EXPLORING THE IMPACT OF A20 POLYMORPHISMS ON TLR4 SIGNALLING

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Abstract

Background. Opioids are powerful analgesics, used widely for pain management. However, they are associated with side-effects causing the development of analgesic tolerance and induction of pain (OIH). There is evidence implicating inflammatory signalling in the CNS, mediated by opioid activation of TLR4, in these side-effects. In the peripheral immune system TLR4 signalling is negatively regulated by A20. A20 is also a crucial regulator of neuroinflammation. Recently, polymorphisms of A20 have been identified that alter its function.

Gap and Aims. Little is known about the spatial organisation and regulatory relationship between A20 and TLR4 in the CNS. Likewise, the impact of the A20 polymorphisms on TLR4 signalling, particularly in the context of opioid-activation is unexplored. I aimed to identify the spatial co-expression of TLR4 and A20 in the CNS and determine the functional consequences of the A20 polymorphisms on (+)-morphine and LPS-induced TLR4 signalling.

Experimental approach. To achieve this, CNS RNA-seq data was analysed, an *in vitro* TLR4 system was optimised, and the impact of four A20 polymorphisms on TLR4 signalling were tested.

Results and conclusions. I was unable to observe (+)-morphine activation of TLR4, nor regulation of LPS-induced TLR4 signalling by A20 or its polymorphisms. However, I have shown that TLR4 and A20 are co-expressed throughout much of the CNS and identified regions which may be susceptible to the A20 polymorphisms. Some of which are implicated in opioid actions. Further optimisation is required to test the polymorphisms, but the spatial co-expression of A20 and TLR4 warrants further study.

1. Introduction

Opioid medications exhibit potent analgesic properties and are widely regarded as the gold standard for the management of moderate to severe pain[1]. The prescription rate of opioids in Australia has increased 11% between 2013 and 2017[2]. However, their efficacy is hindered by unwanted side-effects, including the development of analgesic tolerance, increased pain, and increased sensitivity to painful stimuli (the latter two referred to as Opioid-induced hyperalgesia – OIH)[3, 4]. Management of analgesic tolerance and OIH is particularly challenging since the underlying pain must still be addressed. Current interventions to curb these side-effects focus on switching between opioid medications. Whilst other strategies are being explored, clinical data is limited[5]. Our current understanding of the mechanisms underlying these side-effects is incomplete but is crucial for intervention and avoidance.

There is evidence neuronal mechanisms are involved in both analgesic tolerance and OIH, including glutaminergic signalling[6-8], variations in the Mu-opioid receptor (MOP)[9] and induced alterations of various secondary messengers[10-12]. The glutaminergic signalling hypothesis is supported by the exploration of ketamine, an NMDA receptor antagonist (blocking glutaminergic signalling), which has been shown to alleviate OIH[13] and oppose the reinforcement of analgesic tolerance[14]. However, since neurons are capable of responding to immune signalling molecules[15], studies focusing solely on neuronal mechanisms fail to consider the importance of the cross-talk between resident immune cells of the central nervous system (CNS) and neurons in analgesic tolerance and OIH. The glutaminergic-ketamine hypothesis is a prime example of this, as ketamine exerts extensive regulation on immune signalling within the CNS[16], suggesting the mechanisms underlying these side-effects are more complex than purely neuronal.

Microglia are resident immune cells of the CNS, functioning to maintain homeostasis. As part of their role, they respond to environmental milieu associated with cellular damage and pathogens (DAMPs and PAMPs). Detection of DAMPs and PAMPs by microglia results in the transition to a reactive

phenotype, consistent with an innate immune response (secretion of cytokines, chemokines, reactive species and the recruitment of more immune cells)[17]. This proinflammatory environment can alter neuronal receptor expression, impacting neurotransmission[18] and amplifying mechanisms involved in the transmission of nociception[19].

Interestingly, evidence suggests opioids can also induce the microglial reactive phenotype[20]. It has been observed that opioid-induced microglial signalling via BDNF facilitates neuronal changes inducing OIH[21]. Furthermore, morphine treatment can upregulate proinflammatory cytokines; TNF-α, IL-1β and IL-6[22], which are implicated in OIH and the development of analgesic tolerance[23, 24]. Similarly, it has been demonstrated that morphine exacerbates pain via activation of the NLRP3 inflammasome, a potent inflammatory factor[25]. Converging lines of evidence between the opioid-induced side effects and activation of central immune signalling point towards the involvement of innate immune receptor, Toll-like Receptor 4 (TLR4). TLR4 is a pattern recognition receptor with a binding affinity for various DAMP's and PAMP's[26] and its expression on microglia is irrefutably involved in the transition to the reactive state[27].

The most widely characterised TLR4 ligand is liposaccharide (LPS), a component of the bacterial cell wall. TLR4 activation by LPS is facilitated by multimerisation with co-signalling molecules CD-14 and MD2 at the cell surface, inducing intracellular signalling via the MyD88-dependant and -independant pathways. Activation of the MyD88-dependant pathways eventuates in the phosphorylation (and activation) of NF-kB, a transcription factor responsible for the expression of various pro-inflammatory cytokines[28] (Figure 1a). Interestingly, LPS from *Rhodobacter sphaeroides* (LPS-RS) is a potent antagonist of TLR4[29].

Emerging evidence suggests that TLR4 may be a non-classical site of opioid action[30]. Strong evidence indicates various opioids directly activate TLR4 signalling *in vitro* and morphine analgesia is elevated in TLR4 knockout mice[31]. This is supported by evidence suggesting morphine directly activates TLR4 signalling in a similar fashion to LPS, initiating the dimerization of MD-2 and CD14, and induces a pro-inflammatory response.[32] In addition, plasma from morphine treated mice induces

TLR4 signalling[33]. Interestingly, while the MOP is highly stereoselective, TLR4 may not be, since the MOP inactive morphine isomer, (+)-morphine also exhibits TLR4 activity[34]. Finally, *in silico* docking approaches have also revealed that morphine is capable of binding to TLR4 and activating its signalling[35]. Despite the wealth of literature implicating opioid action at TLR4 in OIH, there is also evidence suggesting otherwise[36]. One possible explanation for the observed discrepancy may lie in the regulation of TLR4 signalling, as experimental systems where TLR4 is tightly regulated may mask opioid activation.

In the peripheral immune system, TLR4 signalling is negatively regulated by A20[37]. A20 primarily functions as a deubiquitinating enzyme, preventing NF-kB mediated signalling. In TLR4 signalling, A20 cleaves the K63-linked polyubiquitin chains at TRAF6 (Figure 1a), preventing protein-protein interaction thereby arresting downstream signalling[38]. In the CNS, A20 has recently been implicated as a crucial regulator of microglial mediated inflammation[39, 40]. Despite this, the regulation of TLR4 by A20 has not been directly explored in the CNS. Recently, several single nucleotide polymorphisms (SNPs) of A20 have been identified in the human population (Figure 1b). These SNPs confer reduced regulation of NF-kB activation in response to TNFα activation[41]. Specifically, these SNP's cause alterations in the phosphorylation of A20 by IKK-β, a necessary process for the activation of A20. The function of these novel polymorphisms is largely unexplored in the signalling pathways A20 acts on, including TLR4. Until very recently, the interaction between A20 and opioid signalling was unexplored. It has now been shown *in vivo* that A20 may enhance opioid analgesia by a mechanism associated with analgesic tolerance and OIH[42].

I hypothesise that understanding the regulatory action of A20 in opioid-driven central immune signalling will identify novel mechanisms underlying side-effects associated with opioid use and a key role for A20 in the regulation of TLR4-mediated neuroinflammation. Therefore, by understanding the functional consequences of the A20 polymorphisms in this system, a potential genetic basis for the opioid-induced side-effects and presentation of neuroinflammatory pathologies

may be elucidated. This is important as it may reveal opportunities for personalised medicine and novel clinical therapeutics.

