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Developmental and Activity-Dependent Expression of LanCL1 Confers Antioxidant Activity Required for Neuronal Survival

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SUMMARY

Production of reactive oxygen species (ROS) increases with neuronal activity that accompanies synaptic development and function. Transcription-related factors and metabolic enzymes that are expressed in all tissues have been described to counteract neuronal ROS to prevent oxidative damage. Here, we describe the antioxidant gene *LanCL1* that is prominently enriched in brain neurons. Its expression is developmentally regulated and induced by neuronal activity, neurotrophic factors implicated in neuronal plasticity and survival, and oxidative stress. Genetic deletion of *LanCL1* causes enhanced accumulation of ROS in brain, as well as development-related lipid, protein, and DNA damage; mitochondrial dysfunction; and apoptotic neurodegeneration. *LanCL1* transgene protects neurons from ROS. LanCL1 protein purified from eukaryotic cells catalyzes the formation of thioether products similar to glutathione S-transferase. These studies reveal a neuron-specific glutathione defense mechanism that is essential for neuronal function and survival.

INTRODUCTION

Structural and functional plasticity of the developing nervous system are modulated by neuronal activity (Flavell and Greenberg, 2008). During this process, neurons are especially vulnerable to oxidative stress because neuronal activity increases oxygen utilization for energy production with the accompanying production of reactive oxygen species (ROS) (Coyle and Puttfarcken, 1993; Ikonomidou and Kaindl, 2011). Excessive ROS causes progressive oxidative damage to lipids, proteins, and DNA in neurons (Finkel and Holbrook, 2000); impairs synaptic function (Massaad and Klann, 2011; Stranahan and Mattson, 2012); and is implicated in developmental-related neurodegenerative diseases, including Alzheimer's and Parkinson's diseases (Andersen, 2004; Kondo et al., 2013). Antioxidant defense is evoked by neuronal activity to control ROS levels (Papadia et al., 2008; Soriano et al., 2011). Most antioxidant mechanisms are under the control of transcription factors *PGC-1α* and *Nrf-2* (Crunkhorn, 2012; St-Pierre et al., 2006). These mechanisms appear widely conserved across species and cell types and may be redundant since the genetic deletion of individual enzymes, or even *PGC-1α*, produces only modest phenotypic alterations without exogenous insult (Carlsson et al., 1995; Ho et al., 2004; Reaume et al., 1996; St-Pierre et al., 2006). Glutathione (GSH) is a major effector of antioxidant defense by virtue of its ability to scavenge free radicals and participate in the reduction of hydrogen peroxide (H_2O_2) (Hayes and McLellan, 1999). The GSH antioxidant defense mechanism involves multiple enzymes, including the GSH-dependent enzymes, GSH peroxidase (GPX) and GSH S-transferase (GST) (Crunkhorn, 2012; St-Pierre et al., 2006).

LanCL1 (Lanthionine synthetase C-like protein 1, also known as P40 or GRP69A) (Bauer et al., 2000) is a mammalian member of the LanC-like protein superfamily encompassing a highly divergent group of peptide-modifying enzymes present in plants and bacteria (LanCs). Prokaryotic LanC is a zinc-containing enzyme that acts in concert with lantibiotic dehydratases to facilitate intramolecular conjugation of cysteine to serine or threonine residues, yielding macrocyclic thioether (Lanthionine) products with potent antimicrobial activity (Chatterjee et al., 2005). Three LanC-like genes—*LanCL1*, *LanCL2*, and *LanCL3*—are present in human genome (Landlinger et al., 2006). Human LanCL1 protein binds zinc ion and GSH and appears to play a regulatory role in axonal growth (Chung et al., 2007; Zhang et al., 2009). Here, we report that LanCL1 is primarily expressed in brain neurons, is developmentally regulated, is induced by neuronal activity, and is essential for mitigating neuronal oxidative stress during normal postnatal development and in response to oxidative stresses. Additionally, LanCL1 transgene expression is protective against oxidative stress. Enzymatic assays demonstrate

