Low-dose Naltrexone (LDN) extends healthspan and lifespan through activation of the transcription factor SKN-1/NRF2 in *C. elegans*.

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Abstract

Aging is a topic of urgency and importance, particularly as the world's aging population continues to grow. Numerous studies have been conducted to identify potential interventions that can improve health and promote longevity, however few are close to implementation. One promising approach to accelerate the implementation is drug repurposing, or using existing drugs for new indications. Here, we selected naltrexone by repurposing existing drugs from the Library of Integrated Network-based Cellular Signatures (LINCS) with several selection criteria. In recent decades, there has been increasing attention and use of low-dose naltrexone (LDN) as an adjunct treatment modality for cancers, autoimmune diseases, chronic pain and mental health issues. We found that a low, but not high dose of naltrexone extended both healthspan and lifespan in *C. elegans* worms. Further analysis revealed that LDN treatment-induced longevity was dependent on SKN-1 (NRF2 in mammals) signaling. Moreover, LDN treatment not only increased the expression of innate immune genes but also activated the oxidative stress response in worms, which could be abolished by inhibition of SKN-1/NRF2. Overall, paired with LDN's low side effects profile, our study highlights the great potential of LDN to be repurposed as a geroprotector for promoting healthy aging and suggests further research in humans is warranted.

Introduction

Many countries are facing a significant challenge in dealing with the unprecedented and rapid pace of population aging. The World Health Organization's report reveals that the number of individuals aged 60 years and above was 1 billion in 2019\(^1\,^2\). Aging is a primary risk factor for various diseases and conditions\(^3\). It is therefore necessary for a concerted effort to reduce health disparities and enhance the quality of life for older individuals by promoting healthy aging.

There is increasing interest in the concept of aging as a druggable target to prevent age-related diseases. However, developing new drugs to address human aging presents challenges in conducting clinical trials. In the absence of validated risk biomarkers, a large and initially healthy population would need to be treated over an extended period, making it difficult to conduct trials\(^4\). Therefore, repurposing existing drugs with a good safety profile is a more practical short-term solution than developing new drugs. For instance, the use of computational screening of drugs with publicly available databases, such as the Library of Integrated Network-based Cellular Signatures (LINCS), provides simple, important platforms available to describe gene expression 'signatures' stimulated by small molecules in cell lines\(^5\,^7\). Genetic approaches have effectively prolonged the lifespan of model organisms\(^8\). These investigations have uncovered conserved genes and pathways that play a crucial role in regulating longevity and promoting healthy aging. Consequently, geroprotective drugs that specifically target these pathways can be identified\(^9\).

Naltrexone is a prescription medication approved by the US Food and Drug Administration (FDA) in 1984 for the treatment of alcohol use disorder and opioid use disorder. It belongs to a class of drugs called opioid antagonists\(^10\). In recent years, there have been several significant findings regarding a specific
dosage of naltrexone called low-dose naltrexone (LDN). LDN has been shown to have immune-modulating properties that could reduce various oncogenic and inflammatory autoimmune processes and alleviate symptoms of certain mental ailments\textsuperscript{11–14}. These studies have demonstrated its potential in promoting general health sustainability. As such, naltrexone may have broader therapeutic applications beyond addiction medicine, with greater unexplored potential.

Here, we studied the potential benefits of low-dose naltrexone (LDN) in promoting healthy aging using \textit{Caenorhabditis elegans} as a model organism. We found that LDN treatment extended both healthspan and lifespan in worms, while high-dose naltrexone did not produce the same effects. Further metabolomics analysis revealed that LDN treatment induced metabolic changes that led to increased activity of both amino acid and glucose metabolism, but the longevity effect was independent of the DAF-16/FOXO3 signaling. We then tested various mutant strains and found that the lifespan extension induced by LDN treatment was dependent on the SKN-1/NRF2 transcription factor. We also observed that LDN treatment not only increased the expression of innate immune genes but also activated the oxidative stress response, in line with a role for SKN-1/NRF2 in LDN's lifespan promoting effects. Inhibiting the nuclear translocation of SKN-1 from the cytosol could attenuate the LDN-mediated innate immune gene expression and oxidative stress response. Overall, our study highlights the potential of LDN as a therapeutic agent for promoting healthy aging and identifies its mechanism of action.

