

The Sustained Phase of Tyrosine Hydroxylase Activation *in vivo*

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Abstract:

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthetic pathway for catecholamine synthesis. Stress triggers an increase in TH activity, resulting in increased release of catecholamines from both neurons and the adrenal medulla. In response to stress three phases of TH activation have been identified (acute, sustained and chronic) and each phase has a unique mechanism. The acute and chronic phases have been studied *in vivo* in a number of animal models, but to date the sustained phase has only been characterised *in vitro*. We aimed to investigate the effects of dual exposure to lipopolysaccharide (LPS) in neonatal rats on TH protein, TH phosphorylation at serine residues 19, 31 and 40 and TH activity in the adrenal gland over the sustained phase. Wistar rats were administered LPS (0.05 mg/kg, intraperitoneal injection) or an equivolume of non-pyrogenic saline on days 3 and 5 postpartum. Adrenal glands were collected at 4, 24 and 48 h after the drug exposure on day 5. Neonatal LPS treatment resulted in increases in TH phosphorylation of Ser40 at 4 and 24 h, TH phosphorylation of Ser31 at 24 h, TH activity at 4 and 24 h and TH protein at 48 h. We therefore have provided evidence for the first time that TH phosphorylation at Ser31 and Ser40 occurs for up to 24 h *in vivo* and leads to TH activation independent of TH protein synthesis, suggesting that the sustained phase of TH activation occurs *in vivo*.

Keywords: *LPS, tyrosine hydroxylase, adrenal gland, sustained phosphorylation*

Abbreviations:

2DG	2-deoxy-D-glucose
LPS	Lipopolysaccharide
PND	Postnatal day
Ser	Serine residue
TBST	Tris-buffered saline with Tween
TH	Tyrosine hydroxylase,

Introduction

The catecholamines, including dopamine, noradrenaline and adrenaline, have many functions and influence the activity of almost every tissue. They are especially important in the stress response where stored catecholamines are secreted from the central nervous system and sympathetic neurons as well as the adrenal medulla [1,2]. However, it has been shown that there is no significant change in catecholamine levels within these cells. This is because in parallel to catecholamine secretion there is a concomitant increase in catecholamine synthesis [3,4]. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthetic pathways for catecholamine synthesis [5]. TH is regulated acutely by TH phosphorylation at serine residues (Ser19, Ser31 and Ser40) and chronically by TH protein synthesis [6]. Both these mechanisms are well established *in vivo* [7-10]. Recently, we have demonstrated a third mechanism for the control of TH activity *in vitro*, referred to as the sustained phosphorylation of TH [11,12]. This mechanism has not been demonstrated to occur *in vivo*.

Stress triggers an increase in TH activity. In response to stress there are three phases of TH activation and each phase is mediated by distinct mechanisms. The acute phase of TH activation is mediated by TH phosphorylation primarily at Ser40, but also to a lesser extent at Ser31, without any changes in TH protein levels. This phase lasts up to approximately 1 h after which TH phosphorylation and TH activity generally return to baseline levels [6]. The sustained phase of TH activation is mediated by TH phosphorylation primarily at Ser40 which occurs from 1 to 24 h and again there are no changes in TH protein levels [12,11]. The sustained phase of TH activation is distinguishable from the acute phase of TH

activation as it only occurs in response to selected stimuli, the protein kinases mediating the two phases are different and it lasts for 24 h. The chronic phase of TH activation is mediated by an increase in TH mRNA expression between 1 to 24 h and subsequent TH protein synthesis which occurs from 6 to 72 h [6].

TH activation in response to stressors has been studied *in vivo* in many different animal models. Most of the studies have focused on changes in TH mRNA and TH protein levels [13-15,10]. We have recently discovered that different stressors lead to different patterns of TH phosphorylation at all three serine residues and TH protein levels [9,7,8,16]. However, to date we have not found any stress protocol *in vivo* that matches the previous findings *in vitro* using chromaffin cell cultures and which leads to increased TH phosphorylation and TH activity at 24 hours, but with no change in TH protein levels.

Here we investigated whether immunological challenge elicited by dual exposure to lipopolysaccharide (LPS) in neonatal rats causes the sustained phase of TH activation *in vivo*. This protocol of LPS challenge on days 3 and 5 postpartum has been previously employed to study immediate and long-term behavioural and physiological alterations, induced by neonatal immune activation [16-20]. To achieve our aims we investigated the effects of dual exposure to LPS on TH phosphorylation at Ser19, Ser31 and Ser40, TH activity and TH protein synthesis in the adrenal gland at 4, 24 and 48 h after the second LPS treatment.