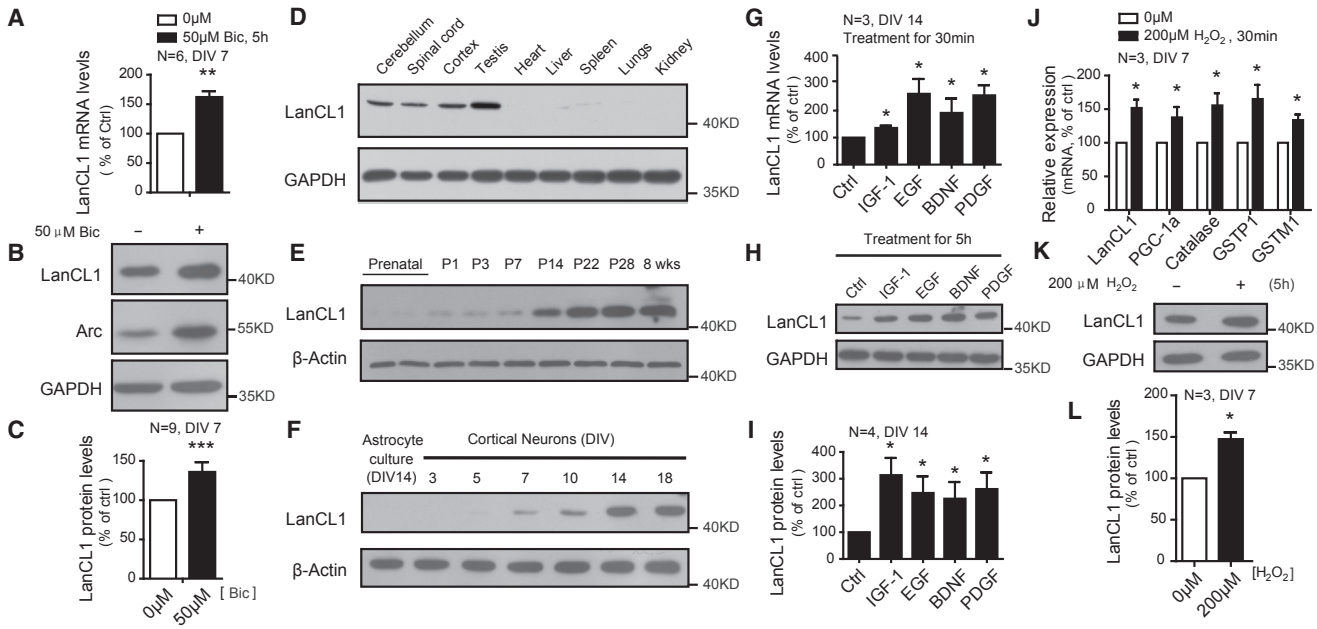

Figure 1. Induction of LanCL1 Expression by Neurotrophic Factors and Oxidative Stress-Inducing Agents

(A) qRT-PCR shows induction of *LanCL1* mRNA in bicuculline-treated (Bic, 5 hr) cortical neurons (DIV 7). Error bars indicate SEM. **p = 0.0016, n = 6. h, hours.

(B and C) Western blots (B) and quantification (C) show increased LanCL1 protein levels in bicuculline-treated (15 hr) cortical neurons (DIV 7). Error bars indicate SEM. *p < 0.0001, n = 9.

(D) Western blots show the expression pattern of LanCL1 in multiple organs.

(E and F) Western blots show the temporal expression pattern of LanCL1 in prenatal and postnatal (P1–P28 and 8 weeks) mouse cortex (E) and neuronal versus astrocyte cultures (F).

(G) qRT-PCR shows induction of *LanCL1* mRNA in neurotrophic factors-treated (30 min) cortical neurons (DIV 14). Error bars indicate SEM. *IGF-1*, *p = 0.0491; *EGF*, *p = 0.0490; *BDNF*, *p = 0.0489; *PDGF*, *p = 0.0265; n = 3.

(H and I) Western blots (H) and quantification (I) show increased LanCL1 protein levels in neurotrophic factors-treated (5 hr) cortical neurons (DIV 14). Error bars indicate SEM. *IGF-1*, *p = 0.0403; *EGF*, *p = 0.0292; *BDNF*, *p = 0.0256; *PDGF*, *p = 0.0224; n = 4.

(J) qRT-PCR shows induction of *LanCL1* mRNA along with oxidative defense genes in cortical neurons (DIV 7) in response to H_2O_2 treatment (30 min). The relative induction fold is normalized against nontreatment control. Error bars indicate SEM. *LanCL1*, *p = 0.0258; *PGC1-α*, *p = 0.0345; *Catalase*, *p = 0.0465; *GSTP1*, *p = 0.0456; *GSTM1*, *p = 0.0276; n = 3.

(K and L) Western blots (K) and quantification (L) show increased LanCL1 protein level in H_2O_2-treated cortical neurons (DIV 7). Error bars indicate SEM. *p = 0.0292, n = 3.

See also Figure S1.

catalysis of GSH conjugation to synthetic substrates similar to the GST (Habig and Jakoby, 1981). These observations indicate that LanCL1 is part of the GSH antioxidant defense mechanism that is uniquely essential for neuronal function.

RESULTS

LanCL1 Expression Is Induced by Neuronal Activity and Oxidative Stress

We identified *LanCL1* based on its neuronal expression and rapid induction by activity. LanCL1 messenger RNA (mRNA) and protein are induced in vivo by maximal electroconvulsive seizure (MECS) (Figures S1B and S1C available online). The induced expression of LanCL1 by activity is also recapitulated in cortical neuron culture by addition of bicuculline that blocks the inhibitory action of GABA receptors (Ueno et al., 1997) (Figures 1A–1C). LanCL1 is primarily expressed in neural tissues and testis (Figure 1D and Figure S1A). The expression of LanCL1 protein in brain is developmentally regulated, increasing during the first postnatal month and remaining high in adult (Figure 1E). Expression in neuron cultures increases between 7 and 14 days in vitro (DIV) (Figure 1F) and parallels the formation of synapses and spontaneous neuronal activity (Kamioka et al., 1996). This pattern of activity-regulated expression is typical of neuronal immediate early genes (Brakeman et al., 1997; Lyford et al., 1995). Consistent with a role for LanCL1 in an induced genomic program to activity, neurotrophic factors such as insulin growth factor 1 (IGF-1), epidermal growth factor (EGF), brain-derived neurotrophic factor (BDNF), and platelet-derived growth factor (PDGF), which modulate synaptic activity and protect neurons against oxidative stress (Cheng and Mattson, 1995; Skaper et al., 1998; Zhang et al., 1993), induce LanCL1 expression (Figures 1G–1I). Furthermore, we found that LanCL1 is induced by agents that evoke oxidative stress, including glutamate (Ratan and Baraban, 1995) and H_2O_2 (Figures 1J–1L and Figure S1D), and this induction occurs concurrently with the canonical oxidative stress response that includes *PGC-1α* and *-β* and ROS-detoxifying enzymes copper/zinc superoxide dismutase (*SOD1*) and manganese SOD (*SOD2*) (St-Pierre et al., 2006).