\textbf{Result}

\textbf{Identification of naltrexone as a geroprotective compound}

To identify potential geroprotective compounds, we previously screened for drugs that could mimic the overexpression of the longevity transcription factor FOXO3 (\textit{daf-16} in worms)\textsuperscript{15}. Pharmaceutical modulation of the FOXO3 signaling pathway has emerged as a promising avenue for promoting healthy longevity\textsuperscript{16,17}. This led us to identify the longevity effects of atracurium, which extends lifespan in \textit{C. elegans} through activation of the DAF-16/FOXO transcription factor\textsuperscript{15}, and zidovudine, which extends lifespan independently of DAF-16/FOXO, acting rather through the ATF4 longevity transcription factor\textsuperscript{18}. In order to expand the range of compounds that can mimic FOXO3 overexpression, we re-analyzed the ranked compounds list obtained from the LINC\textsuperscript{15,18} dataset used in our initial screening for FOXO3 overexpression geroprotectors\textsuperscript{15,18}. We focused on repurposing existing drugs, and non-FDA-approved compounds on the table were excluded (Fig. 1a). This list included atracurium and zidovudine, as previously described\textsuperscript{15,18}, in addition to other compounds that either extend lifespan in model organisms directly, reduce senescence phenotypes, or are associated with lower mortality in humans. For example, cyproheptadine, sirolimus (also known as rapamycin), and temsirolimus have exhibited significant geroprotective properties beyond their current clinical applications\textsuperscript{19–23}. Panobinostat has an anti-senescence role in chemotherapy-treated cancer cells\textsuperscript{24}. PDE5 inhibitors, such as sildenafil, have been associated with lower mortality rates in humans\textsuperscript{25}.  

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Here, we chose to focus our study on naltrexone (NTX), which (a) has been unexplored in relation to longevity effects, (b) is highly ranked in our screen, and (c) may possess low side effect profiles for a greater probability of translation to humans. NTX was used as an opioid antagonist and approved by the FDA in 1985 to treat opiate dependence\(^2\text{6}\) (Fig. 1b). To evaluate the effects of NTX on healthspan and lifespan, we turned to \textit{C. elegans} worms, a well-accepted model for aging research. Using a healthspan assay assessing crawling speed of the worms at eight days of age, a time point directly before mortality starts in the population, we found that lower doses of NTX (2.5 µM and 5 µM) were effective in extending healthspan and lifespan in wild-type (N2) worms. Interestingly, higher doses (50 µM and 100 µM) had no significant impact (Fig. 1c). Similarly, low-dose NTX (2.5 µM) extended worm lifespan with a 17.6% increase in median lifespan relative to control-fed, while high-dose (50 µM) did not (Fig. 1d,e; Supplementary Fig. 1a-h and Supplementary Table S1). Overall, our findings suggest that low doses of NTX are a lifespan-extending agent in \textit{C. elegans}, whereas higher doses do not confer these benefits.

**Metabolomics profiling reveals a metabolic rewiring in worms upon LDN**

To investigate the metabolic effects of LDN and its role in lifespan extension, we collected worms treated with LDN on day 3 of adulthood and performed metabolomics analysis using UPLC-mass spec (Supplementary Table S2). We found a clear separation between the two treatment groups by using partial least squares discriminant analysis (PLS-DA), suggesting a distinct effect of the LDN treatment (Fig. 2a), consisting of 14 metabolites significantly increased and five significantly decreased in abundance (p-value < 0.05) (Fig. 2b). Among these metabolic changes, the most striking is the accumulation of amino acids (Fig. 2c). Indeed, Metabolite set enrichment analysis (MSEA) confirmed that the most altered metabolic pathways in LDN-treated worms were related to amino acid metabolism (Fig. 2d).

In addition to supporting protein synthesis, amino acids also control immune cell function, playing a key role in regulating different steps of innate immunity, which has been previously shown to be of interest regarding LDN’s effects\(^2\text{7,28}\). The amino acids that were increased after LDN treatment included isoleucine, valine, leucine, methionine, tyrosine, lysine, phenylalanine, and tryptophan (Fig. 2b). In the top altered metabolites, we also found that LDN treatment resulted in high levels of ophthalmic acid, an oxidative stress marker (Fig. 2c)\(^2\text{9–31}\). In line with this, we found pathways enriched related to glucose metabolism in the MSEA, such as amino sugar and nucleotide sugar metabolism, fructose and mannose metabolism and galactose metabolism (Fig. 2d). Oxidative stress significantly impacts glucose homeostasis regulation, suggesting a possible link\(^3\text{2,33}\) (Fig. 2d). Together, these metabolic changes suggested an age-related change in oxidative stress response and immune system related changes in LDN treated worms.

