Elevated *P2x7r* and *P2x4r* transcripts levels in the Flinders Sensitive Line Rats, a genetic animal model of depression.

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Abstract

P2X7 and P2X4 receptors (P2X7R and P2X4R, respectively) are ligand-gated ion channels activated by adenosine triphosphate (ATP), which have been associated to dysfunctional processes in stress responses linked to depression, such as neurotransmitter release, cognition, sleep, energy, appetite, immune and endocrine dysfunction. Clinical studies indicate that polymorphisms in the *P2x7r* gene results in increased susceptibility for development of depression. Existing studies have investigated the role of P2X7R and P2X4R in animal models based on stress exposure. Therefore, the present work aimed to investigate the transcript and protein levels of these receptors in a genetic animal model of depression, the Flinders Sensitive Line (FSL) and its control group, the Flinders Resistant Line (FRL) rats. We found that FSL rats have increased transcript levels of P2X7R and P2X4R in frontal cortex (FC), ventral and dorsal hippocampus (vHip and dHip, respectively) compared to FRL rats. There were no alterations in the protein levels in the FC and dHip, but the P2X7R was lower in FSL than in FRL rats in the vHip. The results suggest that increased transcripts levels of *P2x7r* and *P2x4r* in the FSL rats may contribute to the stress-susceptibility observed in these animals.

Key words: P2X7 receptors, P2X4 receptors, FSL/FRL rats.

1 Introduction

Stress exposure is a key factor for development of depression (1) and both are associated with dysfunctions in common neuronal transmitters, circuitries and receptor systems (2). The P2X7 and P2X4 receptors (P2X7R and P2X4R) are ligand-gated ion channels activated by adenosine triphosphate (ATP) which have been indicated to modulate such process (3). Interestingly, existing studies support the importance of P2X7R in depression and stress response. Polymorphisms in the *P2x7r* are associated with increased susceptibility for development of depression (4, 5) and linked to symptom-severity (6). Genetic deletion of P2X7R results in a transgenic mouse with less depressant phenotype, i.e. decreased immobility time in the forced swim test (FST) and tail suspension test (TST) (7). These animals also showed attenuated anhedonia response in the sucrose preference test after bacterial endotoxin challenge (8).

Independently, our research group and Csolle et al. described for the first time that the treatment with P2XR antagonists induce antidepressant-like effect in the TST (9) and in the FST (10). Subesquent studies demonstrate that repeated administration of a P2X7R antagonist attenuated the elevated immobility time in the TST and FST induced by treatment with lipopolysaccharide (11), reversed anhedonia caused by chronic unpredictable stress (12), and helplessness behavior induced by inescapable foot shocks (13).

Similarly, the P2X4R also appears to be involved in response to stress. Acute treatment with a positive allosteric modulator of P2X4R, ivermectin, increased depressive-like behaviour in animals exposed to the FST and TST in low doses (14), but in higher doses induced antidepressant-like effect in the TST (14).

As most studies involving P2X7R and P2X4R link these receptors to stress response, the present study was conducted using the Flinders Sensitive Line (FSL) and the Flinders Resistant Line (FRL) rats, a genetic animal model of depression (15-17). Therefore, the work aimed to investigate the transcript and protein levels of P2X7R and P2X4R in frontal cortex (FC), ventral and dorsal hippocampus (vHip and dHip, respectively), which are key structures in stress and depression neurobiology, of FSL/FRL rats.

2 Materials and Methods

2.1 Animals

Male FSL/FRL rats (10 weeks old) obtained from the breeding colonies at the Translational Neuropsychiatry Unit, Aarhus University, Denmark, were kept in the vivarium, housed in pairs in Euro Standard Type III-H cages, under standard conditions: 22 ± 1°C, lights on from 6:00 a.m. to 6:00 p.m., free access to food and water.

2.2 Experimental design

FSL and FRL (control group) rats were decapitated without prior anesthesia, the brain was removed, FC, vHip and dHip were dissected, frozen on powdered dry-ice and maintained at -80°C until analysis. Transcripts levels of P2X7R and P2X4R were investigated by real-time quantitative polymerase chain reaction (qPCR) while protein expression was analysed by Western blotting (WB).