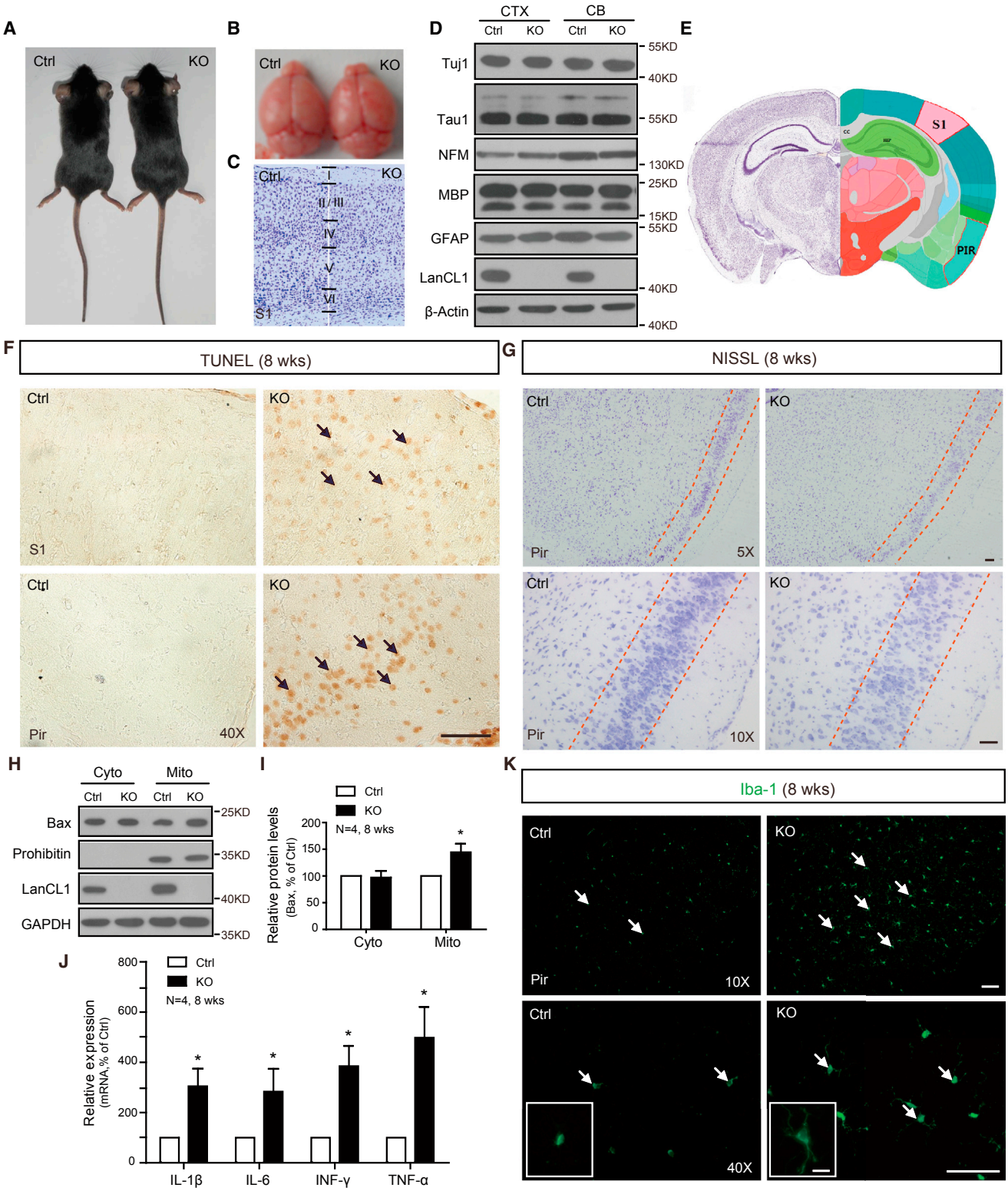

Figure 2. Apoptotic Neuronal Death and Inflammation in *LanCL1* KO Brains

(A and B) Photographs show that the genetic deletion of *LanCL1* does not affect the body (A) or brain size (B) (*LanCL1*$^{-/-}$, KO, hereinafter).

(C) Nissl staining reveals cortical lamination.

(D) Western blots show the normal expression of axonal, astrocytes, and myelination markers.

(E) Illustration (from the Allen Brain Atlas) shows the brain zoning in mice as reference for (F) and (G).

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Loss of LanCL1 Causes Development-Dependent Neuronal Death and Inflammation

To examine the functional role of LanCL1 in the response to oxidative stress, we generated a *LanCL1* knockout (KO) mouse (*LanCL1*$^{-/-}$, KO) (Figures S2A–S2D). *LanCL1*$^{-/-}$ mice are born at an expected Mendelian ratio (Figure S2E) and display normal postnatal viability and growth. At 4 weeks of age, the gross brain morphology, cortical lamination, and expression of select neuronal and glial markers are comparable to those of wild-type (WT) mice (Figures 2A–2D). This indicates that LanCL1 is not essential for embryonic or early postnatal brain development. However, during later postnatal development, *LanCL1*$^{-/-}$ mice demonstrate prominent neuronal degeneration. Brains of 8- to 12-week-old *LanCL1*$^{-/-}$ mice display increased TUNEL-positive staining in cerebral cortex and cerebellum (Figure 2F; data not shown). Apoptotic death is present in the entire cortex but is most prominent in neurons of layers II/III. Nissl staining reveals a loss of cortical neurons in the layers II/III (Figure 2G). BCL-2 family member Bax is increased in the mitochondria fraction from cortex (Figures 2H and 2I), consistent with increased apoptotic death (Rossé et al., 1998). Neuronal death in the brain of 8-week-old *LanCL1*$^{-/-}$ mice is accompanied by neuroinflammatory responses, including increased levels of inflammatory cytokines *IL1*, *IL6*, *TNF*, and *INF* (Figure 2J) and a 50% increase of activated microglia ($p = 0.0045$, $n = 6$) displaying an increased size of soma and number of cellular processes (Figure 2K).