**LDN improves immunity and oxidative stress responses and extends lifespan independent of \textit{daf-16} signaling.**
Previous studies have noted the immunoregulatory and oxidative damage prevention effects of naltrexone though no clear mechanism has been proposed\textsuperscript{14,34,35}. To investigate the specific functional influences of LDN treatment, we evaluated the expression of p38MAPK/ATF-7-dependent innate immune genes in worms and observed an increase in these genes upon NTX treatment, including increases in \textit{C17H12.8, F56D6.2} and \textit{K08D8.5} gene expression\textsuperscript{36,37} (Figs. 3A, B). Furthermore, to determine whether the antioxidant genes \textit{sod-3} and \textit{gst-4} play a role in LDN treatment\textsuperscript{38}, we examined their expression through the use of GFP reporter strains (\textit{gst-4::GFP} and \textit{sod-3::GFP} strains). LDN increased the GFP signal of \textit{sod-3} and \textit{gst-4}, which is a representation of the activated/enhanced oxidative stress response (Fig. 3c,d). Given that the compounds list was screened based on mimicking FOXO3 overexpression, we sought to determine whether the lifespan extension induced by LDN was dependent on \textit{daf-16} (the worm gene orthologous to the FOXO genes in mammals). We therefore assessed the mobility of \textit{daf-16(mu86)} worms to determine their healthspan. On day 5, a significant increase in healthspan was observed in \textit{daf-16(mu86)} worms with LDN treatment, revealing that LDN's healthspan effects were independent of DAF-16/FOXO signaling (Fig. 3e). We subsequently evaluated whether the \textit{daf-16} was required for LDN-induced lifespan extension, and tested the effects of LDN in worms possessing a mutation in the DAF-16/FOXO gene. The results revealed that LDN was still extended lifespan in the \textit{daf-16(mu86)} mutants (Fig. 3f; Supplementary Table S1). These findings confirmed that LDN's healthspan and lifespan extension effects were not dependent on \textit{daf-16}, thus emphasizing the need for further investigation to better understand the underlying pathways involved.

**LDN extends lifespan in C. elegans dependent on SKN-1/NRF2**

We next aimed to uncover the mechanism allowing for LDN's lifespan and healthspan benefits. To do so, we prepared a small-scale screen, testing the effects of LDN in worms deficient in various pathways emerging from our metabolome analysis. We first tested the innate immune response (\textit{pmk-1} strain), the oxidative stress response (\textit{skn-1} strain)\textsuperscript{39,40}, and also investigated nerve synapse signaling (\textit{cdk-5} and \textit{unc-13} strain)\textsuperscript{41–43}, AMPK signaling (\textit{aak-2} strain)\textsuperscript{44,45}, and mitochondrial translation regulation (\textit{atf-4} strain)\textsuperscript{46}. We performed lifespan analysis of control worms and mutants of each of these regulators (Fig. 4a-f; Supplementary Table S1). We found lifespan extension still present upon LDN treatment in \textit{pmk-1(km25)} (Fig. 4a), \textit{cdk-5(ok626)} (Fig. 4b), \textit{atf-4(ok576)} (Fig. 4c), \textit{aak-2(ok524)} (Fig. 4d), \textit{unc-13(n2813)} (Fig. 5e) mutant strains, which suggested that these pathways were not involved in LDN-induced lifespan extension. However, we observed no lifespan extension in \textit{skn-1(mg570)} mutant strain (Fig. 4f), demonstrating that the lifespan extension induced by LDN was dependent on SKN-1. As healthspan plays a major role in longevity, we also measured the mobility in \textit{skn-1(mg570)} mutants. At day 8 of adulthood, the increased age-related mobility induced by LDN in mutant worms was almost completely abrogated when compared with the wild type strain N2 (Fig. 4g,h). Taken together, we conclude that the healthspan and lifespan extension observed with LDN was dependent on the activity of SKN-1.
LDN promotes the translocation of transcription factor SKN-1 from the cytosol to the nucleus and activates the oxidative stress response

SKN-1, the worm gene orthologous to NRF2 in mammals, is a major oxidative stress response regulator\(^47\). During adult stages, SKN-1 accumulates in the intestinal nuclei and promotes longevity and innate immunity by inducing genes involved in detoxification of ROS, such as gamma-glutamine cysteine synthetase \(gcs-1\), glutathione S-transferases \(gst-7\) and \(gst-4\), which play key roles in increasing oxidative stress resistance and extending lifespan\(^47\)–\(^51\). Because SKN-1 has been shown to be transcriptionally regulated by other compounds\(^52\), we next asked if SKN-1 was also transcriptionally regulated by LDN. We therefore measured the mRNA expression of SKN-1 after LDN treatment. However, there was no difference between treating with or without LDN, suggesting an alternative regulation exists (Fig. 5a).