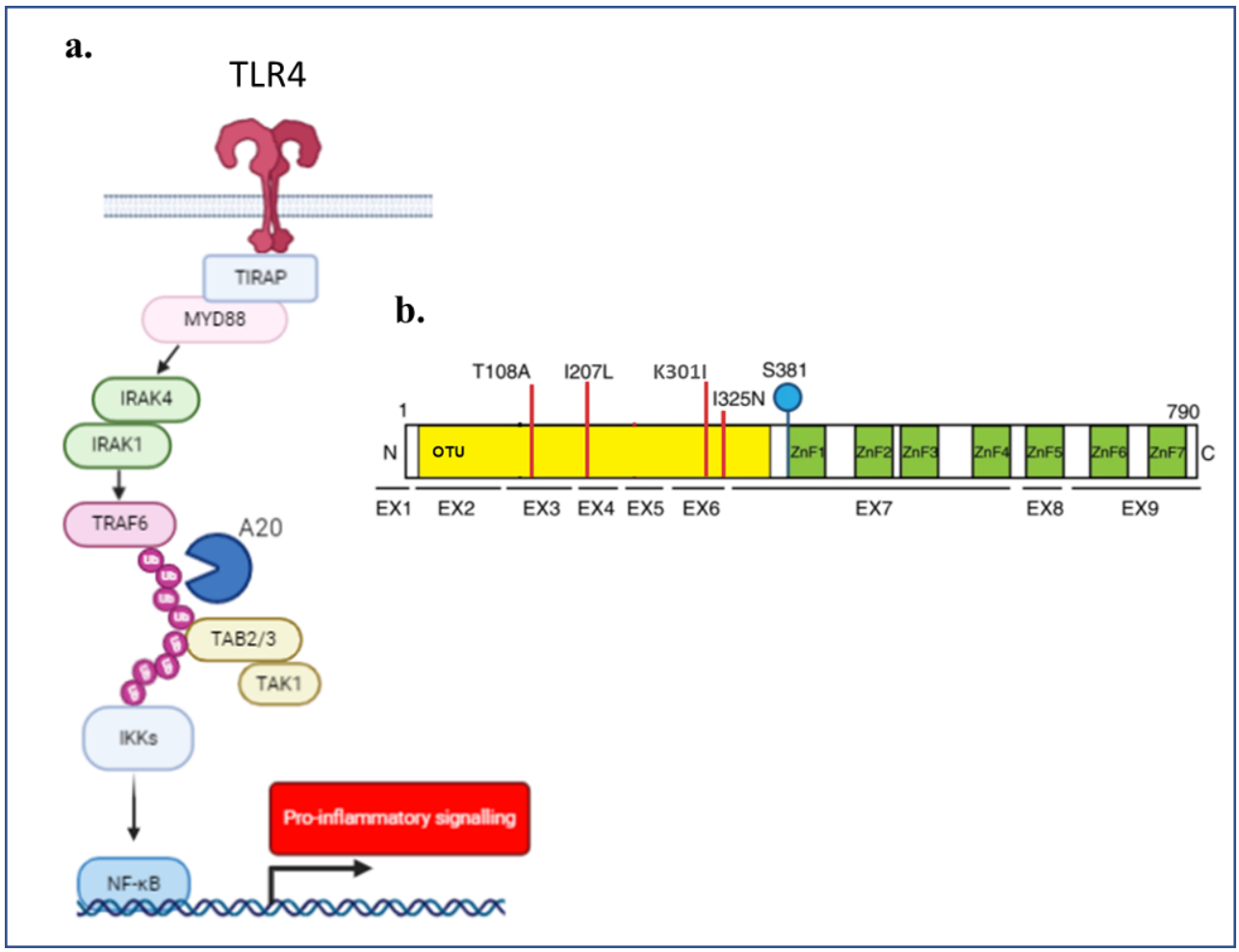

Figure 1a.) TLR4 MyD88-dependant pathway showing where A20 acts **b.)** site of A20 polymorphisms (red lines) and phosphorylation site (blue dot) (**b.** adapted from Zammit et. al.[40]**)**

1.1 Knowledge Gap

Collectively the literature presented highlights several unexplored questions which may be pivotal in understanding the mechanisms causing the opioid-induced side-effects. Firstly, what is the spatial organisation and regulatory relationship between A20 and TLR4 in the CNS? Is A20 crucially involved in regulating opioid activation of TLR4? And therefore, what are the functional consequences of the A20 polymorphisms on the regulation of TLR4 signalling?

1.2 Hypothesis and Aims

Here I aimed to test the hypothesis that regulation of TLR4 by A20 is crucial in the CNS and in regulating opioid-induced side effects. Furthermore, polymorphisms which confer weaker regulatory capacity, will result in increased TLR4 signalling, and exacerbate a TLR4 response to (+)-morphine. To test this hypothesis the following aims were developed.

1. Utilise a bioinformatic approach to identify spatial co-expression of A20 and TLR4 in the CNS
2. Validate an *in vitro* TLR4 signaling system incorporating the A20 polymorphisms.
3. Determine the functional consequences of the A20 polymorphisms on TLR4 signaling.

2. Methods & Materials

2.1 Allen Human Brain Atlas (AHBA) transcriptomic analysis

To characterise the special relationship between the expression of TLR4 and A20 in the brain (Aim 1), RNA-seq expression data from AHBA (n=6 healthy brains- 2 female, 4 male, 414 regions) was extracted into R via ABAEnrichment[43]. A Z-score was then generated for each gene across all brain regions. TLR4 and A20 Z-scores were applied to Density-based spatial clustering with applications of noise algorithm (DBSCAN)(dbscan package[44]). The parameters for minimum number of points in a cluster (minPts) and distance between points (epsilon neighbourhood) were set at 4 and 0.4 respectively based on methods outlined by Ester. Et. al[44]. The plot was generated using the "fviz_cluster" function from "factoextra" package.

2.2 Cell culture

Human embryonic kidney 293 (HEK293) cells stably expressing TLR4, MD2 and CD-14 ("HEK-TLR4") were maintained in high glucoses Dulbecco's Modified Eagle's Medium (DMEM): supplemented with 10%(v/v) foetal bovine serum (FBS), 2nM L-Glutamine, 50U/ml Penicillin, 50µg/ml Streptomycin, 100µg/ml Normocin, 10µg/ml Blasticidin, 10µg/ml Hygromycin. Cells were

passaged by mechanical dissociation 2-3 times per week up-to 20-24 passages and grown at 37ºC in a humidified incubator at 5% CO_2.

2.3 LPS, LPS-RS and (+)-Morphine Treatments

HEK-TLR4s were seeded in a 96-well plate coated with poly-D-lysine at $4.5x10^4$cells/well in 200µL growth media. At the time of treatment, cells were 90-100% confluent. LPS doses (0.1-100ng/ml) were based established lab protocols and (+)-morphine doses (0.1-100µM) were influenced by lab protocols and literature[33]. TLR4 antagonism was conducted by 2h pre-treatment of 200µg/ml LPS-RS. Working concentrations were diluted from stock aliquots (100µg/ml LPS/LPS-RS and 10mM (+)-morphine) in growth media. Treatment volumes were 100µl. (+)-Morphine stocks were in dimethyl sulfoxide (DMSO), so vehicle treatments were media containing 1% DMSO to match %DMSO in the highest treatment. Experiments were conducted in technical triplicate and repeated independently a minimum of three times (n=3).