Materials and Methods

Materials

LPS (*Salmonella enterica*, serotype enteritidis), EGTA, dithiothreitol (DTT), ammonium molybdate, sodium pyrophosphate, sodium vanadate, B-glycerolphosphate, microcystin, sodium chloride, Tris, Tween-20, bovine serum albumin, sodium azide, β -actin antibody, catalase, β -mercaptoethanol and activated charcoal were from Sigma Chemical Co. (St Louis, MO, USA). Protease inhibitor cocktail tablets were from Roche Diagnostics Australia (Castle Hill, NSW, Australia). Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) reagents were from Bio-Rad Laboratories (Hercules, CA, USA). PageRuler Prestained Protein Ladder was from Thermo Fisher Scientific (Rockford, IL, USA). Anti-rabbit immunoglobulin (horseradish peroxidase-linked whole antibody from donkey) and anti-mouse immunoglobulin (horseradish peroxidase-linked whole antibody from sheep) and 3,5- ^3H -L-tyrosine were from GE Health Care (Little Chalfont, UK). Total-TH antibody (tTH) and phospho-specific TH antibodies (pSer19, pSer31 and pSer40) were generated and were tested for specificity as described [21]. Anti-sheep antibody (horseradish peroxidase-linked whole antibody from rabbit) was from Pierce Biotechnology (Rockford, IL, USA). L-tyrosine was from DBH Biochemicals (Poole, UK). Tetrahydrobiopterin was supplied by Dr. Schirck's Laboratory (Jona, Switzerland). Optiphase HiSafe scintillation cocktail was from Perkin-Elmer (Waltham, MA, USA).

Animal Protocols

All animal protocols were approved by the University of Newcastle Animal Care and Ethics Committee and performed in accordance with the New South Wales Animal Research Act and the “Australian code of practice and use of animals for scientific purposes”. Wistar rats were mated at the University of Newcastle Psychology vivarium. Neonatal rats (n=30) were randomly allocated into either saline control or LPS conditions at birth (postnatal day [PND] 1). On PND 3 and PND 5, neonatal rats were removed from their home cages, weighed and administered intraperitoneally with either 0.05 mg/kg LPS or an equivolume of non-pyrogenic 0.9 % saline as describe previously [16]. Neonatal rats were euthanized at 4, 24 or 48 h (n=5 per group) following drug exposure on PND 5. Whole adrenal glands were dissected and were kept frozen at -80°C until further analysis. Adrenal samples were sonicated in 100 µL of homogenizing buffer (2 mM potassium phosphate buffer pH 7.4, 1 mM EGTA, 1 x protease inhibitor cocktail tablet, 1 mM DTT, 80 µM ammonium molybdate, 1 mM sodium pyrophosphate, 1 mM sodium vanadate, 5 mM β-glycerolphosphate, 2 µM microcystin, final concentration) with a microsonicator (UP50H, Hielscher Ultrasonics GmbH, Teltow, Germany) for 3 x 30 sec pulses at 4°C. Samples were centrifuged at 16000 RPM for 20 min at 4°C. The clear supernatants were collected and protein concentrations were determined by Pierce BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Samples were diluted with homogenizing buffer to equalize protein concentrations (5 mg/mL), were aliquoted and were kept frozen at -80°C for further analysis.

Western Blotting

Western blotting were performed as previously described with some modifications [7]. Aliquots (one-half volume) of these samples were mixed with sample buffer (1 % SDS, 10 % glycerol, 0.5 % DTT and minimal bromophenol blue, final concentration). 30 µg of each samples were subjected to SDS-PAGE gel electrophoresis and were transferred to nitrocellulose membranes (Hybond ECL, GE Health Care). Nitrocellulose membranes were stained with Ponceau S (0.5 % ponceau in 1 % acetic acid) to assess the efficacy of the transfer.

Membranes were washed in Tris-buffered saline with Tween (TBST) (150 mM sodium chloride, 10 mM Tris, 0.075 % Tween-20, pH 7.5) and incubated with blocking solution (5 % bovine serum albumin, 0.04 % sodium azide in TBST) for 2 h at 25°C. Membranes were washed in TBST and incubated with primary antibodies (total- and phospho-TH, β -actin) for 1 h at 25°C for adrenal medulla samples. Membranes were washed in TBST and incubated with horse-radish peroxidase-linked anti-IgG secondary specific antibodies for 1 h at 25°C.