It is known that oxidative stress induces SKN-1 to translocate from the cytoplasm to the nucleus in \(C.\) \textit{elegans}\(^53\). Therefore, we aimed to explore whether the effect of LDN was induced by nuclear localization of SKN-1 in the intestine, using GFP-tagged marker strains. The results indicate that LDN treatment caused an accumulation of SKN-1 in the nuclei of the intestine, with nearly a two-fold increase in the number of nuclear translocations (Fig. 5b; Supplementary Table S3). To address whether the oxidative stress response resulting from LDN treatment could be regulated by SKN-1, we examined LDN-induced GFP expression in the \(gst-4::GFP\) and \(sod-3::GFP\) reporter strains while knocking down \(skn-1\). Here, we found the LDN-induced oxidative response was significantly reduced after knockdown of \(skn-1\) (Fig. 5d,e; Supplementary Fig. 2a,b).

Recently, studies have shown that \(skn-1\) not only regulates the oxidative stress response but also influences immunity in \(C.\) \textit{elegans}\(^54,55\). Therefore, we next aimed to assess whether LDN's effect on innate immune gene expression was also regulated by \(skn-1\). Assessing transcript abundances of innate immune gene expression (including \(C17H12.8, C32H11.4, F49F1.6, F56D6.2, K08D8.5\) and \(M02F4.7\)), we found activation of these genes upon LDN treatment was abolished in the \(skn-1(mg570)\) mutant (Fig. 5c). Taken together, these results suggest that both LDN's activation of oxidative stress-induced genes, and activation of innate immunity genes, are dependent on nuclear accumulation of SKN-1.

Discussion

In summary, our study uncovered a geroprotective role of LDN in promoting healthy aging in \(C.\) \textit{elegans} (Fig. 6). Specifically, we show that LDN treatment extended both healthspan and longevity by promoting nuclear translocation of SKN-1, leading to the activation of the oxidative stress response and increased innate immunity gene expression. These findings suggested that LDN treatment may have therapeutic potential as an adjunct treatment modality for age-related diseases.

It is interesting to place these findings in the context of other work on LDN. LDN is to be a potential treatment for cancers, autoimmune diseases, chronic pain and mental health issues\(^56\). Since many of these are related to inflammation, which is partly regulated by NRF2, it would be worth studying if LDNs
benefits come from NRF2 activation in these diseases. The range of diseases for which LDN benefits is so diverse and large, that it may in fact be the case that LDN is affecting basic aging pathways in humans too. This possibility has been realized by others, and recently a clinical trial for LDN as a general treatment to slow aging has been proposed (NCT05307627). Our work suggests that LDN may indeed prove beneficial, and the mechanism rests on NRF2 activation. It would be interesting to study further NRF2 activity in LDN treated older individuals.

Our study possesses several limitations. Firstly, our work has been conducted entirely in C. elegans worms. While worms allow discerning mechanisms and mode of action of compounds more easily in the context of aging, it nonetheless suffers the drawback of being a simple, short-lived invertebrate, and translation of findings to humans may differ. Secondly, we have defined low-dose naltrexone in worms as 2.5µM, with higher doses being 50 and 100µM. We considered this to be a ‘low’ dose because our previous work using other compounds often works with higher doses of drugs to achieve lifespan benefits, including 50 and 100µM\textsuperscript{15,18}. While the dose of 2.5µM was indeed optimal for lifespan extension, it may still be the case that translating this to humans does not match the same low dose used in LDN human studies. Thirdly, while the lifespan extension from 2.5µM naltrexone treatment was robustly associated to lifespan in our work, the median lifespan extension of 17.6% is on the shorter side of lifespan extension possible in worms. While some may view this as a limitation, other recent work from our team has demonstrated that the quantity of lifespan extension in worms from small molecule treatments is directly related to the probability of the small molecule causing large side effects in humans\textsuperscript{57}. Therefore, the lower, though significant lifespan extension we observe with LDN, paired with clear healthspan extension in our study, may in fact be the most optimal result when aiming to translate a longevity drug from worms to humans.

