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Selection of reference genes for gene expression studies in rat oligodendrocytes using quantitative real time PCR

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Abstract

Quantitative real time polymerase chain reaction (qPCR) has become a widely used tool to examine gene expression levels. Reliable quantification, however, depends on a proper normalization strategy. Normalization with multiple reference genes is becoming the standard, although the most suitable reference genes depend on the applied treatment as well as the tissue or cell type studied. In this study the stability of various reference genes was investigated in cultures of oligodendrocytes derived from either mature or neonatal rats, the latter also in the presence of the liver X receptor (LXR) agonist.

The expression stability of ten commonly used reference genes (HPRT, GAPDH, 18S, ActB, CycA, Tbp, Rpl13A, YWHAZ, HMBS, Pgk1) was analyzed using geNorm and NormFinder. When comparing the different types of cell cultures, Rpl13A, CycA, Pgk1 and YWHAZ were identified as most stable genes. After LXR agonist treatment, CycA, Pgk1 and Rpl13A were found to be the most stable by both geNorm and NormFinder. HMBS and the commonly used housekeeping genes GAPDH and 18S turned out to be the most variable according to geNorm and NormFinder. In conclusion, the use of multiple reference genes, instead of only one, in qPCR experiments with rat oligodendrocytes is strongly advised and standard housekeeping genes such as GAPDH and 18S are not recommended as they appear to be relatively unstable under the experimental conditions used. Reference gene selection should always be performed for each individual experiment, since useful reference genes are very specific for every situation.

Keywords glial cells, internal control gene, normalization, geNorm, NormFinder

Introduction

Oligodendrocytes (OLGs) are the myelin-forming glial cells of the central nervous system (CNS). The myelin sheath is a fatty insulating layer that surrounds axons of neurons. Over 25% of the lipid content in myelin consists of cholesterol. Mice that lack the ability to produce cholesterol in their OLGs show impaired myelination (Saher et al., 2005). Another indication of the importance of the cholesterol metabolism in myelinating OLG was found in liver X receptor (LXR) knockout mice. LXRs are oxysterol activated nuclear receptors, which play an important part in the control of cellular and whole-body cholesterol homeostasis (Peet et al., 1998; Repa and Mangelsdorf, 2000). Absence of LXRs in mice leads to disorganized myelin sheaths, which indicates a role for the LXRs in OLG function (Wang et al., 2002). Although OLGs are important cholesterol producing cells in the CNS, most of the research on the CNS cholesterol homeostasis has focused on neurons and astrocytes. We investigate the role of OLGs in CNS cholesterol trafficking by studying the effect of LXR agonist (Schultz et al., 2000) treatment on expression levels of cholesterol related genes.

For measuring molecular responses, qPCR is the most sensitive and reliable method to detect and quantify specific mRNA expression levels. Proper use of this method requires normalization to account for the differences in amount of starting material, variability in RNA quality, variable PCR or cDNA synthesis efficiencies and differences between tissues and cell types in overall transcriptional activity (Vandesompele et al., 2002). The most frequently applied normalization method is the use of internal reference genes. Until recently, "housekeeping genes" (HKGs) were commonly used as reference genes. HKGs encode proteins that provide basic, essential functions that all cells need to survive. They are supposed to have stable expression levels in different cell types and tissues, across developmental stages and various conditions. Several studies, however, have demonstrated

that expression levels of traditional HKGs can vary under different experimental conditions (Deindl et al., 2002; Dheda et al., 2005; Radonic et al., 2004). In addition, normalization to a single control gene can lead to erroneous normalization. The use of at least three reference genes for the correct normalization of qPCR data has been proposed (Vandesompele et al., 2002).

The search for suitable reference genes was complicated by the fact that for the evaluation of the expression stability of a candidate reference gene another one is needed for its normalization. Several algorithms have been developed to deal with this problem. Vandesompele et al. introduced the use of the geometric mean of multiple reference genes in a normalization program named geNorm (Vandesompele et al., 2002). This application defines the gene stability as the average pairwise variation of a particular gene with all other control genes and ranks the genes according to their expression stability. An alternative program, NormFinder (Andersen et al., 2004), ranks the candidate reference genes based on the combined estimates of both intra- and intergroup variations.