Membranes were visualized on Fugifilm Las-3000 imaging system (Fuji, Stamford, CT, USA) using detection reagents (Amersham ECL Plus Western Blotting Detection Reagents, GE Health Care). The density of the bands was measured using a MultiGauge V3.0 (Fuji, Stamford, CT, USA). Total-TH protein levels were normalized to β -actin. Site-specific phospho-TH at pSer19, pSer 31 and pSer40 levels were normalized to total-TH protein levels and were expressed as a fold increase relative to the saline control.

TH Activity Assay

TH activity was measured using a method based on the tritiated water release assay with slight modification [22]. Briefly, aliquots of these samples were mixed in the reaction mixture (50 µg sample, 36 µg catalase, 2 mM potassium phosphate pH 7.4, 0.008 % β-mercaptoethanol, 24 µM L-tyrosine, 1 µCi 3,5-[3H]-L-tyrosine, final volume 50 µL). The 50 µL reactions were initiated with the addition of 100 µM tetrahydrobiopterin in 5 mM HCl. Control representing background reactions were added with 5 mM HCl but did not contain tetrahydrobiopterin. Assays were performed for 20 min at 30°C and were stopped by addition of 700 µl charcoal slurry (7.5 % activated charcoal in 1 M HCl). Mixtures were vortexed for 1 min and were centrifuged at 16000 RPM for 10 min at 30°C. 350 µl supernatants were added to 3 mL scintillation cocktail and were vortexed for 10 sec. Mixtures were assayed by scintillation spectrometry (Wallac1410, Pharmacia, Turko, Finland) for 20 min per sample. TH activity assays which were performed under these conditions were linear. The changes in TH activity were normalized to total-TH protein levels and expressed as a fold increase relative to the saline control.

Statistical Analysis

The data for saline and LPS groups were expressed as a fold increase of the mean ± SEM for each group relative to the mean of the saline group. These data were analysed by using Prism 5 for Windows (Version 5.04, GraphPad Software, Inc., CA, USA). Two-Way ANOVA was used to determine whether there were any

significant effects of LPS treatment and/or time across the groups. Additional Bonferroni post tests were used to analyse differences between saline and LPS groups at each of the time points (4, 24 and 48 h), where an overall LPS treatment or time effects was found. The significant differences shown on the graphs with asterisks (*) refer to the post hoc tests for LPS treatment effects. All differences were considered to be significant at $p < 0.05$.

Results

The adrenal glands were analysed by Western Blotting with antibodies that recognise total-TH (tTH) and site-specific phospho-TH. Total- and phospho-TH appeared as single bands corresponding to molecular masses of approximately 60 kDA (Fig. 1a). The results for total-TH levels were calculated relative to β -actin levels (Fig. 1b). The results for phospho-TH (pSer19, pSer31 and pSer40) levels were calculated relative to total-TH levels because the ratios more accurately represent phosphorylation states and account for variability in total-TH among samples (Fig 1c, d and e). The adrenal glands were also analysed for TH activity levels using a tritiated water release assay. The results for TH activity levels were calculated relative to total-TH levels.

As illustrated in Fig. 1b, there was a significant effect of LPS treatment on total-TH levels ($F_{(1,24)}=4.7, p<0.05$). Post hoc analysis further indicated that LPS caused a significant increase in total-TH levels relative to saline controls at 48 h (1.2 fold, $p<0.05$) (Fig. 1b). There was no effect of LPS treatment or time on pSer19 levels (Fig. 1c). However, a significant effect of LPS treatment ($F_{(1,24)}=5.9, p<0.05$) and time ($F_{(2,24)}=12.7, p<0.001$) was found on pSer31 levels (Fig. 1d). Post hoc analysis indicated that LPS caused a significant increase in pSer31 levels relative to saline controls at 24 h (1.5 fold, $p<0.001$) (Fig. 1d). There was a significant effect of LPS treatment ($F_{(1,24)}=50.8, p<0.001$) and time ($F_{(2,24)}=12.3, p<0.001$) on pSer40 levels (Fig. 1e). Post hoc analysis indicated that LPS caused a significant increase in pSer40 levels relative to saline controls at 4 h (1.5 fold, $p<0.001$) and 24 h (2.0 fold, $p<0.001$) (Fig. 1e). A significant effect of LPS treatment ($F_{(1,24)}=22.1, p<0.001$) and time ($F_{(2,24)}=9.3, p<0.01$) was also

found in regards to TH activity levels (Fig. 2). Post hoc analysis indicated that LPS caused a significant increase in TH activity levels relative to saline controls at 4 h (2.0 fold, $p < 0.01$) and 24 h (2.2 fold, $p < 0.001$) (Fig. 2).

