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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, and 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.

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Author Contributions

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

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 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 glutathione antioxidant defense mechanism involves multiple enzymes, including the glutathione-dependent enzymes, glutathione peroxidase (GPX) and glutathione S-transferase (GST). (Crunkhorn, 2012; St-Pierre et al., 2006)(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 (Champak Chatterjee, 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 glutathione 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 catalysis of glutathione conjugation to synthetic substrates similar to the glutathione S-transferase (Habig and Jakoby, 1981). These observations indicate that LanCL1 is part of glutathione 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 mRNA and protein are induced *in vivo* by maximal electroconvulsive seizure (MECS) (Figure S1B and S1C). 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) (Figure 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 DIV 7–14 (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 IGF-1, EGF, BDNF, and PDGF that modulate synaptic activity and protect neurons against oxidative stress(Cheng and Mattson, 1995; Skaper et al., 1998; Zhang et al., 1993) induce LanCL1 expression (Figure 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 (Figure 1J–1L and Figure S1D), and this induction occurs concurrently with the canonical oxidative stress response that includes P*GC-1*α and β and ROS-detoxifying enzymes copper/zinc superoxide dismutase (*SOD1*) and manganese SOD (*SOD2*) (St-Pierre et al., 2006).

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 mouse (*LanCL1*−/−, ko) (Figure 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 is comparable to WT mice (Figure 2A–2D). This indicates LanCL1 is not essential role for embryonic or early postnatal brain development. However, during later postnatal development, *LanCL1*−/− mice demonstrate prominent neuronal degeneration. Brains of 8- to 12-week *LanCL1*−/− mice display increased TUNEL positive staining in cerebral cortex and cerebellum (Figure 2F and 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 (Figure 2H and 2I), consistent with increased apoptotic death (Rosse et al., 1998). Neuronal death in the brain of 8-week *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 *LanCL1*−/− mice, multiple antioxidant defense genes such as *PGC-1α*, *PGC-1β*, *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 and 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 (Figure 3C, 3D and Figure 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 *LanCL1*−/− mice (Figure 3B). Increased 4-HNE levels are also detected in cultured *LanCL1*−/− neurons (Figure 3E, 3F and Figure 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 (Nystrom, 2005) is increased more than 2 fold (Figure 3H, 3I and Figure S3F). Increased immunoreactivity of 8-Oxoguanine (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 mitochondrial 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 GFP-tagged LanCL1 or GFP with H_2O_2. LanCL1 expressing cells show reduced apoptosis (PI/Hoechst staining; Figure 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 (Figure 4A, 4B and Figure S4D, 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 (Figure S4F, 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 upon crossing with Nestin-Cre driver mice (Figure 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 (Figure S4K and Figure 4C, 4D). Furthermore, accumulation of 4-HNE induced by H_2O_2 treatment is significantly reduced in neuronal cultures of LanCL1 transgenic mice (Figure 4E, 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 glutathione mediated antioxidant defense mechanism. In mammalian cells, GSTs catalyze the reaction of glutathione 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 (Champak Chatterjee, 2005). The antioxidant effect of LanCL1 together with its ability to bind glutathione (Zhang et al., 2009) led to the prediction that mammalian LanCL1 may catalyze the formation of thioether products like GST proteins. Expression of myc-tagged LanCL1 in HeLa cells increased GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) (Figure 4G), a common reporter substrate for GST activity (John D. Hayes, 1995). LanCL1 point mutants (R4A and R322A) that fail to bind glutathione (Zhang et al., 2009) failed to increase GST activity and showed reduced cellular protection (Figure 4H and Figure S4L, 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 and 8-week 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 (Figure 4I, 1E). A similar development-dependent reduction of GST activity in DIV 14 but not DIV 6 LanCL1−/− neuronal cultures parallels the normal developmental expression of LanCL1 protein. (Figures 4J, 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 and Figure S4P). Coomassie staining shows that LanCL1 protein was not co-purified with GSTM1 or GSTP1 and appeared as a single band (Figure 4K, right panel). We assayed the enzymatic activity of purified LanCL1 protein preparation towards two different substrates for GST; CDNB and p-Nitrophenyl acetate (Habig and Jakoby, 1981). The LanCL1 preparation exhibited GST enzymatic activity towards both substrates. (Figure 4L, 4M). Enzymatic kinetics assays revealed a V_{max} and K_m of 1087nmol/min/mg protein and 1.93mM (CDNB), respectively (Figure 4N and 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 (Figure 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 glutathione 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 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, DNAs and mitochondria, and 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 (Figure 4E, 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 (Figure 4L, 4M).