Previous studies that investigated the effect of LXR agonist treatment on gene expression levels mostly made use of one of the traditional HKGs *i.e.* GAPDH, 18S or CycA (Abildayeva et al., 2006; Karten et al., 2006; Liang et al., 2004). Recently, suitable reference genes were described for rat brain tissue and hippocampal neurons under different experimental conditions (Bonefeld et al., 2008; Langnaese et al., 2008; Santos and Duarte, 2008). However, to our knowledge no study has yet been performed on glial cells. In the current study 10 candidate reference genes were selected and evaluated according to their expression stability in different rat glial cell cultures and in neonatal OLGs after LXR agonist treatment.

Materials and Methods

1) Cell culture

Primary mature rat OLGs were isolated from whole brains of adult Wistar rats (Harlan, Horst, The Netherlands) as described by Yong and Antel (1992), with some minor modifications (Vanderlocht et al., 2006). Briefly, brain tissue was subjected to enzymatic dissociation with trypsin (0.25%, Gibco) and DNase I (100 µg/ml, Roche diagnostics, Vilvoorde, Belgium) and mechanical dissociation by passage through a 132 µm mesh. Glial cells where separated from myelin debris and red blood cells by centrifugation on a 30% Percoll gradient. The mixed glial cell fraction was suspended in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Paisley, UK) supplemented with 5% FCS, penicillin (50 U/ml) and streptomycin (50 µg/ml). The cultures where selectively enriched for OLGs by means of differential adhesion to plastic. The non-adherent OLG fraction was plated at a density of $1.5x10^6$ cells/well on poly-L-lysine coated 24-well plates for qPCR. Cells were cultured for 11 days in DMEM supplemented with 5% FCS, and subsequently for 72h in SATO medium (Buttery and ffrench-Constant, 1999) with 0.5% FCS. Four isolations of each six rats were tested.

Primary neonatal rat OLGs were isolated from newborn Wistar rats (postnatal day 1). Forebrains were collected, mechanically dissociated and the homogenate was cultured for 10-12 days on Poly-L-lysine coated (5µg/ml) cell culture flasks (Thermo Fisher Scientific/Nunc, Waltham, MA, USA) in DMEM (Gibco BRL) containing 10% FCS, L-glutamine and penicillin/streptomycin (Gibco BRL). After a pre-shake to remove microglia (180 rpm, 1h), OLG progenitor cells, grown on top of a layer of astrocytes, were collected by shake-off (240 rpm, 20-24 h) as described (McCarthy and de Vellis, 1980) and cultivated for 2 days on poly-L-lysine coated cell culture dishes in a defined SATO medium in presence of PDGF and FGF (Peprotech, Rocky Hill, NJ). OLG differentiation was induced by removing the growth factors

and cultivating the cells for 72h in SATO medium containing 0.5% FCS. Primary astrocytes were collected after OLG progenitor cells were shaken off, and used for further experiments. Primary neonatal rat OLGs were treated with either vehicle alone (ethanol) or 1 μM LXR agonist T0901317 (Cayman Chemical, Tallinn, Estonia) for 72 hours. For the first experiment four preparations with an average of 10 rat pups per preparation were investigated, for the second experiment five preparations were used.

All animals were maintained in accordance with the Belgian and European animal welfare guidelines. The study was approved by the ethical committee for animal experiments of Hasselt University. The animals were housed under temperature controlled conditions, a 12h light/dark cycle, and free access to food and water.

2) RNA isolation and reverse transcription

RNA was extracted from the cells using the RNeasy plus mini kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. Genomic DNA was eliminated by a DNase-on-column treatment supplied with the kit. The RNA concentration was determined spectrophotometrically at 260 nm using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher scientific, Waltham, USA) and RNA purity was checked by means of the absorbance ratio at 260/280 nm. RNA integrity was assessed by electrophoresis on 2% agarose gels as indicated in the MIQE guidelines (Bustin et al., 2009). There was only a significant difference in RNA concentration between the mature oligodendrocytes and astrocytes but there were no significant differences between the other groups. Equal amounts of RNA input (1 μg) were used in the subsequent cDNA synthesis reaction, which was performed using the Reverse Transcription System (Promega, Leiden, The Netherlands). Total RNA was incubated at 70°C for 10 min to prevent secondary structures. The RNA was placed on ice and supplemented

with $MgCl_2$ (25mM), RTase buffer (10X), dNTP mixture (10mM), oligo d(t) primers, RNase inhibitor (20 U) and AMV reverse transcriptase (20 U/µl). A tenfold dilution of the cDNA was made using nuclease-free water and stored at -20°C.