Discussion

The major findings of this study of the adrenal gland *in vivo* were that dual exposure to LPS during neonatal life led to an increase in TH phosphorylation of Ser40 at 4 and 24 h, TH phosphorylation of Ser31 at 24 h, TH activity levels at 4 and 24 h and TH protein levels at 48 h. This is the first study to our knowledge to demonstrate *in vivo* sustained phase of TH activation, as indicated here by increased TH phosphorylation and TH activity at 24 hours, with no change in TH protein levels at this time point,

TH is regulated acutely by phosphorylation of its serine residues and chronically by protein synthesis [6]. In bovine adrenal chromaffin cell cultures, acute incubation (<1 h) with nicotine leads to TH phosphorylation at Ser19, Ser31 and Ser40 [23,24] and chronic incubation (48 h) with nicotine leads to TH protein synthesis [25]. Previous studies *in vivo* showed that TH phosphorylation changes are detectable as early as 10 min in response to a range of stressors in the acute phase of TH activation [8,7,9]. Whereas, TH protein changes are only detectable many hours after exposure to stressors in the chronic phase of TH activation [26-28,8] with the earliest detectable change being 6 h after a 2 h immobilization stress [29]. In this study, we particularly investigated the effects of dual exposure to LPS on TH phosphorylation at three serine residues, TH activity and TH protein synthesis in adrenal gland at 4, 24 and 48 h after the second exposure to LPS. Our data showed that dual exposure to LPS evokes an increase in TH protein in the adrenal gland only after 48 h. These findings suggested that dual exposure to LPS has a delayed effect on TH protein synthesis. However, this increase in TH protein did not lead to an increase in TH activity. Previous studies showed that

chronic cold stress evokes a significant increase in TH protein synthesis without inducing an increase in TH activity [30,31]. Therefore increases in TH protein do not always induce increases in TH activity. This may be because the newly synthesised TH protein binds to catecholamines and becomes inactivated before it is able to be phosphorylated at Ser40 which is required to keep it active. The increase in TH protein without an increase in activity may represent a regulatory mechanism to increase the capacity of the adrenal gland to respond to subsequent stressors.

Recently, we found sustained phosphorylation of TH at Ser40 to be a novel mechanism for maintenance of catecholamine synthesis *in vitro* [11,12]. In bovine adrenal chromaffin cell cultures, sustained incubation (1 - 24 h) with nicotine leads to TH phosphorylation at Ser19, Ser31 and Ser40. Phosphorylation of TH at Ser19 and Ser31 was substantially increased at 10 min to maximum levels and then there was a marked decrease in phosphorylation of TH at these sites between 10 min to 1 h. The level of phosphorylation of TH at Ser31 and Ser19 remained very low up to 24, but was still significantly above the unstimulated control levels. However, phosphorylation of TH at Ser40 was initially increased at 10 min, decreased somewhat from 1 to 8 h and then increased again from 8 to 24 h. Ser40 levels were very significantly above basal levels at all times and no significant differences were observed at 24 h from that observed at 10 min [11]. This increase in Ser40 phosphorylation occurred in response to nicotine, angiotensin, histamine and PACAP but did not occur in response to bradykinin and muscarine [12,11]. We found that the phosphorylation of TH at Ser40 in response to nicotine and PACAP occurred via unique mechanisms and both led to an increase in TH activity. Phosphorylation of TH at Ser19 does not directly influence TH activity, Ser31 increases TH activity

modestly and Ser40 plays a major role, by abolishing the feedback inhibition of TH caused by catecholamine binding, and increases TH activity both *in vitro* and *in vivo* [32,33,24].