LanCL1 Is Required for Mitigating Oxidative Stress under Physiological Conditions

We suspected that the neuronal death in *LanCL1*$^{-/-}$ mice is a result of progressive oxidative damage. In the 4-week-old *LanCL1*$^{-/-}$ mice, multiple antioxidant defense genes such as *PGC-1*α, *PGC-1*β, *SOD1*, *SOD2*, *Catalase*, *Cyt-C*, and *ANT* are upregulated in the cortex (Figure S3A) and cerebellum (data not shown), indicative of oxidative stress (St-Pierre et al., 2006). At this stage, no apparent oxidative damage or neuronal death is evident (Figure S3B; data not shown). By 8 to 12 weeks, *LanCL1*$^{-/-}$ cortex shows an accumulation of ROS and lipid peroxidation, as indicated by fluorescent ethidium labeling (Brennan et al., 2009) (Figure 3A) and 4-hydroxy-2-nonenal (4-HNE) western blot (Figures 3C, 3D, and S3D), and redox imbalance as indicated by a decrease of the NADPH/NADP ratio (Mugoni et al., 2013) (Figure S3C). All these changes indicate development-dependent oxidative damage in *LanCL1*$^{-/-}$ brains. Double labeling with 4-HNE and neuronal marker (NeuN) revealed a widespread increase in 4-HNE in cortical neurons of 8-week-old *LanCL1*$^{-/-}$ mice (Figure 3B). Increased 4-HNE levels are also detected in cultured *LanCL1*$^{-/-}$ neurons (Figures 3E, 3F, and S3E). The increase in 4-HNE is accompanied by an increase in malondialdehyde (MDA) (Figure 3G), implicating lipid peroxidation (Sharma et al., 2004). Oxidative carbonylation of proteins assayed by blot with anti-dinitrophenol (DNP) antibody (Nyström, 2005) is increased more than 2-fold (Figures 3H, 3I, and S3F). Increased immunoreactivity of 8-oxo-guanine (8-oxoG) indicates free radical oxidative damage to DNA (Figure 3J).

Mitochondria are particularly vulnerable to oxidative stress (Yakes and Van Houten, 1997). Mitochondria of *LanCL1*$^{-/-}$ cortical neurons appear impaired based on the JC-1 assay (Smiley et al., 1991) with a 50% increase in the intensity of green fluorescence (Figure 3K; $p = 0.0275$, $n = 3$), and a 40% decrease in the ratio of red/green fluorescence intensity ($p = 0.0031$, $n = 3$), suggesting impairment of the electrochemical gradient across inner mitochondrial membrane. The mitochondrial impairment is also indicated by reduced expression of mitochondria-related genes involved in mitochondrial energy metabolism (Figure S3G). These data suggest that LanCL1 is required for mitigating oxidative stress generated under physiological condition and that oxidative damage is the cause of development-dependent neuronal death in *LanCL1*$^{-/-}$ mouse.

LanCL1 Catalyzes the Formation of Thioether Products

The role of LanCL1 in mitigating neuronal oxidative stress suggests a neuronal protective effect against stressors. To assess its cellular protective effect, we treated HeLa cells expressing green fluorescent protein (GFP)-tagged LanCL1 or GFP with H_2O_2. LanCL1-expressing cells show reduced apoptosis (propidium iodide/Hoechst staining; Figures S4A and S4B) and higher cell viability (CCK-8 assay; Figure S4C). To test its role in neuronal protection, we cultured cortical neurons from WT or *LanCL1*$^{-/-}$ mice and treated them with oxidative stress-inducing agents H_2O_2 or NMDA-type glutamate antagonist MK-801 (Papadia et al., 2008). *LanCL1*$^{-/-}$ neurons exhibit increased cell death (Figures 4A and 4B; Figures S4D and S4E). We also determined that the cellular protective effect of neurotrophic factors EGF and BDNF against H_2O_2 is impaired in cultured cortical neurons from *LanCL1*$^{-/-}$ mice (Figures S4F and S4G). To further confirm its neuronal protective effect, we generated a conditional *LanCL1* transgenic mouse by insertion of a floxed expression construct into the Rosa 26 locus. V5-tagged *LanCL1* is conditionally expressed in the brain on crossing with Nestin-Cre driver mice (Figures S4H–S4K). Cortical neurons derived from transgenic *LanCL1* mice express a higher level of LanCL1 protein and are relatively resistant to H_2O_2-induced neuronal death (Figures 4C and 4D; Figure S4K). Furthermore, accumulation of 4-HNE induced by H_2O_2 treatment is significantly reduced

(F) TUNEL staining shows an increase in apoptosis (brown, indicated by arrows) of the 8-week-old *LanCL1* KO cortex (primary somatosensory cortex [S1] and piriform cortex [PIR]). Scale bar, 50 μm.
(G) Nissl staining reveals reduced density of cortical neurons in the 8-week-old *LanCL1* KO cortex. Scale bar, 50 μm.
(H) Subcellular fractionation shows an increase in *Bax* in the mitochondria of the 8-week-old *LanCL1* KO cortex by western blotting. Prohibitin, the mitochondrial marker; Cyto, cytosol; and Mito, mitochondria.
(I) Quantification of the increase in *Bax* in mitochondrial fraction of *LanCL1* KO relative to WT control. Error bars indicate SEM. *$p = 0.0331$, $n = 4$.
(J) qRT-PCR shows the increase mRNA levels of inflammation factors in *LanCL1* KO cortex. Error bars indicate SEM. *IL-1*, *$p = 0.04$; *IL-6*, *$p = 0.0441$; *INF-*γ, *$p = 0.0417$; *TNF-*α, *$p = 0.0348$; $n = 4$.
(K) *Iba-1* staining shows an increase in the number of activated microglia (indicated by arrows) with increased size of soma and number of cellular processes in the cortex of 8-week-old *LanCL1* KO brains. Scale bar, 50 μm. The insets show enlarged microglia. Scale bars, 10 μm.
See also Figure S2.