The catalytic activity of LanCL1 resembles that of GST proteins, however, LanCL1 is not a member of 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 trans-sulfuration enzyme that functions to increase glutathione synthesis (Zhong et al., 2012). Interestingly, 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 glutathione levels in LanCL1−/− cortex (Figure S4O), however this presumed homeostatic adaptation of glutathione 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 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 wide-spread and prominent neuronal death. This again contrasts with canonical antioxidant defense genes whose contribution to oxidative defense is revealed only upon 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 knockout mice

The targeting construct, in which exon 4 flanked by loxP sites, was made by modifying a BAC clone using recombineering. Homozygous LanCL1 mutants (ko) were generated by intercrossing of heterozygous mutants (LanCL1+/−). More details and the validation of the knockouts are described in the Supplemental Experimental Procedures.

Oxidative stress and cell death analyses in LanCL1 knockout mice

ROS levels were quantitated in LanCL1 wt/ko brain with Ethidium (Eth, Invitrogen). NADPH/NADP ratio assay was performed with NADP/NADPH assay kit (BioVision). Oxidative damage was detected by immunofluorescence on brain section with anti-4-HNE antibody (Abcam) and anti-8-oxoguanine antibody (Millipore). Western blots with anti-dinitrophenol (DNP) antibody (Millipore) were performed to detect carbonylation proteins. Cell apoptosis assay was performed with in situ cell death kit (Roche). More details could 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.

Glutathione S-transferase activity assay and enzymatic kinetics assay

The activity of the glutathione S-transferase was measured with the standard protocol described by Habig *et al*. The enzymatic kinetic assay was performed by fixing the GSH concentration at 5mM, and varying CDNB concentration from 0mM to 6mM. The kinetic data were analyzed by GraphPad Prism with Kcat analysis, the Vmax and the Km values for CDNB were determined from this analysis.

Statistical analysis

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

1. LanCL1 expression is developmentally regulated and induced by neuronal activity.
2. Loss of LanCL1 causes progressive oxidative damage and neuronal death.
3. LanCL1 transgene protects neurons from oxidative stress.
4. LanCL1 catalyzes the formation of thioether products.