2.4 IL-8 ELISA

Sandwich ELISA (Enzyme-linked immunosorbent assay) was used to measure activation of TLR4 (Aim 2 and 3). Supernatants were either added immediately post-treatment or frozen for later use. IL-8 was measured using human IL-8 ELISA kit and reagent set B (BD biosciences; catalogue No.555244, 550534). Assays were performed according to an optimised lab protocol (based on manufacturer's protocol[45], at half volume with 2x recommended dilutions and incubated overnight at 4ºC). Optical density (450nm absorbance with 570nm correction) was measured (CLARIOstar, BMG Labtech). Samples were interpolated from the standard curve in Graphpad Prism 8 using a nonlinear fit. Experiments were conducted in technical triplicate and repeated independently a minimum of three times (n=3).

2.5 Transient Transfection

For A20 experiments (Aim 2 and 3), HEK-TLR4s were transfected with pcDNA3.1+ plasmid vectors containing the wild type (WT), mutant A20 constructs (Kind gift of Professor Shane Grey), or with the empty vector using Lipofectamine 3000 (Thermo Fisher scientific, catalogue No.L3000008). The

nucleic acid substitutions of the four polymorphisms used were; I207L, T108A;I207L, I325N and K301I (referred to as KOI) (Shown in Figure 1b). Transfections were performed in a 6-well plate on cells ~80% confluent, plated in antibiotic-free DMEM according to the manufacturer's protocol[46], using 2.5µl Lipofectamine3000, 1.5µg cDNA and 1.5µl P3000 reagent per well, as optimised (detailed in results). Cells were replated 24h after initial transfection for use in either treatment for ELISA (96-well plate at 45,000 cells/well) or western blot (6-well plate at $1.2x10^6$ cells/well). The duration of the experiment from transfection to treatment was 72h.

2.6 Cell lysis and protein quantification for western blot

For validation of transfections by western blot (Aim 2 and 3), cell samples were lysed using RIPA buffer (supplemented with protease inhibitor). Lysates were stored at -80^{o}C prior to quantification. Protein quantification was conducted using the Pierce^{TM} BCA (bicinchoninic acid) protein assay kit (ThermoFisher scientific, Catalogue No.32335), according to the microplate protocol[47]. Optical density was measured (CLARIOstar, BMG Labtech, absorbance 562nm) and protein concentrations were interpolated from the standard curve. Where the purpose of the western blot was to validate the success of the transfections on cells split for treatments, parallel cells were lysed at the time of treatment.

2.7 Western Blot

Samples for western blot were prepared by adding 30µg of protein sample to 10µl LDS sample buffer, 4µl reducing agent (ThermoFisher Scientific, Catalogue No.NP0007, B0009) brought to a volume of 40ul with Mili-Q water and heated at 99^{o}C for 10 minutes. Samples (40ul) and loading standards (5ul) were resolved on a 4-12% bis-tris gradient gel, MOPS-SDS running buffer (ThermoFischer Scientific, Catalogue No.NP0322PK2 and NP0001) was used for electrophoresis at 120V for 90 minutes in a mini gel tank. Proteins were transferred onto a nitrocellulose membrane (BioRad Catalogue No.162011) via semi-dry transfer stack run at 30V for 60 minutes. Membranes were washed in TBS-tween20 and blocked for 1h in 5% skim milk, then incubated overnight at 4^{o}C on a rotor in a 2% skim milk solution of A20 rabbit mAb (1:1000 dilution, 82kDa detection size) (Cell

Signalling D13H3 Catalogue No.5630S) and GAPDH chicken pAb (1:2000 dilution, 37kDa detection size) (Abcam Catalogue No.ab83957). Membranes were washed in TBS-tween20, re-sealed in 2% skim milk solution of anti-rabbit and anti-chicken secondary antibodies (both 1:10000 dilution) (Li-Cor catalogue No.9263221, No.92668075) and incubated in the dark for 2h at room temperature. Membranes were washed in TBS-Tween20 and imaged (Li-Cor Odyssey-CLx, 700nm and 800nm absorbance). A20 (green, expected size 82kDa) and GAPDH (red, expected size 37kDa). Densitometry analysis was conducted on black and white images with ImageJ on data normalised to GAPDH according to methods described[48, 49].

2.8 Statistical analysis

Two-way ANOVA were conducted on all data using Tukey's post-hoc multiple comparisons, where $p < 0.05$ was used to determine statistical significance, using GraphPad Prism 8.

3. Results

3.1 DBSCAN reveals brains region where regulation of TLR4 by A20 may be functionally important. I used published RNA-Seq data to characterise the expression of TLR4 and A20 in the CNS (Figure 2). The cluster plot shows a positive correlation between A20 and TLR4 expression in the CNS (Pearson's $R = 0.61$), indicating a co-expression trend. The clustering algorithm identifies general co-expression trend (Cluster 1, blue) and brain regions whose relationship between A20 and TLR4 segregates from this trend (Black dots, Cluster 2, and Cluster 3). These regions may indicate where A20 and/or TLR4 are functionally important. Several regions with high segregated co-expression of TLR4 and A20 are implicated in opioid actions (highlighted in bold).