3) Quantitative real time PCR

qPCR was performed in an optical 96-well plate with an ABI PRISM 7500 fast sequence detection system (Applied Biosystems, Carlsbad, California) and universal cycling conditions (10 min 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C). Each 10 µl reaction contained 5 µl SYBR Green Master Mix (Applied Biosystems), 0.3 µl gene-specific forward and reverse primers (10 µM), 2.5 µl pre-diluted cDNA and 1.9 µl nuclease-free water. No template controls contained nuclease-free water instead. Primer efficiency was determined by a standard curve of cDNA samples according to the MIQE guidelines for qPCR (Bustin et al., 2009). Amplification was followed by a melting curve analysis to check PCR product specificity. PCR products were also loaded on 2% agarose gels to confirm specificity of amplification and the absence of primer dimer formation. Data were analyzed using the ΔΔCt method (Livak and Schmittgen, 2001). Expression levels were normalized relative to the sample with the highest expression before geNorm (Vandesompele et al., 2002) v3.5 or NormFinder (Andersen et al., 2004) input.

Results

Ten candidate reference genes were chosen from literature as well as from the geNorm website (HPRT, GAPDH, 18S, CycA, ActB, YWHAZ, Tbp, Rpl13A, HMBS, Pgk1). Full gene name, primer sequences, and amplicon length are shown in Table 1.

In the first part of our study, the stability of these candidate reference genes was tested in primary rat astrocytes and two primary rat OLG cultures. The OLGs were derived from either one day old or mature Wistar rats. The OLG cultures isolated from the adult rats are mature myelinating OLGs, which are differentiated further than the OLG cultures obtained from one day old rats. The latter are situated in the range of immature to non-myelinating mature OLGs (data not shown). geNorm was used to rank the tested genes according to their average expression stability measure (M value) from the most stable (lowest M value) to the least stable (highest M value). All studied genes except HMBS, have a high expression stability with M values below the default limit 1.5 (Vandesompele et al., 2002). geNorm identified CycA and Rpl13A as the most stable combination of reference genes, followed by Pgk1 (Fig 1A, Table 2). Next, the pairwise variation value, $V_{n/n+1}$, was analyzed. $V_{n/n+1}$ measures the stability/variation effect of including an additional reference gene on the normalization factor (that is calculated as the geometric mean of the selected reference genes), starting with genes with the *n* lowest M-value. A small decrease in variation means that the added gene has no significant additional effect, and should not necessarily be included for calculation of a reliable normalization factor. The $V_{n/n+1}$ graph (Fig 1B) shows that three reference genes are sufficient for normalization, since the inclusion of an additional reference gene increases the pairwise variation value. When normalising the genes of interest (cholesterol related genes) with an increasing number (3 to 8) of reference genes, no differences in expression levels were detected (data not shown).

The same dataset was evaluated with the NormFinder software. This algorithm ranks the set of candidate genes according to their expression stability value in a given sample set and a given experimental design. YWHAZ, Pgk1 and Tbp were defined as the three most stable genes (Table 2). This ranking is not identical to the one determined by geNorm. However, both geNorm and NormFinder identified GAPDH, 18S and HMBS as most unstable genes. The NormFinder algorithm available in the GenEx Software also allows for the determination of the optimal number of control genes to be used in the normalization process, through the calculation of the Accumulated Standard Deviation. (Acc.SD). The Acc.SD of the ten reference genes is shown in figure 3A. The optimal number of reference genes is indicated by the lowest value for the Acc.SD. The lowest value, 0.3202, was found when five reference genes were used. However, the improvement of using five reference genes (Acc.SD= 0.3202) instead of only three (Acc.SD= 0.3284) is in this case very small. Therefore, the use of three genes is sufficient for good normalization.

In the second part of this study, the primary neonatal rat OLG culture was treated with the LXR agonist or with the vehicle (ethanol) alone. We opted for this culture since the cholesterol related genes had a higher expression level in the neonatal OLG culture compared to the mature OLG culture (data not shown). Here, geNorm identified CycA, Pgk1 and Rpl13A as the three most stable genes (Fig 2A, Table 3). These are the same genes as in the first part of this study but with a different ranking order. For the LXR agonist treatment of neonatal OLGs, the use of three reference genes for normalization is also sufficient, as the inclusion of an additional reference gene increases the $V_{n/n+1}$ value (Fig 2B) and normalization with more reference genes (3-6) gives the same levels of expression of the target genes (data not shown).