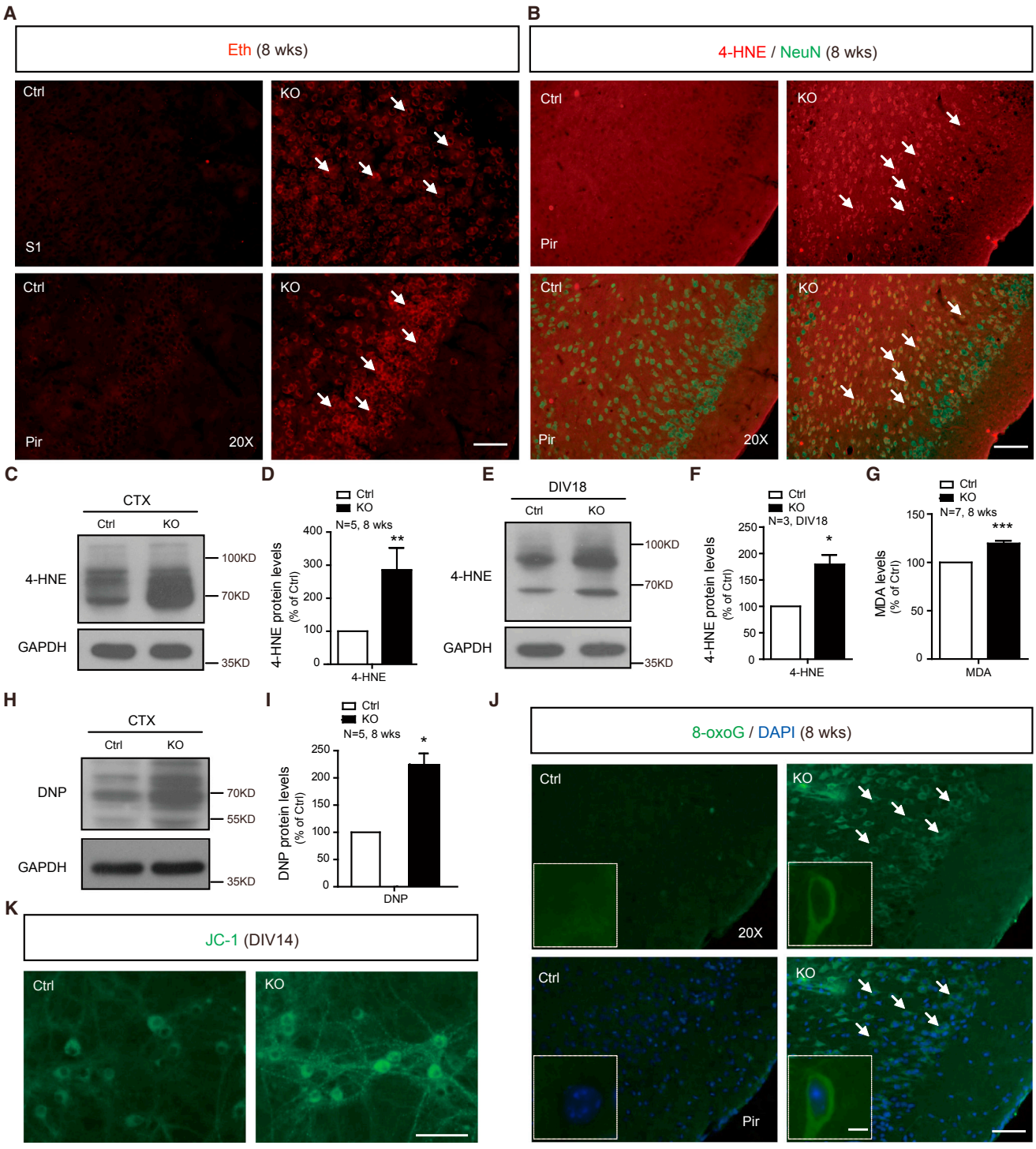

Figure 3. Oxidative Damages and ROS Accumulation in *LanCL1* KO Brains

(A) Representative images of ethidium fluorescence (Eth, red) show the accumulation of ROS (indicated by arrows) in the cortex of 8-week-old *LanCL1* KO brain. Scale bar, 50 μm. Ctrl, control.

(B) Immunostaining with anti-4-HNE (red) and anti-NeuN antibody (green) shows an increase in 4-HNE-positive neurons (indicated by arrows) in the 8-week-old *LanCL1* KO cortex. Scale bar, 50 μm.

(C and D) Western blots (C) and quantification (D) show an increase in the level of 4-HNE in the cortex (CTX) of the 8-week-old *LanCL1* KO brain. Error bars indicate SEM. **p = 0.0033, n = 5.

(E and F) Western blots (E) and quantification (F) show increase in the level of 4-HNE in *LanCL1* cortical culture. Error bars indicate SEM. *p = 0.0236, n = 3.