Open questions remain following our findings. For example, while we demonstrate NRF2 dependency for LDN's lifespan benefits, it is unclear if the direct downstream regulators of lifespan are related to oxidative stress, innate immunity, or both. Future work is necessary to clarify this. Whichever the case, what has emerged as a clear result, is the activation of NRF2 in LDN treatment as a requirement for lifespan and healthspan benefits. This is promising, considering that NRF2 has also been associated with lifespan extension in higher organisms\textsuperscript{58,59}. Taken together, our work suggests that LDN provides a useful strategy for counteracting the diseases of old age.

Declarations

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Author contributions

W.L., RH.H., A.W.G and G.E.J. conceived and designed the project. W.L., R.L.M., and R.K. performed experiments. W.L., B.V.S., A.H.G.L. and G.E.J. analyzed the data. B.V.S. performed, analyzed, and aided in interpretation of metabolomics data. R.H.H. aided in interpretation of data and provided advice, W.L., A.W.G. and G.E.J. wrote the manuscript with contributions from all other authors.

Competing interests

The authors declare no competing interests.

Data and software availability

The reference *C. elegans* metabolic data examined in figure 2 is available at MetaboLights MTBLS7715 (https://www.ebi.ac.uk/metabolights/MTBLS7715). EndNote 7 is used for bibliography insertion.

Materials and methods

C. elegans strains

*Caenorhabditis elegans* strains N2 Bristol, CF1038 [daf-16(mu86)], KU25 [pmk-1(km25)IV], RB814 [cdk-5(ok626)III], RB790 [atf-4(ok576)X], RB754 [aak-2(ok524)X], MT8004 [unc-13(n2813)I], GR2245 [skn-1(mg570) IV], CL2166 [dvls19((pAF15)gst-4p::GFP::NLS)], CF1553 [muls84((pAD76)sod-3p::GFP + rol-6(su1006))], and LD1 [ldls7(skn-1b/c::GFP + rol-6(su1006))] were obtained from *Caenorhabditis* Genetics Center (CGC). Worms were routinely maintained at 20°C with OP50 E. coli on nematode growth media (NGM) agar plates.

Bacterial strains

*Escherichia coli* HT115 (DE3) with the Empty Vector (EV) L4440 was obtained from the *Caenorhabditis* Genetics Center. Bacterial feeding RNAi experiments were performed as described\(^6\). RNAi *E. coli* feeding clone used was *skn-1*(T19E7.2) derived from the Ahringer RNAi library\(^6\). All these clones were confirmed by sequencing and knockdown efficiency was confirmed by qPCR. In all RNAi experiments described in this study, worms were subjected to RNAi bacteria from the time of hatching.

Culture conditions

Naltrexone was obtained from Sigma Aldrich (N3136) and dissolved in water used for treatment and mixed in the NGM agar just before pouring. Worms were treated with compounds or water vehicles from the L4 stage onwards. Plates were changed at least once a week to ensure consistent exposure to the compound.
Mobility analysis

Gravid adult worms were age-synchronized using alkaline hypochlorite treatment and incubated in M9 buffer overnight. L1 stage worms were seeded to NGM plates. Worms were transferred to plates supplemented with compounds and 15 µM 5-fluorouracil (Sigma-Aldrich) at the L4 larval stage. All assays were performed at 20°C, and the L4 stage was counted as day 0 of life. At the stated day of adulthood, ~50 worms were transferred to NGM plates without *E. coli* OP50, stimulated by tapping the plate, and immediately recorded for 200 cycles at room temperature using a Leica (Amsterdam, The Netherlands) M205 FA fluorescent microscope and Leica DFC 365 FX camera. Images were captured using Leica Application Suite X software, then processed with the wrMTrck plugin for ImageJ. Measurements of body size were also derived from these analyses. Data from wrMTrck were analyzed and visualized using RStudio. Statistical analysis compared conditions to their respective control with a one-way ANOVA corrected for multiple testing.

Lifespan measurement

Prior to experiments, all animals were maintained at a permissive temperature and grown for at least two generations in the presence of food to assure health. Lifespan analyses were conducted at 20°C. Synchronized L1 worms were fed with *E. coli* OP50, grown to the L4 stage, and then 100 worms were transferred to a new plate contains with 15 µM 5-fluorouracil (5-FU). L4 stage was counted as day 0 of life. Animals were counted every other day and scored as dead when they did not respond to the platinum wire pick. Worms that were missing, displaying internal egg hatching, losing vulva integrity, and burrowing into NGM agar were censored. In Fig. 1D, all replicates treated with or without 2.5 µM naltrexone in N2 worms were merged to make a single lifespan curve. Survival plots, p values (Log-Rank), and proportional hazards were determined by using GraphPad Prism 9 software.