Previous studies *in vivo* have investigated TH phosphorylation at 24 h but no evidence for the sustained phase of TH activation were found as TH phosphorylation at Ser40 was decreased and TH protein was not altered 24 h after exposure to a 1 h social defeat protocol [9] and TH phosphorylation at Ser40 was not altered and TH protein was increased 24 h after exposure to glucoprivation stress elicited by 2DG [8]. Here we found that TH phosphorylation occurs at Ser40 at 4 h leading to an increase in TH activity without any changes in phosphorylation of TH at Ser19 or Ser31. This suggests that there is a sustained phase of TH activation *in vivo* that extends beyond the acute changes that are generally over by 1 h after exposure to a stressor. In addition, we showed that dual exposure to LPS also evokes an increase in phosphorylation of TH at Ser31 and Ser40 at 24 h and these changes led to an increase in TH activity, without an increase in TH protein. The appearance of Ser31 phosphorylation at 24 h but not at 4 h suggests activation of different signalling pathways occurs later in the stress response and implies that there is a unique pattern of protein kinase activation at 24 h. Overall, we have provided evidence for the first time that TH phosphorylation occurs for up to 24 h and leads to TH activation independent of TH protein synthesis, suggesting that the sustained phase of TH activation occurs *in vivo*.

Acknowledgments

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Conflict of Interests

The authors declare no conflicts of interest.

References

1. Axelrod J, Reisine TD (1984) Stress hormones: their interaction and regulation. *Science* 224 (4648):452-459
2. Flatmark T (2000) Catecholamine biosynthesis and physiological regulation in neuroendocrine cells. *Acta Physiol Scand* 168 (1):1-17. doi:aps596 [pii]
3. Zigmond RE, Schwarzschild MA, Rittenhouse AR (1989) Acute regulation of tyrosine hydroxylase by nerve activity and by neurotransmitters via phosphorylation. *Annu Rev Neurosci* 12:415-461. doi:10.1146/annurev.ne.12.030189.002215
4. Wakade AR, Wakade TD, Malhotra RK (1988) Restoration of catecholamine content of previously depleted adrenal medulla in vitro: importance of synthesis in maintaining the catecholamine stores. *J Neurochem* 51 (3):820-829
5. Nagatsu T, Levitt M, Udenfriend S (1964) Tyrosine Hydroxylase. The Initial Step in Norepinephrine Biosynthesis. *J Biol Chem* 239:2910-2917
6. Kumer SC, Vrana KE (1996) Intricate regulation of tyrosine hydroxylase activity and gene expression. *J Neurochem* 67 (2):443-462
7. Ong LK, Guan L, Stutz B, Dickson PW, Dunkley PR, Bobrovskaya L (2011) The effects of footshock and immobilization stress on tyrosine hydroxylase phosphorylation in the rat locus coeruleus and adrenal gland. *Neuroscience* 192:20-27. doi:S0306-4522(11)00811-6 [pii] 10.1016/j.neuroscience.2011.06.087
8. Bobrovskaya L, Damanhuri HA, Ong LK, Schneider JJ, Dickson PW, Dunkley PR, Goodchild AK (2010) Signal transduction pathways and tyrosine hydroxylase regulation in the adrenal medulla following glucoprivation: an in vivo analysis. *Neurochem Int* 57 (2):162-167. doi:S0197-0186(10)00170-1 [pii] 10.1016/j.neuint.2010.05.009
9. Ong LK, Bobrovskaya L, Walker FR, Day TA, Dickson PW, Dunkley PR (2011) The effect of social defeat on tyrosine hydroxylase phosphorylation in the rat brain and adrenal gland. *Neurochem Res* 36 (1):27-33. doi:10.1007/s11064-010-0255-7
10. Sabban EL, Kvetnansky R (2001) Stress-triggered activation of gene expression in catecholaminergic systems: dynamics of transcriptional events. *Trends Neurosci* 24 (2):91-98. doi:S0166-2236(00)01687-8 [pii]
11. Bobrovskaya L, Gilligan C, Bolster EK, Flaherty JJ, Dickson PW, Dunkley PR (2007) Sustained phosphorylation of tyrosine hydroxylase at serine 40: a novel mechanism for maintenance of catecholamine synthesis. *J Neurochem* 100 (2):479-489. doi:JNC4213 [pii] 10.1111/j.1471-4159.2006.04213.x
12. Bobrovskaya L, Gelain DP, Gilligan C, Dickson PW, Dunkley PR (2007) PACAP stimulates the sustained phosphorylation of tyrosine hydroxylase at serine 40. *Cell Signal* 19 (6):1141-1149. doi:S0898-6568(06)00340-8 [pii] 10.1016/j.cellsig.2006.12.006

