-LO, significantly reduced 3-NP-induced HD-like symptoms in rats [25], suggesting either one or both enzymes are involved in 3-NP-induced neurotoxicity.

Conclusion

In brain, L-12/15 LO can be either beneficial or detrimental. In striatum, our data suggest that L-12/15 LO signaling may be part of an adaptive response that protects against insults resulting from altered bioenergetics or primary mitochondrial dysfunction. More broadly, present results are consistent with the conclusion that brain region-specific effects may determine the ultimate outcome of L-12/15 LO activation.

Acknowledgments

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Highlights

- Increased incidence of 3-NP-mediated striatal lesions in mice deficient in L-12/15 lipoxygenase (L-12/15 LO) as compared to wild-type mice.
- L-12/15 LO deficient mice show enhanced morbidity to systemic 3-NP treatment as compared to their wild-type littermate controls.
- L-12/15 LO is beneficial in the setting of chronic, systemic 3-NP.

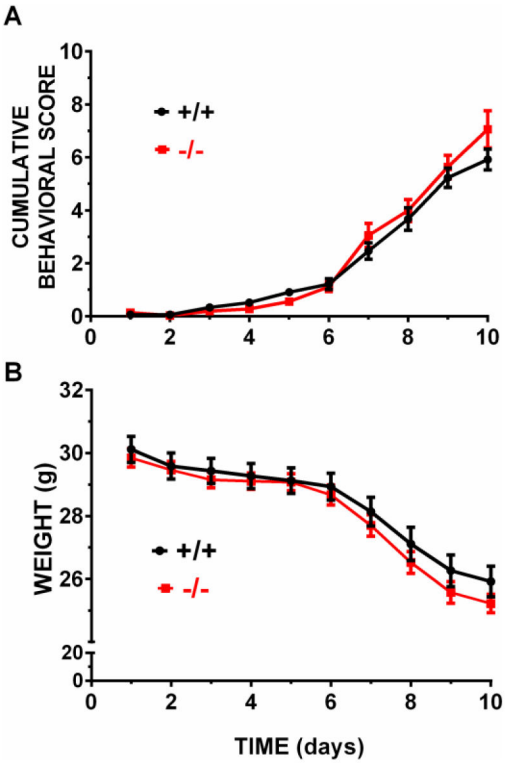

Figure 1. Comparision of behavioral motor deficits and weight loss between wild-type and L-12/15 LO deficient mice

L-12/15 LO null mutant mice (−/−, n = 36) and their wild-type littermates (+/+, n = 33) were injected twice daily with 3-NP as described in methods. (**A**) Behavioral motor scores were recorded just prior to each 3-NP injection. The 2nd score for each animal was used to generate the graph (mean ± SEM). **(B)** Each mouse was weighed daily prior to the first injection and the collective mass reported as mean ± SEM. There is no statistically significant difference between genotypes in either behavior or weight as assessed by two-way ANOVA.

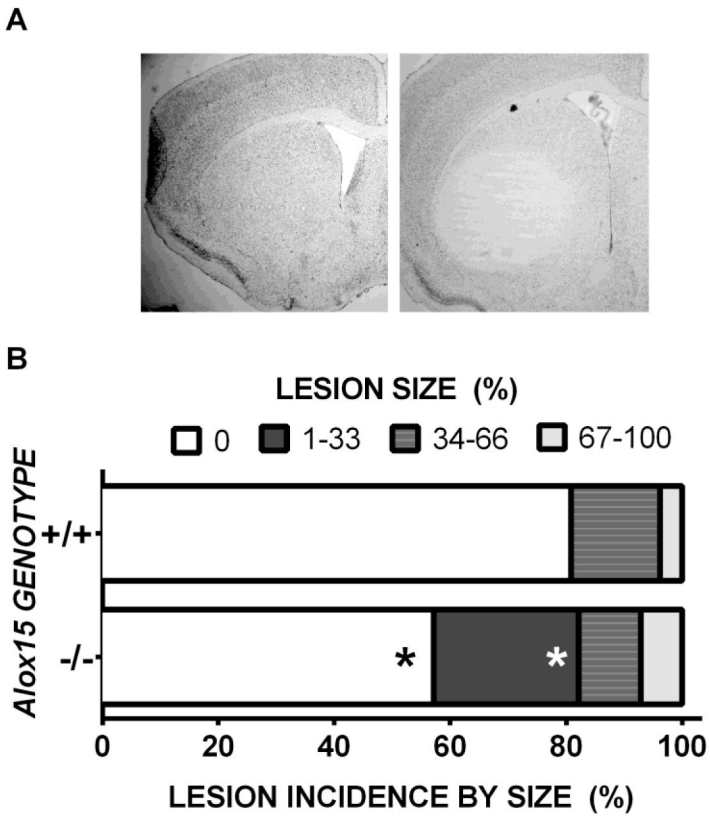

Figure 2. Comparison of lesion incidence between wild-type and L-12/15 LO deficient mice