2.3 RNA and protein isolation

RNA and protein were isolated from the samples of frontal cortex, ventral and dorsal hippocampus with PARIS™ kit (Ambion, TX, USA), according to the manufacturer's specifications and previous studies (18, 19).

2.3.1 Real-time qPCR

Transcript levels were determined in samples of frontal cortex, ventral and dorsal hippocampus from FSL/FRL rats with real-time qPCR, as described before (18, 19).

The RNA concentration and the purity were determined by a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific). Before cDNA synthesis, the RNA concentration of the samples was adjusted to match the sample with the lowest concentration (111.08 ng/µL). RNA was reversely transcribed using random primers and Superscript IV Reverse Transcriptase (Invitrogen,CA) following manufacturer's instructions. The cDNA samples were stored undiluted (61.09 ng/µL) at -80 °C until real-time qPCR analysis.

The samples were diluted with DEPC water prior to real-time qPCR analysis (1:40). The real-time qPCR reactions were carried out in 96-well PCR-plates using an Mx3005P (Stratagene, USA) and SYBR Green.

The expression of eight different reference genes (*18sRNA, ActB, CycA, Gapd, Hmbs, Hprt1, Rpl13A, Ywhaz*) and 2 target genes (*P2x7r* and *P2x4r*) were investigated. The reference genes were selected as described in (20). Essential gene data about primer sequence and amplicon sizes are given in Table 1. The primers were obtained from Sigma-Aldrich, Denmark.

Each SYBR Green reaction (10 µl total volume) contained 1x SYBR Green master mix (Sigma-Aldrich, Denmark), 0.5 µM primer pairs, and 3 µl of diluted cDNA and were carried out as described previously (21). The mixture was heated initially to 95°C for 3 min in to activate hot-start iTaq DNA polymerase and then 40 cycles with denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s were applied. To verify that only one PCR product was detected the samples were subjected to a heat dissociation protocol; after the final cycle of the PCR, the reactions were heat-denatured by increasing the temperature from 60°C to 95°C. The samples and the standard curve were run in duplicate. A standard curve was generated on each plate.

Initially, the mRNA levels were determined for the 8 reference genes. Stability comparison of the expression of the reference genes was then conducted with the Normfinder software. Values of the target genes were subsequently normalized with the geometric mean of the two optimal reference genes (frontal cortex: *Hprt* and *CycA*; ventral hippocampus: *Rpl13A* and *CycA*; dorsal hippocampus: *Hmbs* and *CycA*), based on the NormFinder mathematical algorithm (22).

Table 1. Characteristics of gene-specific real-time qPCR primers (Elfving 2017).

Gene Symbol	Gene Name	Accession No.[1]	Primer Sequence	Amplicon size[2]
Reference genes				
18s rRNA	18s subunit ribosomal RNA	M11188	(+) acggaccagagcgaaagcat (-) tgtcaatcctgtccgtgtcc	310
ActB	Beta-actin	NM_031144	(+) tgtcaccaactgggacgata (-) ggggtgttgaaggtctcaaa	165
CycA	Cyclophilin A	XM_345810	(+) agcactggggagaaaggatt (-) agccactcagtcttggcagt	248
Gapd	Glyceraldehyde-3-phosphate dehydrogenase	NM_017008	(+) tcaccaccatggagaaggc (-) gctaagcagttggtggtgca	168
Hmbs	Hydroxy-methylbilane synthase	NM_013168	(+) tcctggctttaccattggag (-) tgaattccaggtgagggaac	176
Hprt1	Hypoxanthine guanine phosphoribosyl transferase 1	NM_012583	(+) gcagactttgctttccttgg (-) cgagaggtccttttcaccag	81
Rpl13A	Ribosomal protein L13A	NM_173340	(+) acaagaaaaagcggatggtg (-) ttccggtaatggatctttgc	167
Ywhaz	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	BC094305	(+) ttgagcagaagacggaaggt (-) gaagcattggggatcaagaa	136
Target genes				
P2x4r	purinergic receptor P2X4	NM_031594	(+) ctcatccgcagccgtaaagt (-) aacacccacccgatgacgta	80
P2x7r	purinergic receptor P2X7	NM_019256	(+) accgtcttttcctacgttagcttt (-) caaagggagggtgtagtcgg	186

[1] Genbank accession number of cDNA and corresponding gene, available at http://www.ncbi.nlm.nih.gov/.
[2] Amplicon length in base pairs.