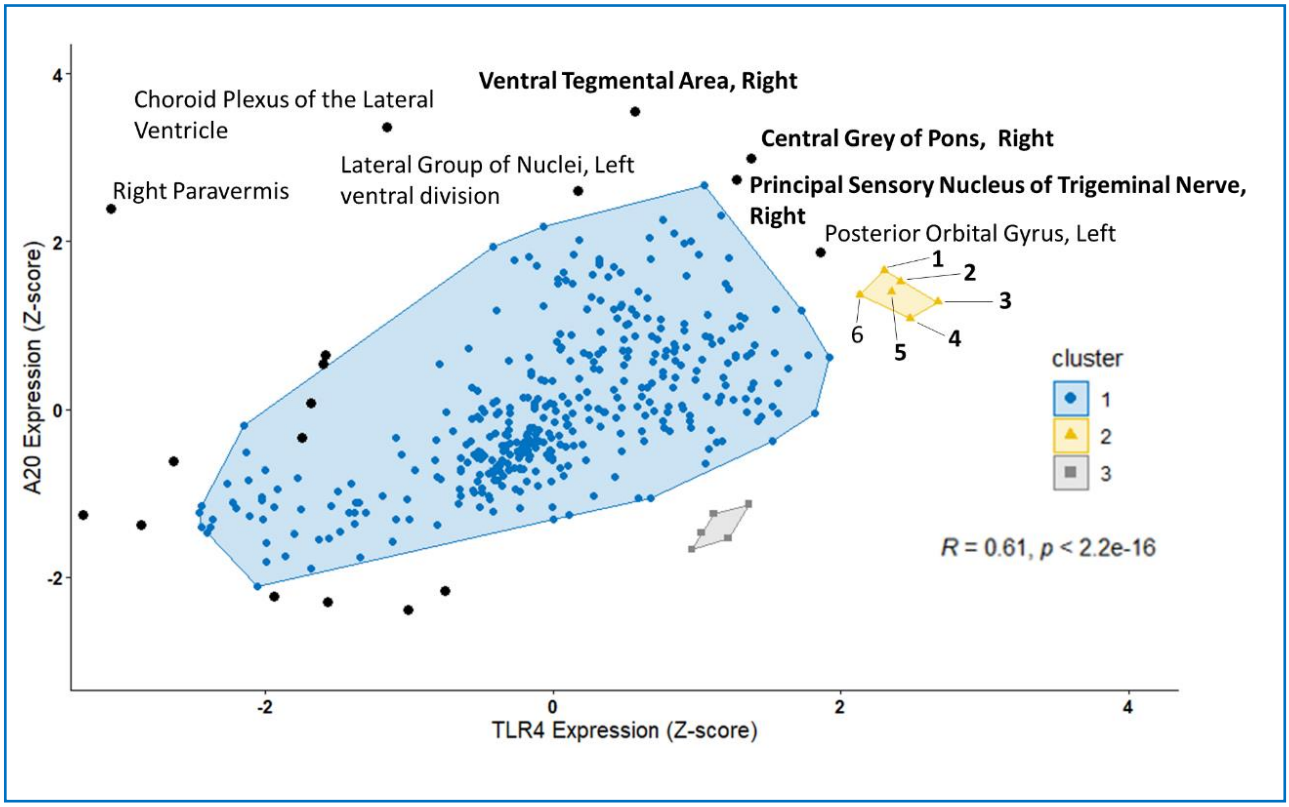

Figure 2. A cluster plot mapping the spatial relationship between the expression of A20 and TLR4 in the CNS. *Each point represents an individual brain region (414 total regions mapped). Identified here is the general co-expression trend, (Cluster 1, blue) and individual regions (black dots) or clusters of regions (Cluster 2, orange, and Cluster 3, grey) whose co-expression segregates from the general trend. The labelled regions are areas of high A20 expression (Cluster 2 is annotated by Table 1). Regions labelled in bold are associated with opioid actions.*

Table 1: Annotated regions of Cluster 2 (orange).

	Cluster 2
1	**Globus Pallidus, External, Right**
2	**Globus Pallidus, External, Left**
3	**Globus Pallidus, Internal, Left**
4	**Substantia Nigra, Pars Reticulata, Left**
5	**Globus Pallidus, Internal, Right**
6	Supraoptic Nucleus Left

3.2 TLR4 antagonism abolishes an LPS response in HEK-TLR4 but (+)-Morphine stimulation induces no response. It was demonstrated that LPS stimulation (2h) induced a robust IL-8 response in HEK-TLR4's across a concentration range (1-50ng/ml). The response from the 10ng/ml LPS treatment was abolished with a 2h pre-treatment of TLR4 antagonist LPS-RS (200ng/ml) ($p < 0.0001$). Abolishment of an LPS-induced IL-8 response following TLR4 antagonism indicates that TLR4 specific signalling can be achieved in our hands. (+)-morphine did not induce an IL-8 response across a concentration range (1-100µM) (Figure 3)

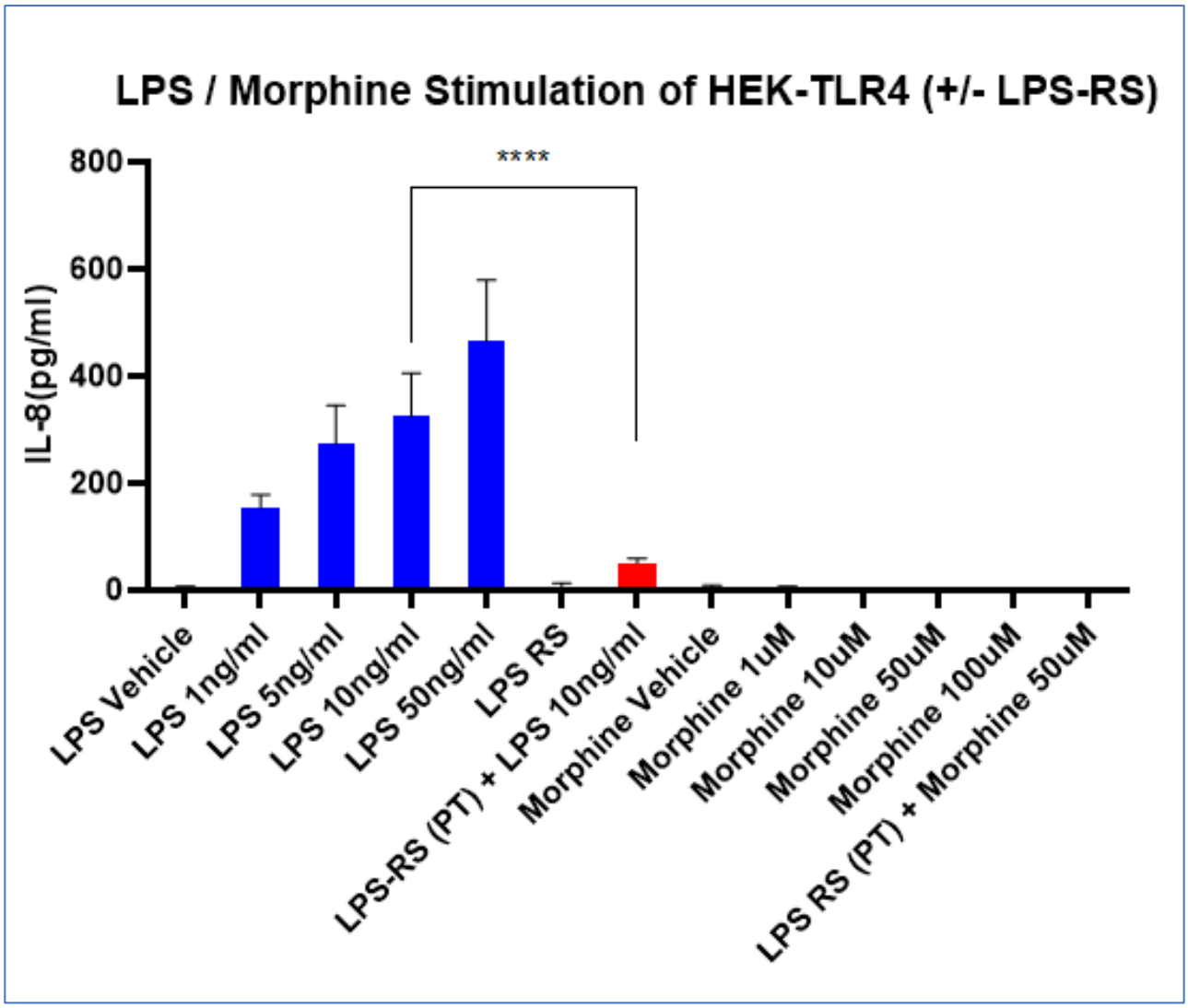

Figure 3. Measured IL-8 production following 2-hour stimulation with LPS or (+)-Morphine of HEK-TLR4. *TLR4 antagonism was conducted by 2h pre-treatment (PT) with 200ng/ml LPS-RS. Pre-treatments were then removed and LPS or (+)-morphine treatments were added. n=3, represented as Mean ±SEM. ****= $p < 0.0001$*

3.3 Lipofectamine 3000 transfection protocol optimisation. Transfections were optimised using the wild-type (WT) A20 plasmid construct, maintaining a constant quantity of cDNA and P3000 reagent outlined in manufacturer's protocol and varying the volume of Lipofectamine3000 reagent. Expression of A20 when transfected with 1.25µl, 2.5µl, 3.75µl and 7.5µl of Lipofectamine3000 respectively is below maximum expression (5.35µl), with A20 (green) and GAPDH (red) detected at their respective sizes (82kDa and 37kDa) (Figure 4a, 4b). 2.5µl and 3.75µl Lipofectamine3000 protocols were selected for further optimisation. Using these volumes of Lipofectamine3000, the amount of cDNA and P3000 reagent were then varied. The results suggest transfections C (1.5µg cDNA + 1.5 µl P3000) or F (1.5µg cDNA + 3µl P3000) are the most suitable protocols preventing saturation (Figure 4c, 4d, 4e). To be conservative of reagents, protocol C was selected to determine the functional consequences of the polymorphisms.