(**A**) Representative photomicrographs depicting striatal outcomes of 3-NP exposure in wild-type (left) and L-12/15 LO null mutant mice (right) at +0.50 from the bregma. (**B**) Comparison of lesion incidence between +/+ (n=26) and −/− (n=28) mice. The graph depicts the percent of mice from each genotype in 4 defined groups based on lesion size (0, 1–33%, 34–66% and 67–100%) determined by dividing the number in each group by the total number of mice analyzed. The black asterisk (*) denotes a significant difference in overall lesion incidence ($p = 0.031$), whereas a white asterisk depicts a between-group size difference ($p = 0.006$), both determined by Chi-squared test. Sample sizes differ from those described in Fig. 1 as some slices were lost during procurement and/or staining. Mice found dead were also excluded from analysis.

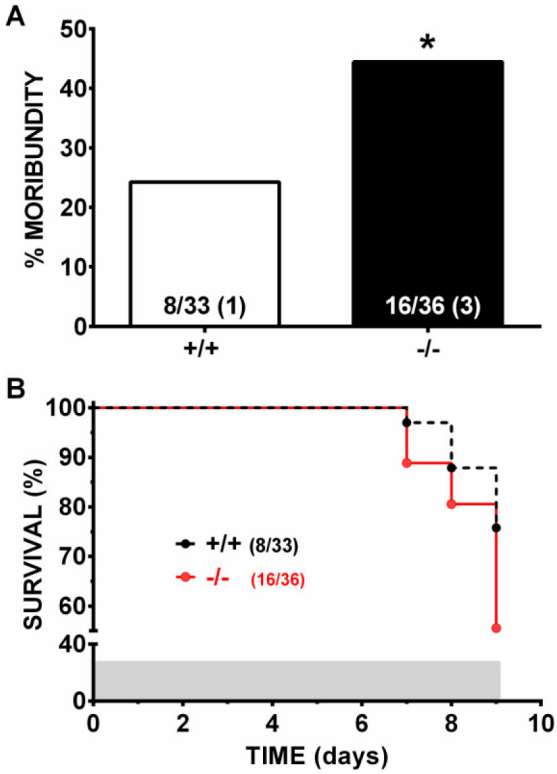

Figure 3. Toxicity of 3-NP is more severe in L-12/15 LO null mutant mice

(**A**) The number of L-12/15 LO +/+ and −/− mice determined to be moribund are expressed as the percent of the total number of mice subjected to the systemic injection paradigm (fraction within bars). Moribund is defined as any mouse found dead (number shown in parentheses within the bars) or mice sacrificed due to excessive weight lost (>20%) or severe motor behavioral deficits (score ≥ 9). (*) significantly different from +/+ group ($p = 0.039$, Chi-squared test). (**B**) A Kaplan–Meier survival curve depicts the rate at which the mice were removed from study. The shaded region represents the 9-day dosing paradigm. No genotypic differences in rate of removal was determined by Mantel-COX log-rank test.

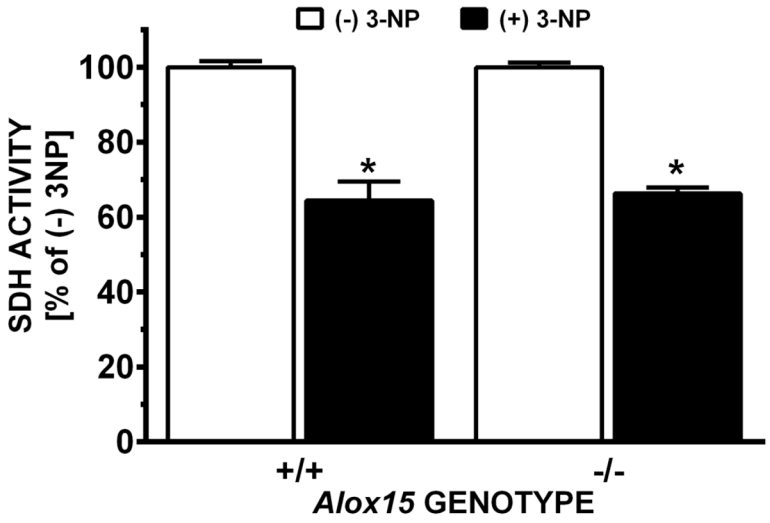

Figure 4. Striatal SDH activity after acute 3-NP injection

L-12/15 LO null mutant mice (−/−,) and their wild type littermates (+/+) were injected with 200 mg/kg 3-NP or saline (n = 3 each). Two hr later, SDH activities were measured as described in methods. Data were normalized to each genotype's control group (− 3-NP) and expressed as mean + SEM. Significant between-group differences (*;+/− 3-NP treatment), but no within-group (i.e., genotype) differences were found by two-way ANOVA followed by Bonferroni's t test.