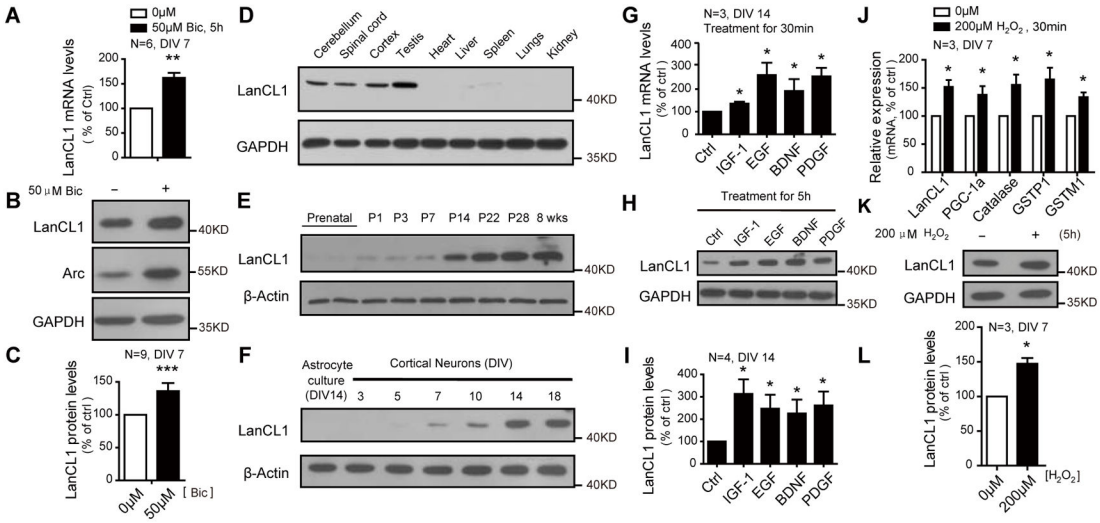

Figure 1. Induction of LanCL1 expression by neurotrophic factors and oxidative stress inducing agents

(A) qRT-PCR shows induction of *LanCL1* mRNA in bicuculline-treated (Bic, 5h) cortical neurons (DIV7). Error bars indicate SEM. **p= 0.0016. n=6.

(B and C) Western blots and quantification show increased LanCL1 protein levels in bicuculline-treated (15h) 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 the postnatal mouse cortex and neuronal versus astrocyte cultures.

(G) qRT-PCR shows induction of *LanCL1* mRNA in neurotrophic factors-treated (30min) cortical neurons (DIV14). 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 and quantification show increased LanCL1 protein levels in neurotrophic factors-treated (5h) 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 of DIV7 in response to H_2O_2 treatment (30min). The relative induction fold is normalized against non-treatment 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 and quantification 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