2.3.2 Western blotting

The concentration (µg/µL) of total proteins was determined in each sample using the Pierce BCA Protein Kit (Thermo Scientific) according to the manufacturer instructions. Tissue samples (prepared using the PARIS purification kit) were mixed with SDS sample buffer (125mM Tris-HCl, pH 6.8; 20% glycerol; 4% SDS; 0.02% bromophenol blue; and 125mM dithiothreitol) (ratio 2:1) and 23 µg of total protein were separated on polyacrylamide gels (Criterion TGX Precast Gel, 26 well comb, 15 µL, 1.0 mm; Bio Rad, #567-1035) and transferred to nitrocellulose membranes (Midi format, 0.2 µm nitrocellulose, single application; Bio Rad, #1704159) using the Trans-Blot Turbo system (Bio Rad, USA; 7 minutes, 25V).

The membranes were washed in TBS (50 mM Tris HCl pH 7.6; 150 mM NaCl) for 5 minutes and incubated with Odyssey Blocking Buffer (OBB; LI-COR Bioscience, #927-5000) for 1 hour at room temperature (RT). Following, they were incubated with primary antibodies to one of the target proteins (rabbit anti-P2X7, 1:200, Alomone Labs, #APR-004; rabbit anti-P2X4, 1:200, Alomone Labs, #APR-002) and to the normalizing protein (mouse anti-β-actin, 1:3000, Licor, #926-42212) overnight at 4°C.

On the following day, the membranes were washed 3 times with 0.1% TBST and incubated with secondary antibody (goat anti-rabbit, 1:10000, Licor, IRDye 800CW; or goat anti-mouse, 1:10000, Licor, IRDye 680RD) diluted in OBB in 0.1%

TBST (0.1 % Tween-20 in TBS) (1:2) with 0.01% SDS (to decrease nonspecific background) for 1 hour at RT and protected from light. Still protected from light, the membranes were washed with 0.1% TBST, (3 times, 5 minutes each) and TBS (3 times, 5 minutes each). Infrared signals were detected using the Odyssey CLx infrared imaging system, and bands were quantified using Image Studio software (LI-COR Biosciences). Values from the target proteins were normalized to the β-actin value in each sample.

2.4 Data analysis and statistical methods

In the real-time qPCR and WB analysis, respective mRNA levels and normalized fluorescence values were expressed as percentage of control group (FRL rats) and compared by *Student's t* test. The critical value considered to indicate significant difference between groups was 5% ($p<0.05$).

3 Results and discussion

As illustrated in Figure 1, FSL rats display increased mRNA levels (A: $t_{12}=2.465$, $p<0.0297$, n=7) of *P2x7r* in FC, but no changes were observed at protein levels (B: $t_{14}=1.422$, p=0, n=8) (Panel 1). Similarly, FSL rats present increased mRNA levels (A: $t_{12}=6.156$, $p<0.0001$, n=7) of *P2x4r* in FC, with no alterations at protein levels (B: $t_{14}=0.7788$, p=0.4491, n=8) (Panel 2).

FSL rats also show increased mRNA levels (A: $t_{13}=4.248$, p=0.0010, n=7-8) of *P2x7r* in vHip accompanied by a decrease in protein levels (B: $t_{13}=2.694$, p=0.0184, n=7-8) (Panel 3). Panel 4 shows that FSL rats display increased mRNA levels (A: $t_{13}=2.690$, p=0.0186, n=7-8) of *P2x4r* in vHip with no changes on protein levels (B: $t_{12}=1.124$, p=0.2830, n=6-7).