(G) Quantification shows increase of MDA in cortex of 8-week *LanCL1* KO. Error bars indicate SEM. ***p = 0.0003, n = 7.

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in neuronal cultures of LanCL1 transgenic mice (Figures 4E and 4F), demonstrating a role for LanCL1 in mitigating neuronal oxidative stress.

How does LanCL1 exert its neuronal protective effect? Since LanCL1 binds GSH, we wondered if LanCL1 is part of the GSH-mediated antioxidant defense mechanism. In mammalian cells, GSTs catalyze the reaction of GSH with a wide range of electrophilic compounds to form thioethers (Hayes and Pulford, 1995) and play a role in mitigating oxidative stress. Similar with GSTs, prokaryotic LanC protein catalyzes the formation of thioether products (Chatterjee et al., 2005). The antioxidant effect of LanCL1, together with its ability to bind GSH (Zhang et al., 2009), led to the prediction that mammalian LanCL1 may catalyze the formation of thioether products such as GST proteins. Expression of myc-tagged LanCL1 in HeLa cells increased GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB) (Figure 4G), a common reporter substrate for GST activity (Hayes and Pulford, 1995). LanCL1 point mutants (R4A and R322A) that fail to bind GSH (Zhang et al., 2009) failed to increase GST activity and showed reduced cellular protection (Figure 4H; Figures S4L and S4M). The finding that LanCL1 contributes to GST activity predicts that loss of LanCL1 will reduce GST activity in the brain of *LanCL1*$^{-/-}$ mouse. Accordingly, we assayed the GST activity of the extracts from 2-week-old and 8-week-old cortex of *LanCL1*$^{-/-}$ mice and noted a modest reduction in GST activity (16.99 ± 3.99%, n = 4, p = 0.0118) at 2 weeks and a more pronounced reduction (31.65 ± 3.69%, n = 6, p = 0.0004) at 8 weeks. This suggests that LanCL1 contributes to the developmental increase of GST activity and parallels the expression profile of LanCL1 in the cortex of WT mice (Figures 4I and 1E). A similar development-dependent reduction of GST activity in *LanCL1*$^{-/-}$ neuronal cultures 14 DIV, but not 6 DIV, parallels the normal developmental expression of LanCL1 protein (Figures 4J and 1F). The major brain GST enzymes GSTM1 and GSTP1 (Mitchell et al., 1997) are expressed at WT levels in *LanCL1*$^{-/-}$ brain (Figure S4N).

To assess if LanCL1 itself possesses enzymatic activity to catalyze the formation of thioether productions like GSTs, we expressed His-tagged LanCL1 and purified it from HeLa cells using nickel affinity chromatography. A negative control expressed myc-tagged LanCL1 that lacked the His tag (Figure 4K, left panel; Figure S4P). Coomassie staining shows that LanCL1 protein was not copurified with GSTM1 or GSTP1 and appeared as a single band (Figure 4K, right panel). We assayed the enzymatic activity of purified LanCL1 protein preparation toward two different substrates for GST: CDNB and p-nitrophenyl acetate (Habig and Jakoby, 1981). The LanCL1 preparation exhibited GST enzymatic activity toward both substrates (Figures 4L and 4M). Enzymatic kinetics assays revealed a V_{max} and K_{min} of 1,087 nmol/min/mg protein and 1.93 mM (CDNB), respectively (Figure 4N; Figure S4S). In the same assay, the activity of LanCL1 was measured in parallel with a canonical GST that is highly expressed in brain, GSTP1 (Figures S4Q and S4R) (Czerwinski et al., 1996). LanCL1 preparation catalysis was ~45% (V_{max}) of GSTP1 (Figure S4S). These data indicate that recombinant LanCL1 functions to catalyze thioether formation and support the conclusion that reduced GST activity in *LanCL1*$^{-/-}$ brain is a direct consequence of loss of LanCL1.

DISCUSSION

The present study identifies *LanCL1* as an antioxidant defense gene that is part of the GSH defense pathway. LanCL1 appears essential for a normal cellular response to stress in developing neurons. Loss of LanCL1 causes oxidative stress followed by development-dependent neuronal death. Antioxidant defense genes are upregulated in the 4-week-old *LanCL1*$^{-/-}$ mice (before the onset of neuronal death) (Figure S3A), and this is followed within the next 4–8 weeks by progressive oxidative damage to lipids, proteins, DNA, and mitochondria, as well as apoptotic cell death. Conversely, increased LanCL1 expression confers neuronal resistance to oxidative stress, as neuronal survival is significantly improved with reduced lipid oxidation under H_2O_2 treatment (Figures 4E and 4F). In further support of a direct role of LanCL1 in mitigating oxidative stress, we demonstrate catalytic activity of purified recombinant LanCL1 in the formation of thioether products using both CDNB and p-nitrophenyl acetate as substrates (Figures 4L and 4M).

The catalytic activity of LanCL1 resembles that of GST proteins; however, LanCL1 is not a member of a GST superfamily. LanCL1 does not share sequence or structural similarity with canonical GSTs (Sheehan et al., 2001; Zhang et al., 2009). An earlier study reported that LanCL1 interacts with cystathionine β-synthase (CBS), a transsulfuration enzyme that functions to increase GSH synthesis (Zhong et al., 2012). It is interesting that transient knockdown of LanCL1 in cultured neurons resulted in elevated CBS activity and modest protection of neurons from oxidative stress (Zhong et al., 2012). We noted an increase in CBS activity in the brain extracts of *LanCL1*$^{-/-}$ mice (data not shown) and increased GSH levels in *LanCL1*$^{-/-}$ cortex (Figure S4O); however, this presumed homeostatic adaptation of GSH cannot compensate for loss of LanCL1 in *LanCL1*$^{-/-}$ brain or cultured neurons.