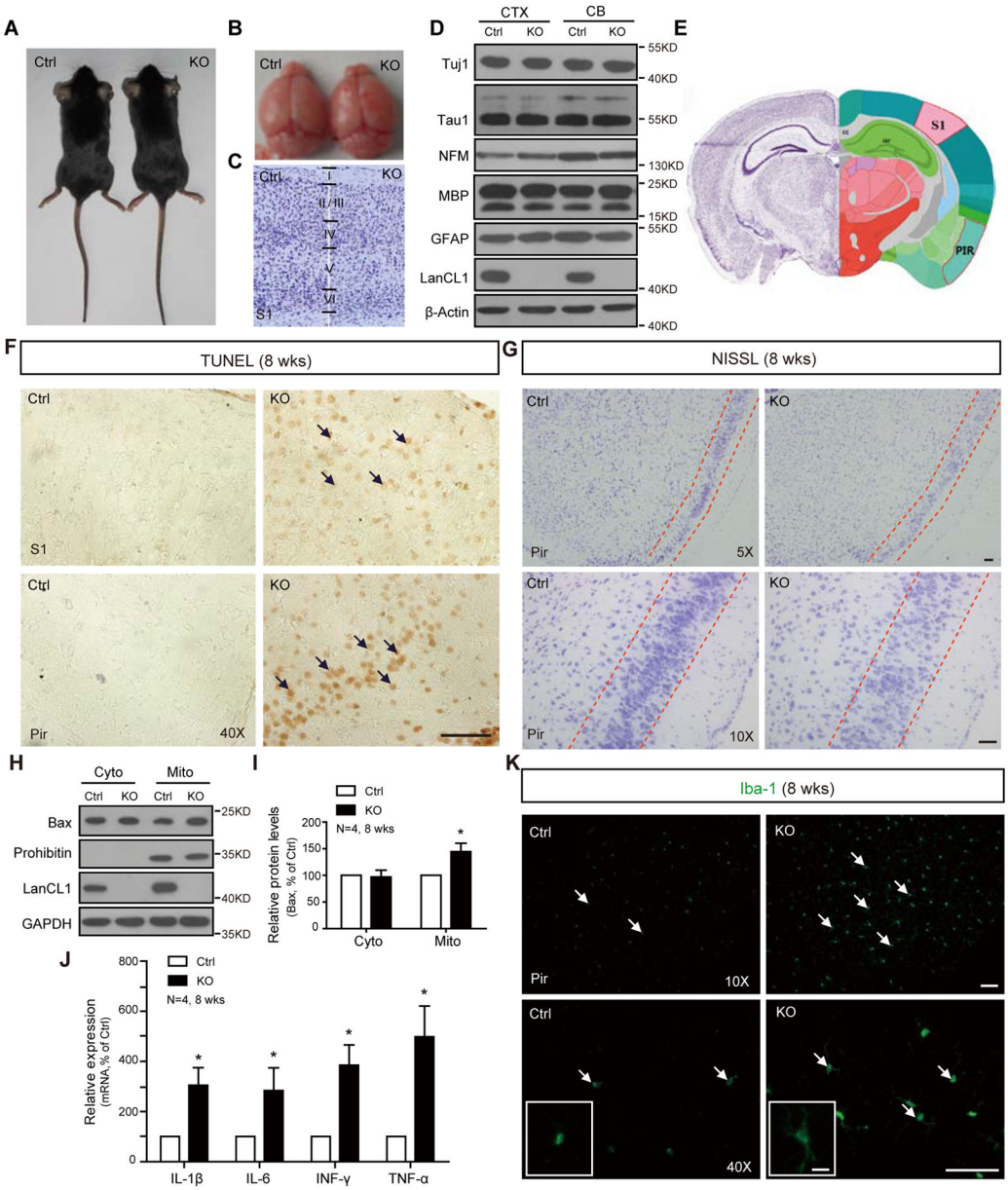

Figure 2. Apoptotic neuronal death and inflammation in *LanCL1* ko brains

(A and B) Photographs show that the genetic deletion of *LanCL1* does not affect the body or brain size (*LanCL1* −/−, ko, hereafter).

(C) Nissl staining reveals cortical lamination.

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

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

(F) TUNEL staining shows an increase in apoptosis (brown) of the 8-week *LanCL1* ko cortex (CTX, S1 & Pir). Bar, 50μm.

(G) Nissl staining reveals reduced density of cortical neurons in the 8-week *LanCL1* ko cortex. Bar, 50μm.

(H) Subcellular fractionation shows an increase in *Bax* in the mitochondria of the 8-week *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 with increased size of soma and number of cellular processes in the cortex of 8-week *LanCL1* ko brains. Bar, 50μm. The insets show enlarged microglia. Bar, 10μm.