Confocal microscopy

Worms expressing SKN-1B/C::GFP were synchronized, grown and treated with compounds from L4 stage as described. To test SKN-1 nuclear localization, day 3 animals were collected and washed 3 times with M9 buffer for DAPI staining. After that animals were immobilized for 30 s in 40 mM tetramisole (Sigma Aldrich) in M9 buffer and mounted on 2% agarose pads. Nuclear localization of SKN-1B/C::GFP was visualized using a Leica DMI6000 inverted confocal microscope containing a 40×, 1.30 Oil CS2 objective lens and a Leica TCS SP8 SMD camera. Images were captured using Leica Application Suite X software. All samples were imaged at room temperature. Images of 20–30 worms per condition were taken over two independent experiments, and results are compiled from both replicates. The calculation of nuclear translocations was determined by randomly selecting 10 images from each condition and manually counting the number of nuclei with translocalization and the total number of nuclei (Table S3).

Fluorescent microscopy
CL2166 [dvIs19((pAF15)gst-4p::GFP::NLS)] and CF1553 [muls84((pAD76)sod-3p::GFP + rol-6(su1006))] strains were grown in NGM plates seeded with *E. coli* OP50 and NTX (2.5 µM) until day 3 (∼96 h after L4 stage). Worms were prepared for fluorescence microscopy, by preparing NGM plates without *E. coli* OP50 and adding a drop of tetramisole (40 mM). Worms were oriented using a picking tool. Images of vehicle and NTX-treated animals were using the same respective settings. Fluorescence exposure time was set to a value where vehicle-treated worms were barely visible and the same settings were used to analyze the NTX-treated worms. For the purpose of showing the results, the same level of brightness and contrast was applied to vehicle- and LDN-treated animals, with no impact on the fluorescence quantification of the images.

**Total RNA isolation**

*N2 C. elegans* from L4 stage were exposed to either vehicle (water) or 2.5 µM naltrexone (Sigma-Aldrich, USA). On adult day 3, nematodes were collected by washing off the plates with 3 times M9 buffer and 2 times water before being snap frozen in liquid nitrogen. For isolation of total mRNA, whole worms were homogenized with a 5 mm steel bead using a TissueLyser II (QIAGEN) for 5 min at frequency of 30 times/second. Cleanup of the RNA was performed according to RNeasy Mini Kit on the QIAcube per the manufacturer’s protocol (QIAGEN). Contaminating genomic DNA was removed using RNase-Free DNase (QIAGEN RNeasy Mini Kit on the QIAcube per the manufacturer’s protocol (Qiagen, California). RNA concentration and quality was measured by Nanodrop (Thermo Scientific; Breda, The Netherlands) and stored at −80°C until use.

**Q-RTPCR**

1 µg of extracted RNA was reverse transcribed into cDNA according to the instructions of the QuantiTect Reverse Transcription Kit (QIAGEN; Venlo, The Netherlands). Quantitative gene expression analysis was performed using the LightCycler® 480 SYBR Green I Master (Roche; Woerden, The Netherlands) and measured using the LightCycler 480 Instrument II (Roche). Gene-specific primers were synthesized according to the sequences in Table S4. The N0 values of target genes were normalized to the geometric mean of reference genes *pmp-3* and *cdc-42*.

**Metabolomics profiling**

To evaluate changes of the metabolome in that with and without naltrexone-treated worms, whole body tissues of adult Day 3 worms were collected (treated from L4). Six replicates were prepared for each group, each of ∼2500 worms. Worm samples were then freeze-dried overnight.