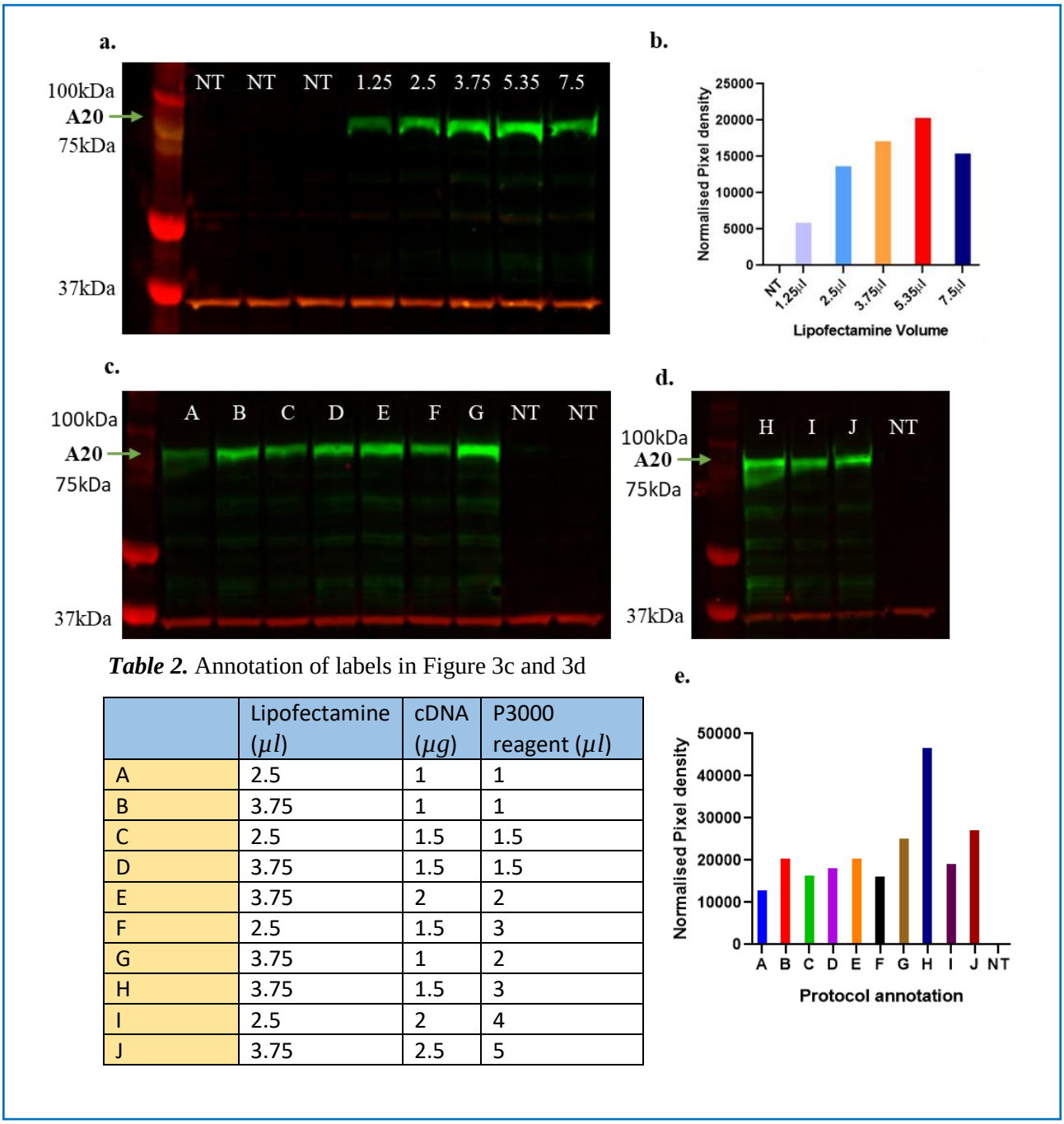

Table 2. Annotation of labels in Figure 3c and 3d

	Lipofectamine (μl)	cDNA (μg)	P3000 reagent (μl)
A	2.5	1	1
B	3.75	1	1
C	2.5	1.5	1.5
D	3.75	1.5	1.5
E	3.75	2	2
F	2.5	1.5	3
G	3.75	1	2
H	3.75	1.5	3
I	2.5	2	4
J	3.75	2.5	5

Figure 4. Optimisations of WT A20 construct transfections. a.) *Western blot of WT A20 lysates using varying volumes of lipofectamine 3000 denoted by the figure annotations (in µl)* ***b.)*** *Densitometry analysis (of* ***a****.)* ***c. and d.)*** *Western blots of WT A20 lysates using various transfection protocols. (Annotations for described in* ***Table 2.)*** ***e.)*** *Densitometry analysis (of* ***c. and d.****) n=1*

3.4 Neither WT nor A20 polymorphisms impact the TLR4-mediated IL-8 response under the tested conditions. HEK-TLR4 transfected with four A20 variants or WT were stimulated with LPS or (+)-morphine and compared with empty vector (pcDNA3.1+) and un-transfected (NT) controls. Neither the WT nor A20 polymorphisms altered the IL-8 response following high dose stimulation with either LPS (100ng/ml) or (+)-morphine (100µM) at 2 or 4h relative to controls (Figure 5a and 5b). To explore the possibility that the selected LPS concentration saturating TLR4 activation, lower LPS concentrations (0.1-50ng/ml) were explored. Since no (+)-morphine response was observed, lower concentrations (0.1-10µM) were explored to test the possibility that the high dose was cytotoxic. (Figure 6a and 6b). Neither the A20 WT nor polymorphisms exerted any regulation over the induced IL-8 response at these doses relative to controls. In addition, no significance was observed between any (+)-morphine treatments and the corresponding vehicle treatment. Successful construct transfection was confirmed in both experiments, with A20 and GAPDH at the expected size. (Figure 5c, 5d, 6c, 6d)