See also Figure S2

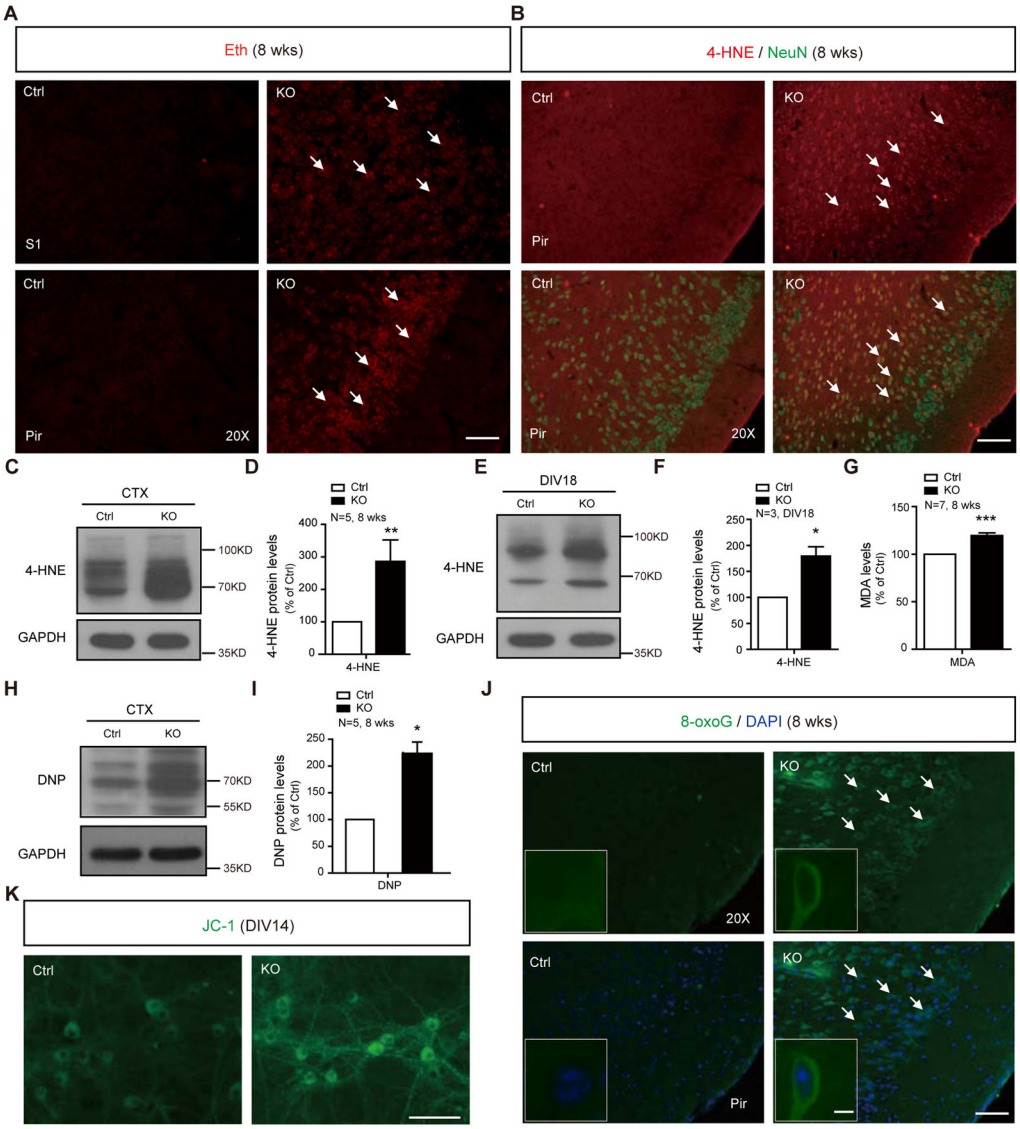

Figure 3. Oxidative damages and ROS accumulation in *LanCL1* ko brains

(A) Representative images of ethidium fluorescence (Eth, red) show the accumulation of reactive oxygen species (ROS) in the cortex of 8-week *LanCL1* ko brain. Bar:50μm.

(B) Immunostaining with anti-4-Hydroxynonenal (4-HNE, red) and anti-NeuN antibody (green) shows an increase in 4-HNE positive neurons in the 8-week *LanCL1* ko cortex. Bar, 50μm.

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

(E and F) Western blots and quantification 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 malondialdehyde (MDA) in cortex of 8-week *LanCL1* ko. Error bars indicate SEM, ***p=0.0003, n=7.

(H and I) Western blots with DNP antibody and quantification show increase of DNP levels in the cortex of 8-week *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) in the 8-week *LanCL1* ko cortex. Bar, 50μm. The insets show enlarged neurons. Bar, 5μm.

(K) JC-1 staining shows an increase in the fluorescence intensity of monomeric form of JC-1 (green) in *LanCL1* ko cultures (DIV14). Bar, 50μm.