In a 2 mL tube, the following amounts of internal standard dissolved in water were added to each sample of freeze-dried worms: adenosine-\(^{15}\)N\(_5\)-monophosphate (5 nmol), adenosine-15N5-triphosphate (5 nmol), D4-alanine (0.5 nmol), D7-arginine (0.5 nmol), D3-aspartic acid (0.5 nmol), D3-carnitine (0.5 nmol), D4-citric acid (0.5 nmol), 13C1-citrulline (0.5 nmol), 13C6-fructose-1,6-diphosphate (1 nmol), 13C2-glycine (5 nmol), guanosine-15N5-monophosphate (5 nmol), guanosine-15N5-triphosphate (5 nmol), 13C6-glucose...
(10 nmol), 13C6-glucose-6-phosphate (1 nmol), D3-glutamic acid (0.5 nmol), D5-glutamine (0.5 nmol), D5-glutathione (1 nmol), 13C6-isoleucine (0.5 nmol), D3-lactic acid (1 nmol), D3-leucine (0.5 nmol), D4-lysine (0.5 nmol), D3-methionine (0.5 nmol), D6-ornithine (0.5 nmol), D5-phenylalanine (0.5 nmol), D7-proline (0.5 nmol), 13C3-pyruvate (0.5 nmol), D3-serine (0.5 nmol), D6-succinic acid (0.5 nmol), D4-thymine (1 nmol), D5-tryptophan (0.5 nmol), D4-tyrosine (0.5 nmol), D8-valine (0.5 nmol). Subsequently, solvents were added to achieve a total volume of 500 µL methanol and 500 µL water. A 5 mm stainless steel bead was added and a Qiagen TissueLyser II was used to homogenize each sample, before the addition of 1 mL chloroform. After thorough mixing, samples were centrifuged for 10 min at 14,000 rpm. The polar top layer was transferred to a new 1.5 mL tube and dried using a vacuum concentrator at 60°C. Dried samples were reconstituted in 100 µL 6:4 (v/v) methanol:water.

Metabolites were analyzed using a Waters Acquity ultra-high performance liquid chromatography system coupled to a Bruker Impact II Ultra-High Resolution Qq-Time-Of-Flight mass spectrometer. Samples were kept at 12°C during analysis and 5 µL of each sample was injected. Chromatographic separation was achieved using a Merck Millipore SeQuant ZIC-cHILIC column (PEEK 100 × 2.1 mm, 3 µm particle size). Column temperature was held at 30°C. Mobile phase consisted of (A) 1:9 (v/v) acetonitrile:water and (B) 9:1 (v/v) acetonitrile:water, both containing 5 mmol/L ammonium acetate. Using a flow rate of 0.25 mL/min, the LC gradient consisted of: Dwell at 100% Solvent B, 0–2 min; Ramp to 54% Solvent B at 13.5 min; Ramp to 0% Solvent B at 13.51 min; Dwell at 0% Solvent B, 13.51–19 min; Ramp to 100% B at 19.01 min; Dwell at 100% Solvent B, 19.01–19.5 min. Equilibrate column by increasing flow rate to 0.4 mL/min at 100% B for 19.5–21 min. MS data were acquired using negative and positive ionization in full scan mode over the range of m/z 50–1200. Data were analyzed using Bruker TASQ software version 2021.1.2.452. All reported metabolite intensities were normalized to total protein content in samples, determined using a PierceTM BCA Protein Assay Kit, as well as to internal standards with comparable retention times and response in the MS. Metabolite identification has been based on a combination of accurate mass, (relative) retention times, ion mobility data and fragmentation spectra, compared to the analysis of a library of standards. Processed metabolomics data can be found in Table S2. Partial least-squares discriminant analysis (PLS-DA) was performed using mixOmics72 setting a variable of importance (VIP) score of greater than 1 as significant. Metabolite Set Enrichment Analysis (MSEA) was analyzed using MetaboAnalyst 3.0 software under the p-value < 0.05. The metabolomics data are available at MetaboLights MTBLS7715 (https://www.ebi.ac.uk/metabolights/MTBLS7715). The fully processed metabolomics can be found in Table S1.

**Statistical analysis**

All data presented are specified in the respective figure legends and results section. Statistical tests are indicated in the Figure legends. P values of less than 0.05 were considered significant. Statistical analyses were performed using the Prism 9 software (GraphPad Software, La Jolla, CA, USA) and R v4.2.2 as described in each respective methods section.

**References**


**Figures**

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**Figure 1**

*Low-dose naltrexone (LDN) promotes healthspan and lifespan in C. elegans.* (a) List of the top 10 FDA-approved drugs that have similar effects at the transcriptional level as observed in FOXO3 overexpression cells, retrieved from the LINCS database. (b) Molecular structure of opioid antagonist naltrexone (NTX). (c) Mobility of N2 worms upon feeding with either water or various doses of NTX, measured at day 8 of adulthood. NTX promotes healthspan in a dose-dependent manner in wild-type (N2) worms. Significance was determined using One-way ANOVA. (d) NTX extends worm lifespan at 2.5 μM. N=880 worms, a lifespan composite of 8 independent lifespan assays (see Figure S1) (e) There is no effect on the lifespan when treating worms with 50 μM NTX. N=120 worms. The statistical analysis of the survival curves is performed using log-rank test. ****p < 0.0001, ***p < 0.001, **p <0.01, *p < 0.05, NS: not significant.
Figure 2