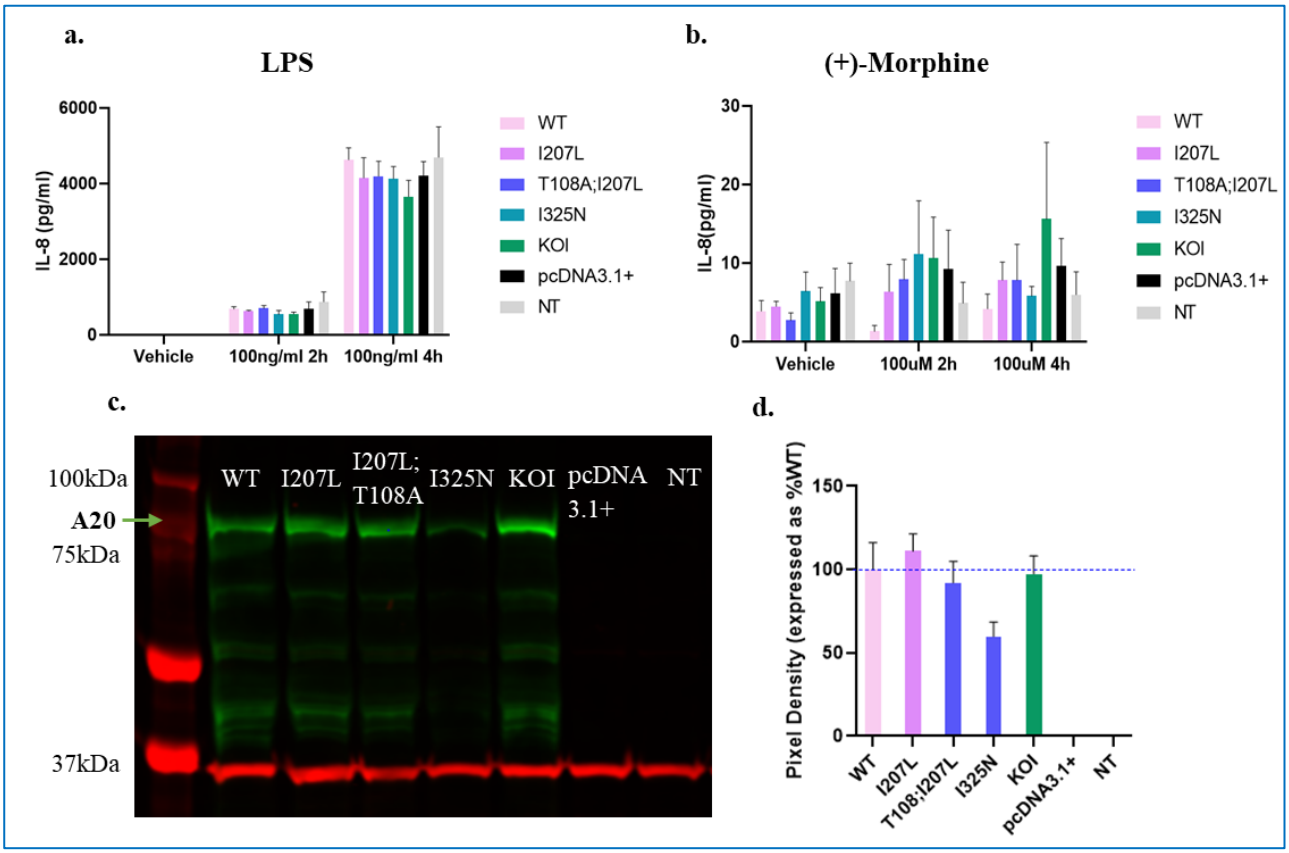

Figure 5. A20 polymorphisms exert no regulation of TLR4 signalling at high dose 4h stimulation. a.) *IL-8 production by transfected HEK-TLR4 following 2 or 4h stimulation with 100ng/ml LPS (n=3, represented as mean ±SEM) or* ***b.)*** *100uM (+)-morphine (n=4, represented as mean ±SEM)* ***c.)*** *Representative image of western blots (from n=4) (on lysates from parallel cells used for treatments in* ***a. and b.)*** ***d.)*** *Densitometry analysis of western blots (represented as mean % of WT ± % SEM.)*

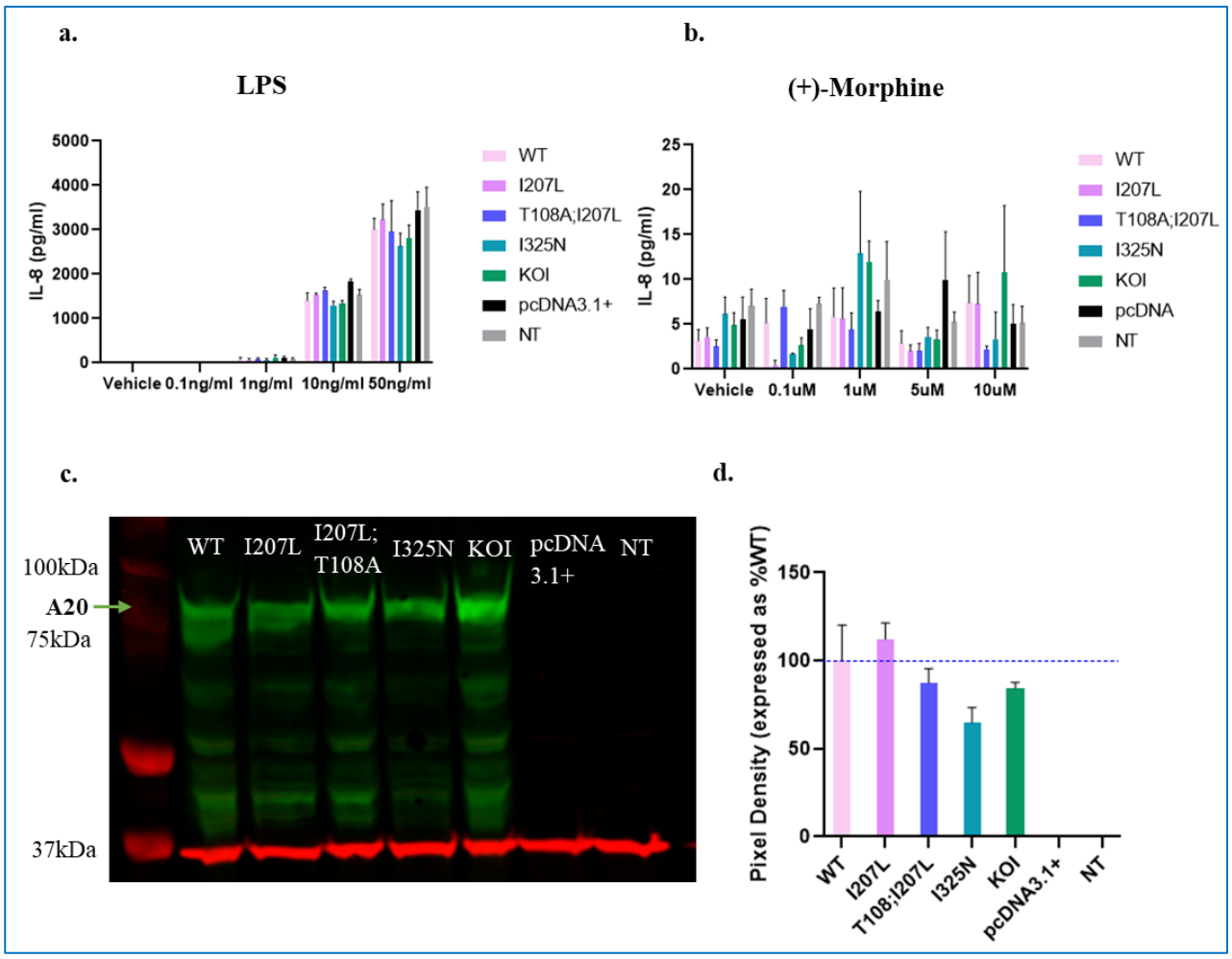

Figure 6. A20 polymorphisms exert no regulation of TLR4 signalling across a dose range. a.) *IL-8 produced from transfected HEK-TLR4 following 4h stimulation with a range of LPS doses (n=3, represented as mean ±SEM) or* ***b.)*** *range of (+)-morphine doses (n=3, (1uM n = 5), represented as mean ±SEM)* ***c.)*** *representative image of western blots (from n=4) (on lysates from parallel cells used for treatments in* ***a. and b.)*** ***d.)*** *Densitometry analysis of western blots (represented as mean % of WT ± % SEM).*

3.5 Further optimisation reveals WT A20 regulates TLR4 signalling. HEK-TLR4 cells transfected with varying quantities of cDNA (50ng, 0.1µg, 0.5µg and 1.5µg) were treated with 100ng/ml LPS for 4h. The results show cells transfected with 0.5µg and 1.5µg WT A20 respectively, produced statistically less IL-8 than the pcDNA3.1+ control (0.5µg, p=0.018. 1.5µg,

p=0.046) (Figure 7a), indicative of regulation. It is also seen that A20 expression in transfections with 50ng and 0.1µg respectively is relatively undetectable by western blot (Figure 7b, 7c).