For this data set, NormFinder identified the same genes as geNorm as most stable genes (Pgk1, CycA, Rpl13A) (Table 3). The Acc.SD reached its lowest value (0.1919), with three reference genes (Fig 3B).

To test the effect of different normalization factors on the expression levels of the gene of interest, one of the cholesterol related genes (Apolipoprotein E, ApoE) was normalised to the three most stable genes suggested by geNorm and Normfinder (CycA, Pgk1, Rpl13A). This result was compared with the result obtained after normalization with two of the commonly used "HKGs", GAPDH and 18S, which were identified as the most unstable genes by both algorithms. When the expression levels of the gene of interest after LXR agonist treatment were normalized with CycA, Pgk1 and Rpl13A, a significant upregulation of the gene compared to untreated cultures was found (Fig 4A). This effect disappears when GAPDH and 18S were used for normalization of ApoE mRNA expression levels (Fig 4B). A strong increase in standard error was detected in the control group after normalization with GAPDH and 18S compared to the other normalization factor (Fig 4A and B).

Discussion

Over the past several years, qPCR has become the method of choice for sensitive and accurate quantification of gene expression levels. Reliable quantification requires appropriate normalization, to correct for variation introduced by RNA quality and enzymatic efficiencies in PCR and reverse transcription. The most commonly used procedure to perform normalization is to relate the measured mRNA concentration for the gene of interest to the mRNA of a reference gene, that is expected to exhibit stable expression levels across various developmental and experimental conditions. Since no single gene is universally suitable for all purposes, these reference genes must be validated for each experimental setting to avoid misleading results (Dheda et al., 2004; Schmittgen and Zakrajsek, 2000; Vandesompele et al., 2002).

The current study is, to our knowledge, the first detailed description of the stability of rat reference genes in different glial cell types and after LXR agonist treatment in primary OLGs. The expression stability of ten commonly accepted candidate reference genes was analyzed. Two public programs, geNorm and NormFinder, were used for the evaluation of gene stability.

In a first study, the transcriptional stability was checked in neonatal and mature derived OLG cultures, and in a primary rat astrocyte culture. geNorm identified Rpl13A, CycA and Pgk1 as the most stable genes. The NormFinder output was not identical to the order of genes proposed by geNorm. This is not unexpected since both programs rely on different mathematical approaches (Andersen et al., 2004; Vandesompele et al., 2002). The pairwise comparison approach, geNorm, tends to select those genes with the highest degree of similarity of expression profiles across the sample set. While NormFinder ranks the candidates with minimal estimated intra- and intergroup variation, and therefore selects the

genes taking into account the different experimental conditions used. The top five genes in both algorithms, however, are comparable. What is remarkable is that both geNorm and NormFinder rank HMBS as well as the traditional reference genes GAPDH and 18S as most unstable genes. Several other studies also described that GAPDH as well as other traditional reference genes are not suitable as internal reference genes (Dheda et al., 2004; Dheda et al., 2005). Therefore, we strongly advise to check the expression stability of these genes before using them for normalization purposes. Beside stability values, geNorm also calculates the pairwise variation value which determines the number of reference genes that should be used for normalization. Our data demonstrate that the use of the three most stable genes is sufficient for accurate normalization, since adding a fourth gene increases the pairwise variation value. The Acc.SD calculated by NormFinder indicated the use of five reference genes. However, since there are only minor differences between five (Acc.SD= 0.3202) and three (Acc.SD= 0.3284) genes, three genes will be sufficient for normalization. When comparing the pairwise variation value and the Acc. SD., both geNorm and NormFinder show that the use of three reference genes is sufficient for good normalization and that addition of the most unstable gene, HMBS, increases the pairwise variation value and the Acc. SD.

In a second part of the study, the gene expression stability was examined in primary neonatal rat OLGs after LXR agonist treatment. The ranking of geNorm and NormFinder is identical. The most stable genes, CycA, Pgk1, Rpl13A, were also identified as most stable ones in untreated OLGs. In previous studies investigating total rat brain tissue under different experimental conditions, CycA, Pgk1 and Rpl13A were also identified as relatively stable genes (Bonefeld et al., 2008; Langnaese et al., 2008). These data suggest that the proposed genes are probably the most suitable reference genes for expression studies on rat brain or specific brain cells, although a careful screen for reliable reference genes is indispensable for each experimental design. For investigation of LXR agonist treatment in OLGs the use of

three reference genes appears to be sufficient for appropriate normalization, since adding a fourth gene increases the pairwise variation value. The Acc. SD., identified by NormFinder, is lowest with 3 reference genes. Thus, both geNorm and NormFinder suggest using 3 reference genes for normalization. The Acc. SD. also increases when more than 6 genes are used, which is comparable to an increasing pairwise variation value when using 7 genes or more.