13. Kvetnansky R, Sabban EL, Palkovits M (2009) Catecholaminergic systems in stress: structural and molecular genetic approaches. *Physiol Rev* 89 (2):535-606. doi:89/2/535 [pii]
10.1152/physrev.00042.2006
14. Wong DL, Tank AW (2007) Stress-induced catecholaminergic function: transcriptional and post-transcriptional control. *Stress* 10 (2):121-130. doi:778862206 [pii]
10.1080/10253890701393529
15. Tank AW, Xu L, Chen X, Radcliffe P, Sterling CR (2008) Post-transcriptional regulation of tyrosine hydroxylase expression in adrenal medulla and brain. *Ann N Y Acad Sci* 1148:238-248. doi:NYAS1148054 [pii]
10.1196/annals.1410.054
16. Sominsky L, Walker AK, Ong LK, Tynan RJ, Walker FR, Hodgson DM (2012) Increased microglial activation in the rat brain following neonatal exposure to a bacterial mimetic. *Behav Brain Res* 226 (1):351-356. doi:S0166-4328(11)00645-0 [pii]
10.1016/j.bbr.2011.08.038
17. Walker AK, Hiles SA, Sominsky L, McLaughlin EA, Hodgson DM (2011) Neonatal lipopolysaccharide exposure impairs sexual development and reproductive success in the Wistar rat. *Brain Behav Immun* 25 (4):674-684. doi:S0889-1591(11)00009-2 [pii]
10.1016/j.bbi.2011.01.004
18. Walker AK, Nakamura T, Byrne RJ, Naicker S, Tynan RJ, Hunter M, Hodgson DM (2009) Neonatal lipopolysaccharide and adult stress exposure predisposes rats to anxiety-like behaviour and blunted corticosterone responses: implications for the double-hit hypothesis. *Psychoneuroendocrinology* 34 (10):1515-1525. doi:S0306-4530(09)00160-7 [pii]
10.1016/j.psyneuen.2009.05.010
19. Walker AK, Nakamura T, Hodgson DM (2010) Neonatal lipopolysaccharide exposure alters central cytokine responses to stress in adulthood in Wistar rats. *Stress* 13 (6):506-515. doi:10.3109/10253890.2010.489977
20. Shanks N, Larocque S, Meaney MJ (1995) Neonatal endotoxin exposure alters the development of the hypothalamic-pituitary-adrenal axis: early illness and later responsivity to stress. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 15 (1 Pt 1):376-384
21. Gordon SL, Bobrovskaya L, Dunkley PR, Dickson PW (2009) Differential regulation of human tyrosine hydroxylase isoforms 1 and 2 in situ: Isoform 2 is not phosphorylated at Ser35. *Biochim Biophys Acta* 1793 (12):1860-1867. doi:S0167-4889(09)00244-4 [pii]
10.1016/j.bbamcr.2009.10.001
22. Briggs GD, Gordon SL, Dickson PW (2011) Mutational analysis of catecholamine binding in tyrosine hydroxylase. *Biochemistry* 50 (9):1545-1555. doi:10.1021/bi101455b
23. Haycock JW (1993) Multiple signaling pathways in bovine chromaffin cells regulate tyrosine hydroxylase phosphorylation at Ser19, Ser31, and Ser40. *Neurochem Res* 18 (1):15-26
24. Dunkley PR, Bobrovskaya L, Graham ME, von Nagy-Felsobuki EI, Dickson PW (2004) Tyrosine hydroxylase phosphorylation: regulation and consequences. *J Neurochem* 91 (5):1025-1043. doi:JNC2797 [pii]