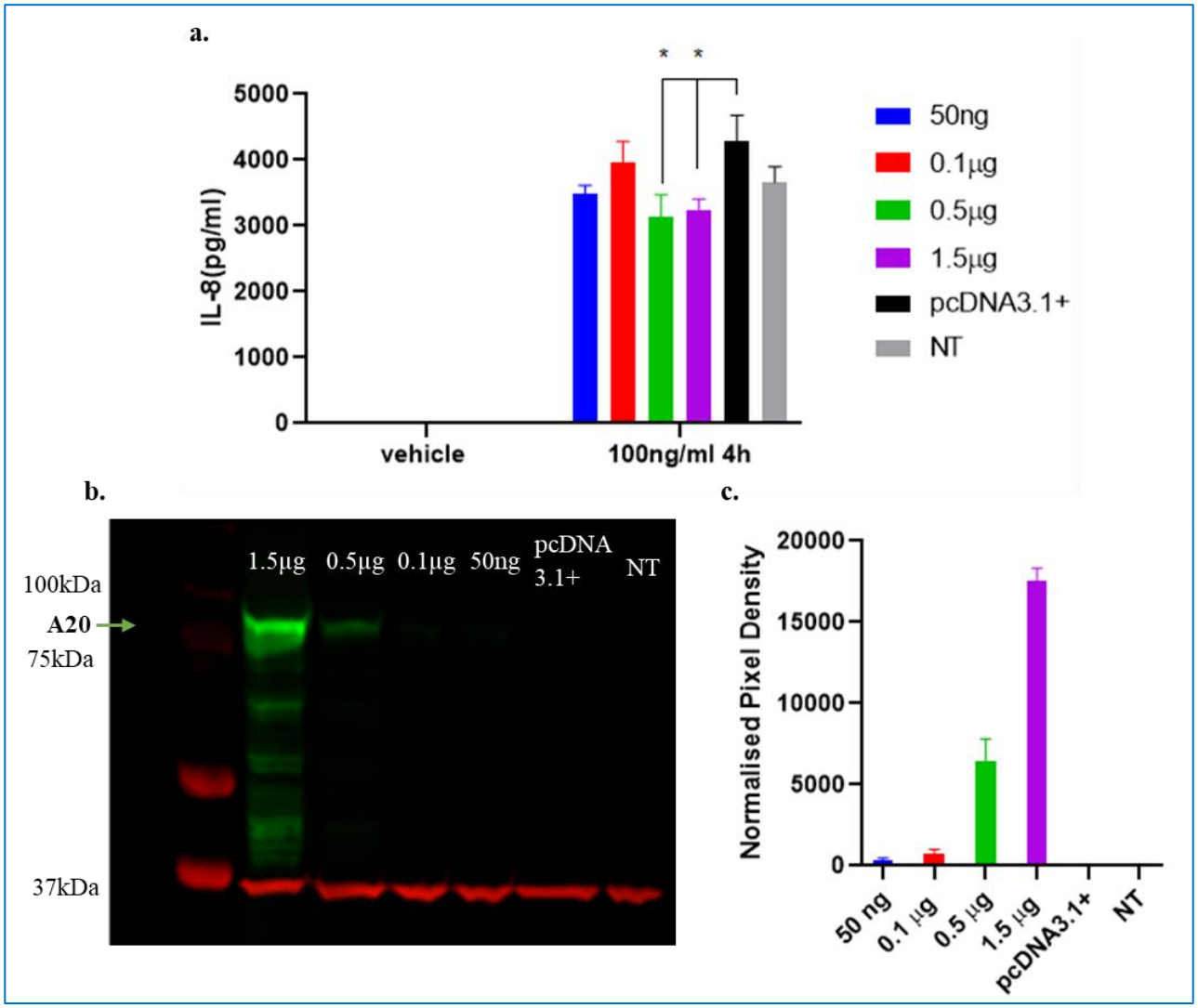

Figure 7. Transfections with 1.5µg and 0.5µg WT A20 cDNA reduced LPS induced TLR4 response. a.) *IL-8 response of HEK-TLR4 transfected with varying quantities of WT cDNA following 4h treatment with 100ng/ml LPS. Total volume of cDNA in all transfections was 1.5µg, in transfections with less, the difference was made up with pcDNA3.1+. * = $p < 0.05$ (n=3 represented as mean ±SEM.).* ***b.)*** *Representative western blot (from n=3).* ***c.)*** *Densitometry analysis of western blot images (n=3, represented as mean ±SEM).*

4. Discussion

This research aimed to explore the knowledge gap surrounding the impact of A20 polymorphisms on TLR4 signalling in the context of the negative side-effects of opioid treatment.

Using the Allan Human-Brain Atlas data I observed that TLR4 and A20 are co-expressed throughout much of the CNS (Figure 2). The approach conducted here was novel, with no literature to date exploring this relationship at a similar level of spatial resolution. In regions which segregate based on high TLR4 and A20 co-expression, it can be assumed that they are both functionally important. The importance of the segregated regions assumes that genes are not expressed needlessly and therefore there is the potential for a unique functional role for A20 and TLR4 in these regions. Since TLR4 is regulated by A20[37], it is likely TLR4 regulation by A20 is crucial in these regions. Furthermore, in the segregated regions where A20 expression is dominant, it may be assumed that these are regions which require tight inflammatory control. Collectively these are all regions where the A20 polymorphisms could have detrimental effects.

In addition, almost all regions of elevated co-expression are implicated in OIH and analgesic tolerance (highlighted in bold Figure 2 and spatially annotated Figure 8). Specifically, the ventral tegmental area, is the origin of dopaminergic signalling in the mesolimbic pathway[50], heavily implicated in opioid signalling, development of OIH and analgesic tolerance [51]. Similarly, the central grey of pons is a primary region of nociceptive processing and a key site of analgesic action by opioids, it too is heavily implicated in OIH and analgesic tolerance[52]. The trigeminal nerve is also involved in nociceptive signalling and implicated in OIH and analgesic tolerance[53, 54]. The globus pallidus (interna and externa) and the substantia nigra are key regions of the nigrostriatal pathway, one of the primary dopaminergic pathways, involved in the reward system associated with opioid addiction[55].

Collectively, these findings extend the existing literature suggesting A20 is an important inflammatory mediator in the CNS[39, 40] and the role of central immune signalling in these regions on OIH and analgesic tolerance[56, 57], by providing neuro-spatial implications for the regulation of TLR4 by A20 . This claim is strengthened as the regions identified are within broad anatomical structures

associated with neuroimmune signalling[58]. By identifying these regions, I have begun to fill the knowledge gap, suggesting that regulation of TLR4 signalling by A20 may be important in mediating OIH and analgesic tolerance, and by identifying regions of the CNS which may be susceptible to the A20 polymorphisms.