FSL rats showed increased mRNA levels of *P2x7r* in dHip (A: $t_{14}=9.633$, $p<0.0001$, n=8) with no alterations in protein levels (B: $t_{14}=1.135$, p=0.2756, n=8) (Panel 5). And lastly, FSL rats increased mRNA levels of *P2x4*r in dHip of FSL rats (A: $t_{14}=5.700$, $p<0.0001$, n=8), with no changes in protein levels (B: $t_{14}=0.09557$, p=0.9252, n=8), as showed in Panel 6.

The main finding in the present study is that FSL rats present increased transcript levels for *P2x7r* and *P2x4r* in FC, ventral and dHip when compared to FRL rats. Despite that, no alterations were observed on protein expression in the

analysed structures, except for P2X7R in vHip, which is lower in FSL than in FRL rats.

The activation of the P2X7R (23-31) and P2X4R (32, 33) in the central nervous system is associated to stress response and they can be prevented or reverted by the antidepressant treatment (34-38). However, since no changes were identified at protein levels, the increased mRNA levels can provide an additional level of regulation to stimulate the P2X7R/P2X4R signalling during stress exposure. In line with this suggestion, the mRNA transcripts levels have extremely low predictive value for the expression of their associated proteins, especially in brain samples (39).

Therefore, the functionally dormant transcriptional state of *P2x7r* and *P2x4r* mRNA on FC and Hip of FSL rats could be proposed to be responsible for the increased stress-susceptibility of these animals and the stress exposure could stimulate a rapid synthesis of P2X receptors in FSL rats. Increased levels of these receptors were not observed in the present study, most likely because the animals were not pre-exposed to stress. However, further studies are warranted to further confirm this proposal.