10.1111/j.1471-4159.2004.02797.x

25. Craviso GL, Hemelt VB, Waymire JC (1992) Nicotinic cholinergic regulation of tyrosine hydroxylase gene expression and catecholamine synthesis in isolated bovine adrenal chromaffin cells. *J Neurochem* 59 (6):2285-2296
 26. Watanabe Y, McKittrick CR, Blanchard DC, Blanchard RJ, McEwen BS, Sakai RR (1995) Effects of chronic social stress on tyrosine hydroxylase mRNA and protein levels. *Brain Res Mol Brain Res* 32 (1):176-180. doi:0169328X95000813 [pii]
 27. Rusnak M, Jelokova J, Vietor I, Sabban EL, Kvetnansky R (1998) Different effects of insulin and 2-deoxy-D-glucose administration on tyrosine hydroxylase gene expression in the locus coeruleus and the adrenal medulla in rats. *Brain Res Bull* 46 (5):447-452. doi:S0361-9230(98)00033-1 [pii]
 28. Tank AW, Meligeni J, Weiner N (1984) Cyclic AMP-dependent protein kinase is not involved in the in vivo activation of tyrosine hydroxylase in the adrenal gland after decapitation. *J Biol Chem* 259 (14):9269-9276
 29. Nankova B, Kvetnansky R, McMahan A, Viskupic E, Hiremagalur B, Frankle G, Fukuhara K, Kopin IJ, Sabban EL (1994) Induction of tyrosine hydroxylase gene expression by a nonneuronal nonpituitary-mediated mechanism in immobilization stress. *Proc Natl Acad Sci U S A* 91 (13):5937-5941
 30. Baruchin A, Weisberg EP, Miner LL, Ennis D, Nisenbaum LK, Naylor E, Stricker EM, Zigmond MJ, Kaplan BB (1990) Effects of cold exposure on rat adrenal tyrosine hydroxylase: an analysis of RNA, protein, enzyme activity, and cofactor levels. *J Neurochem* 54 (5):1769-1775
 31. Fluharty SJ, Snyder GL, Stricker EM, Zigmond MJ (1983) Short- and long-term changes in adrenal tyrosine hydroxylase activity during insulin-induced hypoglycemia and cold stress. *Brain Res* 267 (2):384-387. doi:0006-8993(83)90895-8 [pii]
 32. Jedynek JP, Ali SF, Haycock JW, Hope BT (2002) Acute administration of cocaine regulates the phosphorylation of serine-19, -31 and -40 in tyrosine hydroxylase. *J Neurochem* 82 (2):382-388
 33. Nunez C, Laorden ML, Milanes MV (2007) Regulation of serine (Ser)-31 and Ser40 tyrosine hydroxylase phosphorylation during morphine withdrawal in the hypothalamic paraventricular nucleus and nucleus tractus solitarius-A2 cell group: role of ERK1/2. *Endocrinology* 148 (12):5780-5793. doi:en.2007-0510 [pii]
- 10.1210/en.2007-0510