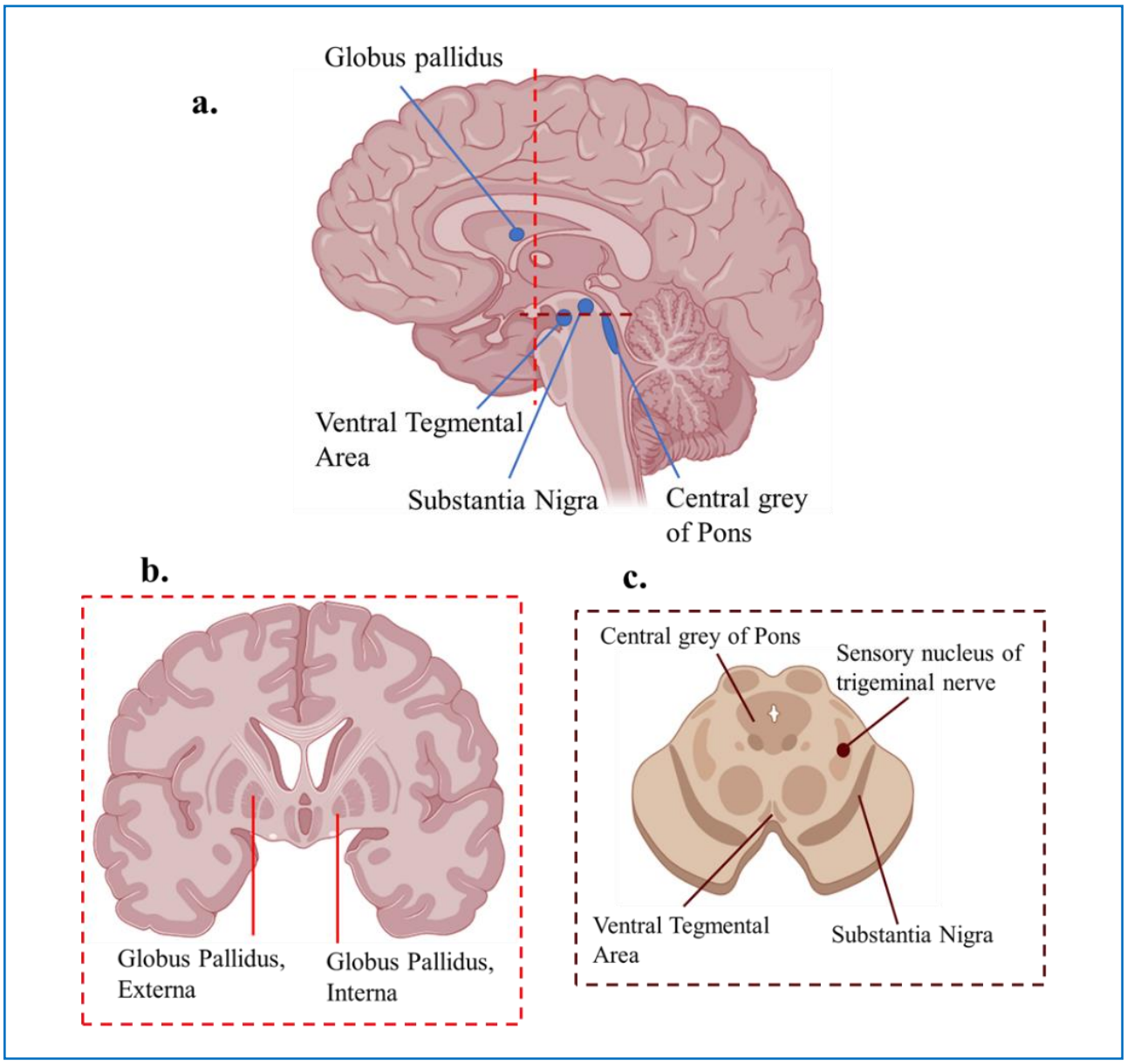

Figure 8. Spatial Annotation of identified key regions. a. *Mid-sagittal section* ***b.*** *Coronal section (denoted by red line in* ***a.****)* ***c.*** *Cross-section (denoted by dark red line in* ***a.****)*

Interestingly, neurodegeneration of the nigrostriatal pathway is heavily implicated in Parkinson's disease (PD), the results presented here support evidence implicating TLR4 and inflammation in the pathology[59, 60]. Therefore, this analysis also points to the potential impact of the A20 polymorphisms in PD.

While these findings are novel and physiologically relevant, limitations of the approach need to be considered. Expression of a single gene is a weakly powered approach to infer a biological role. Ideally further exploration would analyse gene-sets associated with A20 and TLR4 respectively, then identify enriched regions. Based on sample size, the Allan Human Brain Atlas (n=6) is appropriately powered based on previous studies[61, 62], but the conclusions would benefit from an increased sample size, for example, to address the underrepresentation of female samples (2:4). Finally, the use of DBSCAN here is novel and the thresholds were statistically guided, but despite being regarded as a powerful clustering model[63], it's possible some outliers could simply be anomalies in the dataset. Recognising these limitations, to test the hypothesis that the A20 polymorphisms would alter regulation of TLR4 signalling, the TLR4 signalling system was optimised.

Using this system I was able to demonstrate LPS-mediated TLR4 signalling by abolishing the IL-8 response with TLR4 antagonist LPS-RS, however, no response was observed from (+)-morphine (Figure 3), contradicting results observed in previous literature[31]. This discrepancy may be explained by different measures of TLR4 activation, where the published study measured NF-kB activation, compared with IL-8 production measured here. I continued with the selected cell line to explore if the A20 polymorphisms may have functional consequences on TLR4 regulation which reveal morphine activation of TLR4 signalling.

In testing the impact of A20 polymorphisms on the regulation of TLR4 induced IL-8 response, it was observed that neither WT A20 nor the polymorphic variants exerted regulation of IL-8 production under the conditions tested. Also (+)-morphine did not activate TLR4 signalling. (Figure 5a, 5b, 6a, 6b). Although the possibility that the polymorphisms have no impact on the regulation of TLR4 cannot be ruled out, the lack of observed TLR4 regulation by WT A20 opposes much of the

literature[37]. The expression system was optimised based on sub-maximal A20 expression[64] (Figure 4), however, these results suggest this may be inadequate (Figure 5, 6). Hence the physiological effects of the A20 polymorphisms may be undetectable in the current system. Although regulation by WT A20 was observed after re-optimisation (Figure 7), with the benefit of hindsight, more functional optimisation experiments should have been conducted initially. The results observed in Figure 7, contradict those observed in Figure 5. This observed variation is puzzling and ultimately further experimentation is required to unravel it.

One explanation may be the inherent variability within an *in vitro* system, such as, passage number, growth rate, density, and health. Although attempts to control for this were made by ensuring experimental consistency, subtle changes may have impacted transfection efficiency and signalling events. Subtle alterations in transfection efficiency may have been unobservable as western blot band intensity cannot differentiate between a small number of cells highly over expressing A20 or widespread low-level protein expression. Ideally, this could be overcome by the incorporation of a fluorescent marker in the plasmids, making it possible to view protein localisation and transfection efficiency real-time. This would also remove the need to re-plate cells following transfection, removing any variation in cell handing at this step.

In the functional assays it is possible the action of A20 is sensitive to intracellular levels of IKK-β. Phosphorylation of A20 by IKK-β is crucial for its activation[65] and a crucial aspect altered in the polymorphisms[41]. IKK-β may be limited in this *in vitro* system, perhaps meaning A20 is only functional under optimal physiological cellular conditions. Further experimentation should explore whether co-transfection with IKK-β is important and measure phosphorylated A20.

Finally, future experiments using direct measurement of NF-kB activation though the use of a reporter system may be more sensitive and specific. Similarly, measurement of mRNA expression from a range of LPS induced genes including IL-8 but also IL-6, TNFα and IL-1β would provide beneficial insight. Determination of the A20 polymorphic impacts on TLR4 signalling in OIH and analgesic

tolerance may benefit from using neurologically relevant cells or an animal pain model, where full TLR4 physiology is conserved.

In summary, the results observed here shed new light on aspects of the identified knowledge gaps. The novel transcriptomic analysis presented suggests there are specific regions of the CNS where regulation of TLR4 by A20 may occur, and therefore regions which may be critically susceptible to the A20 polymorphisms. Some of these regions are involved in OIH and analgesic tolerance suggesting a role for the regulation of TLR4 by A20 as hypothesised. Interestingly several of the regions identified are implicated in Parkinson's disease, providing support for the inflammatory hypothesis. Although it was not possible to definitively determine whether the A20 polymorphisms have a functional consequence on TLR4 signalling *in vitro*, the experiments presented here will inform future experimentation.

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