The LanC family is evolutionarily ancient. While further enzymology is required, it appears that the ability to form thioethers is retained yet used for widely different purposes in prokaryotic cells and neurons. LanCL1 and LanCL2 are prominently expressed in brain and provide the precedent that antioxidant mechanisms can be cell type specific. This contrasts with canonical antioxidant genes that are ubiquitously expressed in all cell types (Muller et al., 2007). Selective expression in neurons may be understood to result from the special demands of neurons for protection from ROS. LanCL1 deletion alone is sufficient to cause widespread and prominent neuronal death. This again

(H and I) Western blots with DNP antibody (H) and quantification (I) show increase of DNP levels in the cortex of 8-week-old *LanCL1* KO cortex. Error bars indicate SEM. *p = 0.0176, n = 5.

(J) Immunostaining with 8-oxoG antibody shows an increase in 8-oxoG-positive cells (green, indicated by arrows) in the 8-week-old *LanCL1* KO cortex; scale bar, 50 μm. The insets show enlarged neurons; scale bar, 5 μm.

(K) JC-1 staining shows an increase in the fluorescence intensity of monomeric form of JC-1 (green) in *LanCL1* KO cultures (DIV 14). Scale bar, 50 μm.

See also Figure S3.

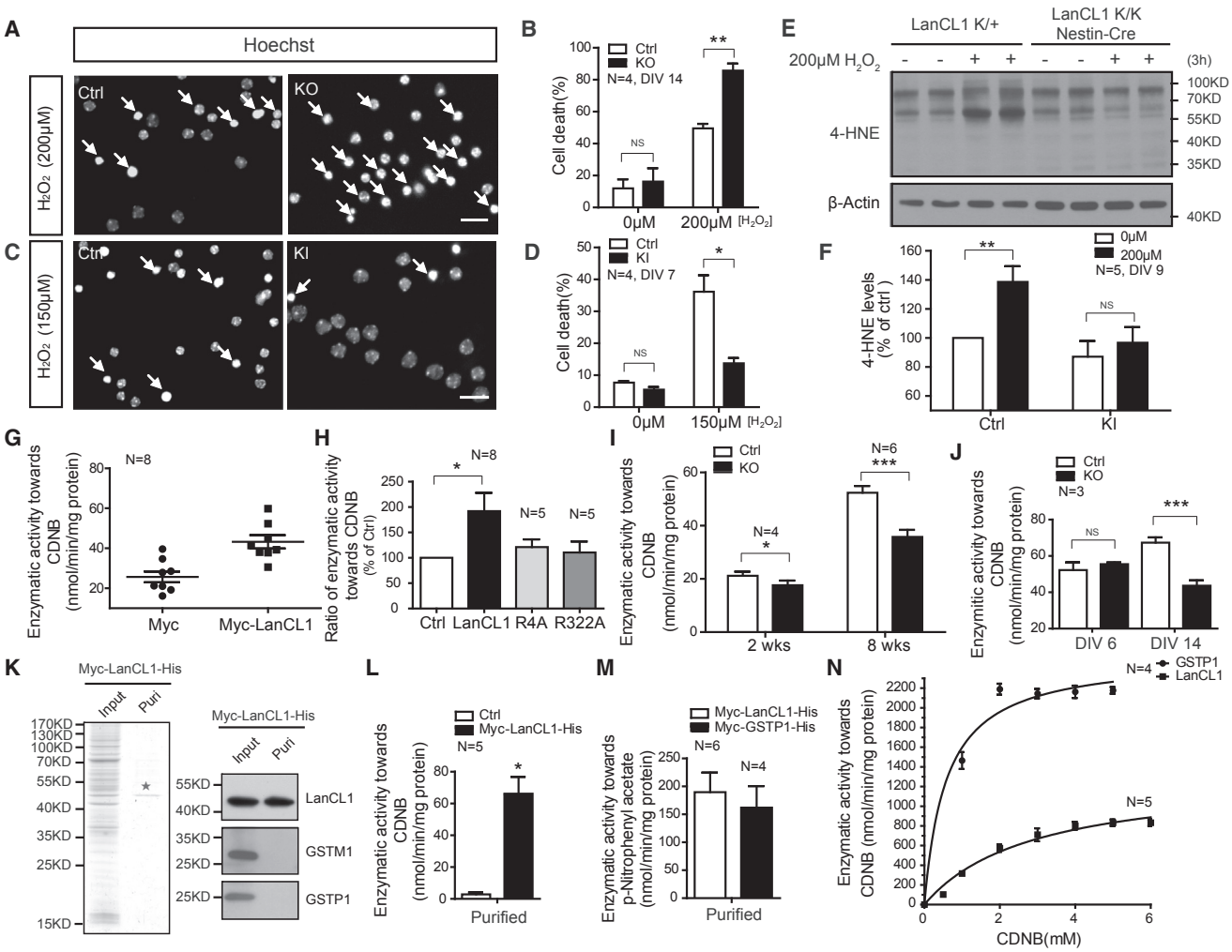

Figure 4. LanCL1 Possesses Catalytic Activity for Thioether Formation and Protects Cells against Oxidative Stress

(A–D) Hoechst staining shows that deletion of LanCL1 increased neuronal death induced by H_2O_2 (12 hr treatment, DIV 14, Control (Ctrl): *LanCL1*$^{+/+}$, KO: *LanCL1*$^{-/-}$) (A and B), and LanCL1 transgene reduced neuronal death (12 hr treatment, DIV7, Ctrl: *LanCL1* K/K, KI: *LanCL1* K/K Nestin-Cre) (C and D). The data represent the mean ± SEM from four independent experiments, with a total number of 2,000 neurons analyzed for each group. Scale bar, 50 μm. Error bars indicate SEM. *p = 0.0135; **p = 0.0069; n = 4. KI, knockin.