Figures

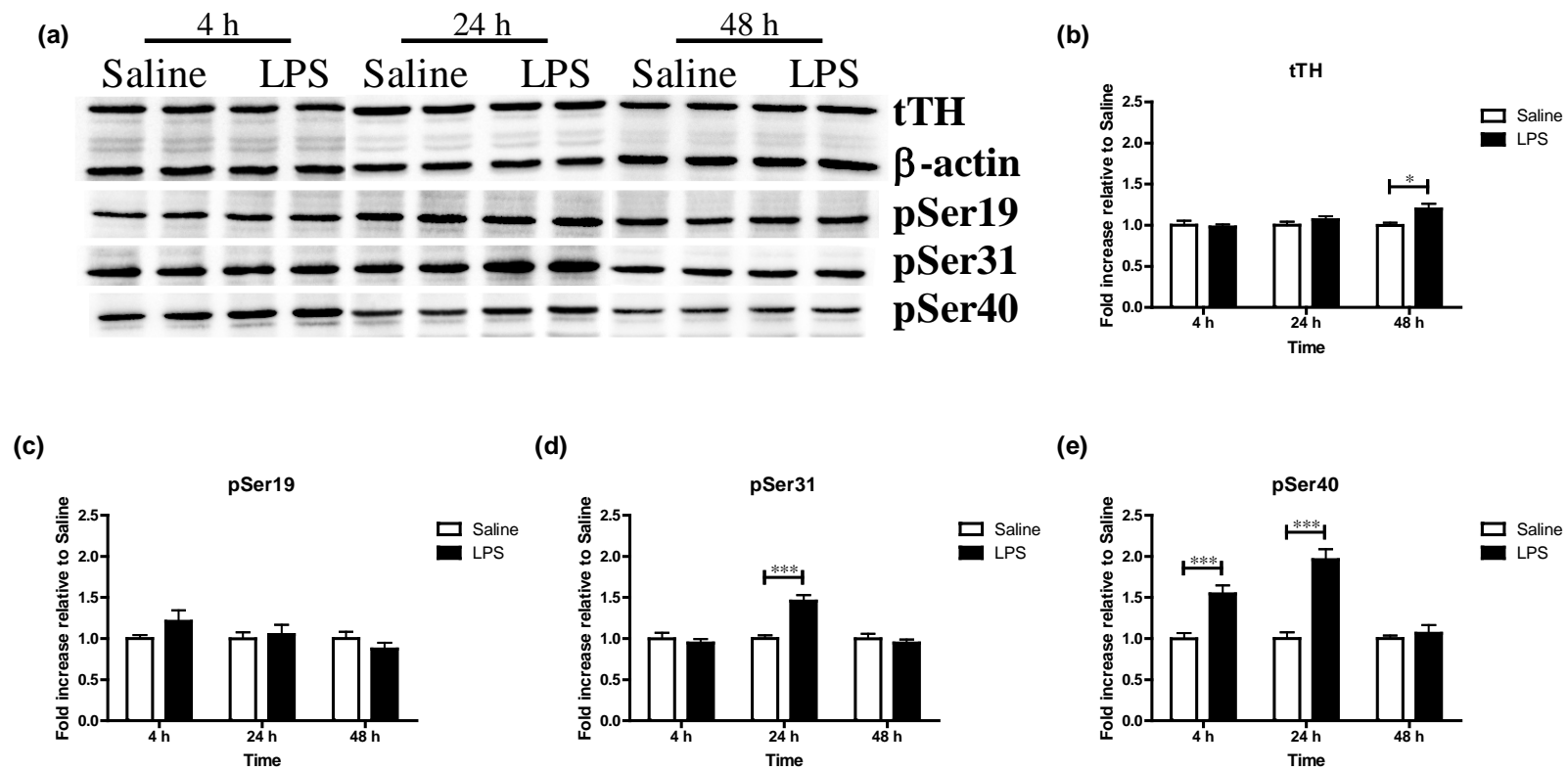


Fig. 1

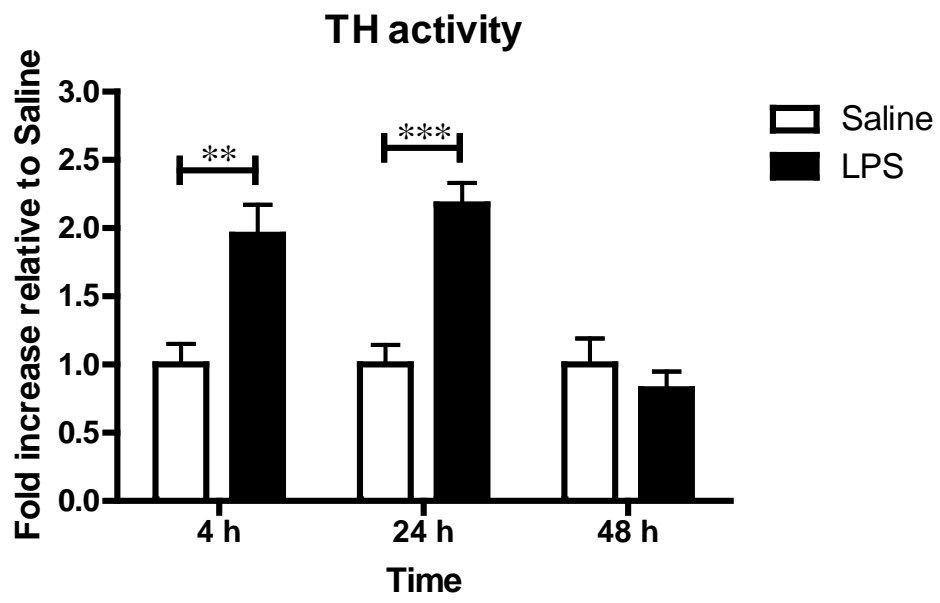


Fig. 2

Fig. 1: Effect on total- and phospho-TH (pSer19, pSer31 and pSer40) levels in adrenal glands 4, 24 and 48 h after LPS treatment (n=5 per group). (a) Representative immunoblots show the effect of LPS treatment on total- and phospho-TH. The loading controls were performed by analysis of β -actin. *p<0.05, **p<0.01, ***p<0.001

Fig. 2: Effect on TH activity levels 4, 24 and 48 h after LPS treatment (n=5 per group). **p<0.01, ***p<0.001