Metabolomics profiling reveals a metabolic rewiring in worms upon LDN. (a) Partial least squares discriminant analysis (PLS-DA) showing group separation on the basis of the metabolite profiles in day 3 adult worms treated with NTX (2.5 µM) compared with control worms (n = 6 biological samples per condition). (b) Differential analysis showing significantly altered metabolites (p < 0.05). Statistical testing of differential expression was performed using empirical Student's t-test. (c) The top 20 altered metabolites ranked according to their variable of importance (VIP) derived from the PLS-DA. (d) Metabolite Set Enrichment Analysis (MSEA) shows the most altered metabolic pathways in NTX-treated worms compared with control worms. MSEA was analyzed using online tools (MetaboAnalyst 3.0 software). Color intensity (yellow to red) reflects increasing statistical significance. Metabolomics data sets can be found in Table S2.
**Figure 3**

LDN improves innate immune and oxidative stress responses and extends worm lifespan independent of *daf-16* signaling. (a) Pathway for activation of p38MAPK. (b) The mRNA level of innate immune genes in 2.5 μM NTX-treated worms was determined by qRT-PCR. Bar graphs indicate mean ± SD. Significance was determined by a student’s t-test. Reference genes: *pmp-3, cdc-42*. (c) Representative fluorescence microscopy images and quantification of day 3 adult worms showing GST-4::GFP expression treated with or without 2.5 μM NTX. Scale bar: 0.3 mm (d) Representative fluorescence microscopy images and quantification of SOD-3::GFP fluorescence treated with or without 2.5 μM NTX for 3 days. Scale bar: 0.3 mm. (e) Mobility of *daf-16(mu86)* worms treated with or without 2.5 μM of NTX at day 5 of adulthood. Significance was determined using student’s t-test. (f) Survival of *daf-16(mu86)* worms treated with or without 2.5 μM NTX. N= 120 worms. The statistical analysis of the survival curves was performed by log-rank test. ***p < 0.001, **p <0.01, *p < 0.05, NS: not significant.
**Figure 4**

**LDN-mediated lifespan extension is dependent on SKN-1 signaling.** (a-f) Survival of *pmk-1(km25) (A), cdk-5(ok626) (B), atf-4(ok576) (C), aak-2(ok524) (D), unc13(n2813) (E), skn-1(mg570) (F)* worms treated with control and 2.5 μM NTX. N=100 worms. The statistical analysis is performed by the log-rank test. (g) Mobility testing at day 8 of adulthood of N2 worms upon feeding with either water vehicle or 2.5 μM NTX. Avg. speed = average speed. Significance was determined using the student’s t-test. (h) Mobility of *skn-1(mg570)* worms treated with 2.5 μM of NTX at day 8 of adulthood. Significance was determined using the student’s t-test. ***p < 0.001, **p <0.01, *p < 0.05, NS: not significant.
Figure 5

LDN promotes the translocation of transcription factor SKN-1 from the cytosol to the nucleus and activates the oxidative stress response. (a) mRNA level of skn-1 gene in control worms and worms treated with 2.5 μM NTX, determined by qRT-PCR. Expression of skn-1 does not change upon NTX treatment. Bar graphs indicate mean ± SD. Significance was determined by an unpaired student's t-test. (b) Representative images of day 3 adult worms showing the translocation of the transcription factor SKN-1 from cytosol to the nucleus in worms fed with or without 2.5 μM NTX. (c) The mRNA level of innate immune genes in either N2 or skn-1(mg570) worms treated with or without 2.5 μM NTX, determined by qRT-PCR. Reference gene: pmp-3, cdc-42. Bar graphs indicate mean ± SD. Significance was determined by a One-way ANOVA between vehicle and NTX treatment comparisons. (d) Representative images of day 3 adult worms showing SOD-3::GFP expression in N2 or skn-1(mg570) worms fed with or without 2.5 μM NTX. Scale bar: 0.3 mm. (e) Representative images of day 3 adult worms showing GST-4::GFP expression in N2 or skn-1(mg570) worms fed with or without 2.5 μM NTX. Scale bar: 0.3 mm. ***p < 0.001, **p < 0.01, *p < 0.05, NS: not significant.
Figure 6

Schematic model of LDN’s mechanism. LDN treatment results in nuclear translocation of SKN-1, leading to the activation of the oxidative stress response, increased innate immunity gene transcription, and healthspan and lifespan extension.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- tableS2metabolism.xlsx
- supplementaryfiguresandtables.docx
- rs.pdf