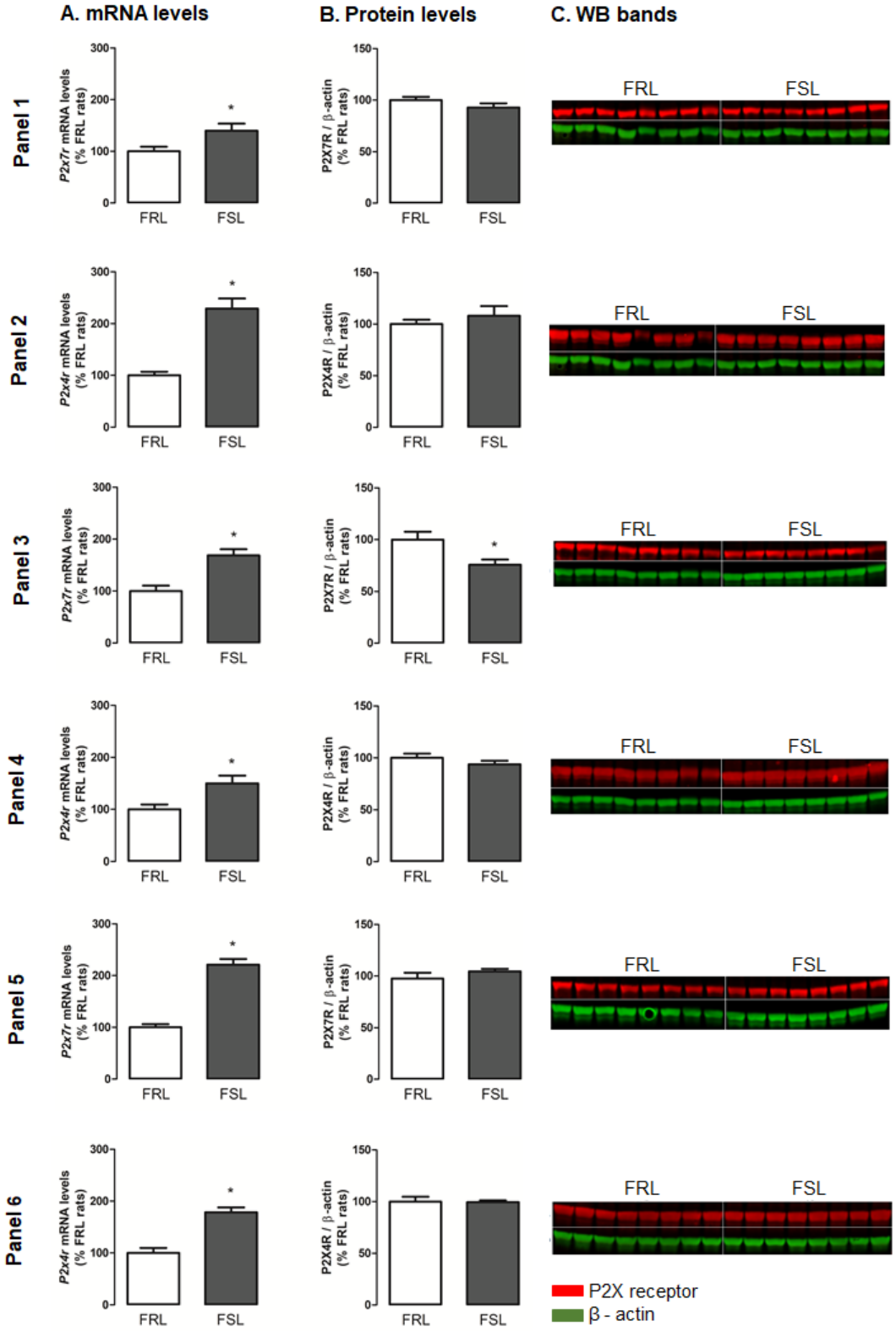

Figure 1. mRNA levels (column A), protein expression (column B) and WB bands (column C) of P2X7R and P2X4R in frontal cortex (Panels 1 and 2), ventral (Panels 3 and 4) and dorsal (Panels 5 and 6) hippocampus of FSL/FRL rats. FSL and FRL rats were decapitated after 1 hour of room habituation and had frontal cortex, ventral and dorsal hippocampus dissected for real-time qPCR and WB analysis. Bars represents mRNA levels of *P2x7r/P2x4r* expressed as percentage of control group (FRL rats) (A) or optical density of these receptors normalized by β-actin also expressed as percentage of FRL rats (B); values are mean ± SEM; asterisks indicate significant differences from FRL rats (*p<0.05), n=7-8 animals/group. WB bands were obtained with Image Studio Lite software (version 5.2) are represented in (C).

4 List of abbreviations

ATP: adenosine triphosphate

dHip: dorsal hippocampus

FC: frontal cortex

FRL: Flinders Resistant Line

FSL: Flinders Sensitive Line

FST: forced swim test

OBB: Odyssey Blocking Buffer

P2X4R: P2X4 receptors

P2X7R: P2X7 receptors

qPCR: quantitative polymerase chain reaction

RT: room temperature

TBS: tris buffered saline

TBST: Tween-20 in TBS

TST: tail suspension test

vHip: ventral hippocampus

WB: Western blotting

5 Declarations

5.1 Ethics approval and consent to participate

All the procedures were approved by the Danish Animal Experiments Inspectorate (Protocol Number 2012-15-2934-00254). Consent to participate is not applicable to this study since it was conducted in animals.

5.2 Consent for publication

Not applicable.

5.3 Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

5.4 Competing interests

Gregers Wegener declares having received lecture/consultancy fees from H. Lundbeck A/S, Servier SA, Astra Zeneca AB, Eli Lilly A/S, Sun Pharma Pty Ltd, and Pfizer Inc., Shire A/S, HB Pharma A/S, Arla Foods A.m.b.A., Alkermes Inc, and Mundipharma International Ltd., and research funding from the Danish Medical Research Council, Aarhus University Research Foundation (AU-IDEAS initiative (eMOOD)), the Novo Nordisk Foundation, the Lundbeck Foundation, and EU Horizon 2020 (ExEDE). All other authors declare that they have no conflict of interest.

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5.6 Authors' contributions

Deidiane Elisa Ribeiro developed this study as part of her PhD study, she performed the WB and wrote the first version of the manuscript. Heidi Kaastrup Müller helped with the WB analysis. Betina Elfving carried out the real time qPCR experiments. Samia R. L. Joca and Gregers Wegener supervised the study. Gregers Wegener also provided financial and structural support for the development of this project in Aarhus University.

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