See also Figure S3

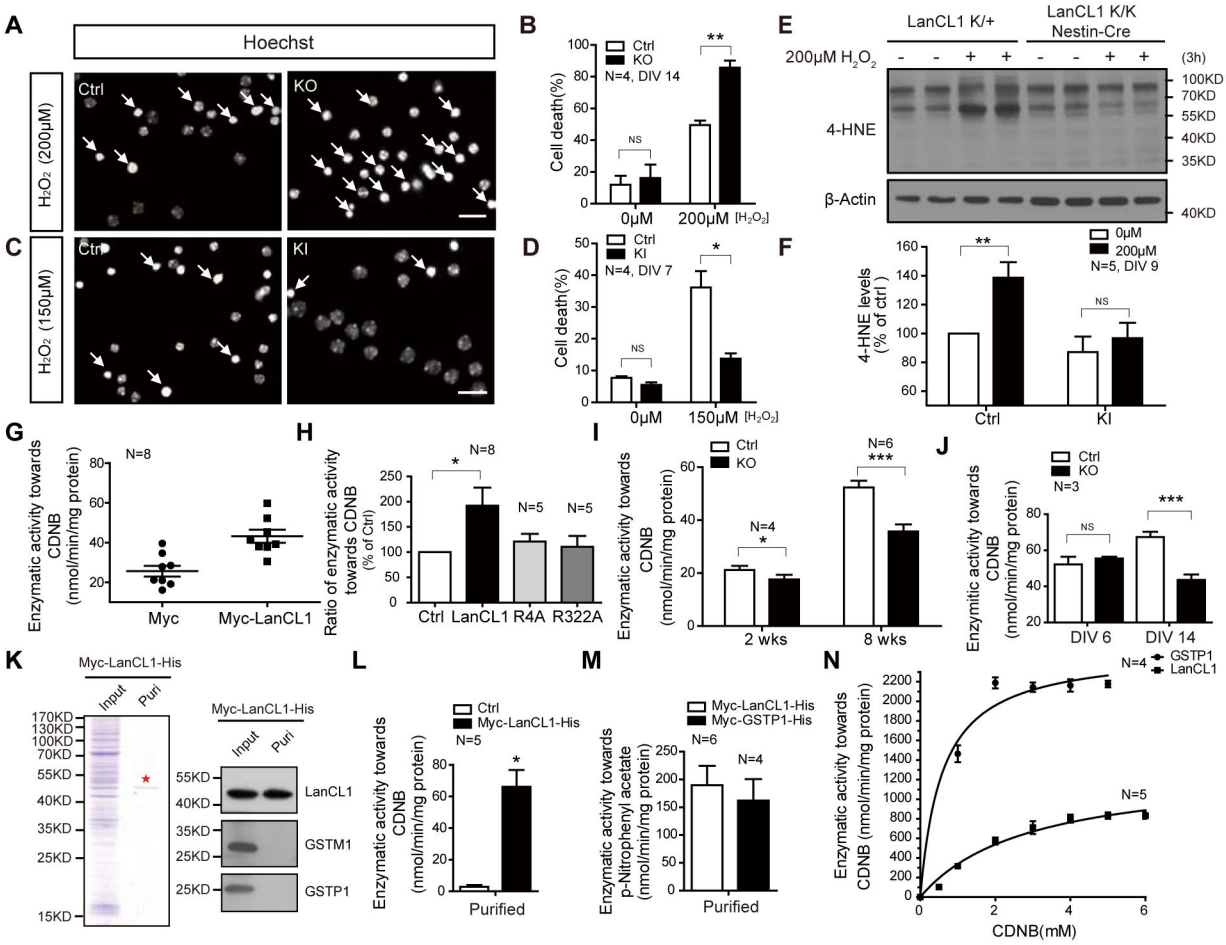

Figure 4. LanCL1 possesses catalytic activity for thioether formation and protects cells against oxidative stress

(A–D) Hoechst staining shows that deletion of LanCL1 increased neuronal death induced by H_2O_2 (12 hour treatment, DIV14, Ctrl: *LanCL1* +/+, KO: *LanCL1* −/−) (A, B), and LanCL1 transgene reduced neuronal death (12 hour treatment, DIV7, Ctrl: *LanCL1* K/K, KI: *LanCL1* K/K Nestin-Cre) (C, D). The data represent the mean ± SEM from four independent experiments, with a total number of 2,000 neurons analyzed for each group. Bar, 50μm. Error bars indicate SEM, *p= 0.0135, **p= 0.0069. n=4.

(E and F) Western blots and quantification 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 ctrl cells or LanCL1 point mutants that lack glutathione binding. 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-weeks to 8-week mice. Error bars indicate SEM. *p=0.0118 for 2-week sample, n=4; ***p=0.0004 for 8-week sample, n=6.

(J) Quantification shows a correlation of a reduction in GST activity in *LanCL1* ko culture neurons with its protein level in DIV6 to DIV14 cultures. 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 glutathione to 1-Chloro-2, 4-dinitrobenzene (CDNB). GSH: 0.5mM; CDNB, 0.5mM. Error bars indicate SEM, *p=0.0243, n=5.

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

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

See also Figure S4