(E and F) Western blots (E) and quantification (F) show that LanCL1 KI culture neurons are more resistant to H_2O_2-induced 4-HNE accumulation (DIV9, Ctrl: *LanCL1* K/+, KI: *LanCL1* K/K Nestin-Cre). Error bars indicate SEM. **p = 0.0030; NS, p = 0.5079; n = 5.

(G and H) Quantifications show increased GST activity in LanCL1-overexpressing HeLa cells compared with control cells (G) or LanCL1 point mutants that lack GSH binding (H). Ctrl: Myc; LanCL1: Myc-LanCL1; R4A: Myc-LanCL1(R4A); R322A: Myc-LanCL1(R322A). Error bars indicate SEM. *p = 0.0189; n = 8.

(I) Quantification shows a correlation between the reduction in GST activity in *LanCL1* KO cortex with LanCL1 expression level in 2- to 8-week-old mice. Error bars indicate SEM. *p = 0.0118 for 2-week-old sample; n = 4. ***p = 0.0004 for 8-week-old sample; n = 6.

(J) Quantification shows a correlation of a reduction in GST activity in *LanCL1* KO culture neurons with its protein level in cultures 6 to 14 DIV (DIV6 and DIV14, respectively). Error bars indicate SEM. ***p = 0.0002; n = 3.

(K) Coomassie brilliant blue staining and western blots show the affinity-purified, polyhistidine-tagged LanCL1 preparation from HeLa cells. GSTM1 and GSTP1 were depleted from purified LanCL1. ★ indicates the LanCL1 band. Puri, purification.

(L) Affinity-purified LanCL1 catalyzes conjugation of the GSH to CDNB. GSH: 0.5 mM; CDNB, 0.5 mM. Error bars indicate SEM. *p = 0.0243; n = 5.

(M) Affinity-purified LanCL1 and GSTP1 catalyze conjugation of the GSH to p-nitrophenyl acetate. GSH: 0.5 mM; p-nitrophenyl acetate: 0.2 mM. Error bars indicate SEM. LanCL1, n = 6; GSTP1, n = 4.

(N) Kinetics assay of affinity-purified LanCL1 and GSTP1. GSH was 5 mM with varying CDNB concentrations from 0 mM to 6 mM. Error bars indicate SEM. LanCL1, n = 5; GSTP1, n = 4.

See also Figure S4.

contrasts with canonical antioxidant defense genes whose contribution to oxidative defense is revealed only on challenge by exogenous oxidative stressors. It is possible that LanCL1 is uniquely effective for detoxification of critical substrates and for S-glutathionylation of critical proteins (Dalle-Donne et al., 2009). Our study indicates that ROS generated during normal developmental physiology are toxic in the absence of sufficient oxidative defense. The LanCL family will be important to

integrate into understandings of synaptic physiology, stress response, and the selective vulnerability of neurons in aging and neurodegenerative diseases.

EXPERIMENTAL PROCEDURES

Generation of LanCL1 KO Mice

The targeting construct, in which exon 4 was flanked by loxP sites, was made by modifying a bacterial artificial chromosome clone using recombineering. Homozygous LanCL1 mutants (KO) were generated by intercrossing of heterozygous mutants (*LanCL1*$^{+/-}$). All mouse work was done in accordance with the Animal Care and Use Committee guidelines of Johns Hopkins University School of Medicine and Sichuan University West-China Hospital. More details and the KO validation are described in the Supplemental Experimental Procedures.

Oxidative Stress and Cell Death Analyses in LanCL1 KO Mice

ROS levels were quantitated in LanCL1 WT/KO brain with Ethidium (Eth, Invitrogen). NADPH/NADP ratio assay was performed with an NADP/NADPH assay kit (BioVision). Oxidative damage was detected by immunofluorescence on brain section with anti-4-HNE antibody (Abcam) and anti-8-oxoG antibody (Millipore).Western blots with anti-DNP antibody (Millipore) were performed to detect carbonylation proteins. Cell apoptosis assay was performed with an in situ cell death kit (Roche). More details can be found in the Supplemental Experimental Procedures.

Western Blotting, RNA Extraction and PCR Methods, Induction of LanCL1, In Vitro Cell Death Assay, and Affinity Purification of Polyhistidine-Tagged LanCL1 Protein

Please see Supplemental Experimental Procedures for detail description.

GST Activity Assay and Enzymatic Kinetics Assay

The activity of GST was measured with the standard protocol described by Habig and Jakoby (1981). The enzymatic kinetic assay was performed by fixing the GSH concentration at 5 mM and varying the CDNB concentration from 0 mM to 6 mM. The kinetic data were analyzed by GraphPad Prism with K_{cat} analysis, the V_{max}, and the K_m values for CDNB were determined from this analysis.

Statistical Analysis

Data represent the mean and SEM. Student's t test (one-tailed for western blot, ratio quantification, and quantitative RT-PCR [qRT-PCR]; two-tailed for the others) was performed for all statistical significance analysis using GraphPad Prism software (*$p < 0.05$ and **$p < 0.01$).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.06.011.

AUTHOR CONTRIBUTIONS

M.C. initiated this project and provided the foundation of this work. C.H. and D.P. did the bulk of biochemical and cellular experiments.

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