Different target gene expression results were obtained when performing normalization with the three best genes of geNorm and NormFinder (CycA, Pgk1, Rpl13A) compared to normalization with the commonly used HKGs GAPDH and 18S which were defined as the most unstable genes. Similarly, when comparing normalization using ActB, with a normalization factor generated by geNorm, Bonefeld et al. demonstrated that in rat brain tissue the different approaches yield different qPCR results (Bonefeld et al., 2008). These findings again emphasize the importance of evaluating all genes used for normalization, even if they are traditionally used in other studies.

In conclusion, we evaluated ten candidate reference genes for normalization of gene expression levels in astrocytes and mature and neonatal OLGs and after LXR agonist treatment. Data suggest the use of a combination of CycA, Pgk1, Rpl13A or YWHAZ for normalization in the different cell cultures. During treatment of neonatal OLGs with the LXR agonist CycA, Pgk1 and Rpl13A can be used. Normalization with HMBS or the “traditional” housekeeping genes GAPDH and 18S should be avoided in both experimental setups. Although these results could be an indication which reference genes could be used in glial cells, reference genes should always be evaluated for each individual experiment, since useful reference genes are very specific for every situation.

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Tables

Table 1 Reference genes selected for evaluation of expression stability

Symbol	name	forward and reverse primer	Amplicon size	Primer efficiency	Cq value (average±SD)	Reference*
HPRT	Hypoxanthine phosphoribosyl-transferase	CTCATGGACTGATTATGGACAGGAC GCAGGTCAGCAAAGAACTTATAGCC	123	93	23.23 ± 1.52	(Peinnequin et al., 2004)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	ACC ACA GTC CAT GCC ATC AC TCC ACC ACC CTG TTG CTG TA	452	100	24.22 ± 3.36	*
18S	18S subunit ribosomal RNA	ACG GAC CAG AGC GAA AGC AT TGT CAA TCC TGT CCG TGT CC	310	107	21.05 ± 2.86	Bonefeld et al., 2008
CycA	Cyclophilin A	TAT CTG CAC TGC CAA GAC TGA GTG CTT CTT GCT GGT CTT GCC ATT CC	126	93	17.15 ± 1.32	Langnaese et al., 2008
ActB	Beta actin	TGT CAC CAA CTG GGA CGA TA GGG GTG TTG AAG GTC TCA AA	165	107	17.53 ± 2.12	Bonefeld et al., 2008
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	GAT GAA GCC ATT GCT GAA CTT G GTC TCC TTG GGT ATC CGA TGT C	117	100	20.32 ± 2.03	Langnaese et al., 2008
Tbp	TATA box binding protein	TGG GAT TGT ACC ACA GCT CCA CTC ATG ATG ACT GCA GCA AAC C	131	93	26.33 ± 2.62	Langnaese et al., 2008
Rpl13A	Ribosomal protein L13A	GGA TCC CTC CAC CCT ATG ACA CTG GTA CTT CCA CCC GAC CTC	132	94	17.76 ± 1.63	Langnaese et al., 2008
HMBS	Hydroxymethyl-bilane synthase	TCC TGG CTT TAC CAT TGG AG TGA ATT CCA GGT GAG GGA AC	176	99	24.62 ± 2.49	Bonefeld et al., 2008
Pgk1	Phosphoglycerate kinase 1	ATG CAA AGA CTG GCC AAG CTA C AGC CAC AGC CTC AGC ATA TTT C	104	97	21.07 ± 1.58	Langnaese et al., 2008
ApoE	Apolipoprotein E	CCT GAA CCG CTT CTG GGA TT GCT CTT CCT GGA CCT GGT CA	65	107	16.96 ± 1.68	(Vanmierlo et al., 2009)

* Primers were designed by our laboratory using Oligo6 software.

Table 2 Candidate reference genes for normalization in different glial cell cultures ranked according to their expression stability by geNorm and NormFinder

geNorm			NormFinder		
Ranking order	Gene name	Average M value	Ranking order	Gene name	Stability value
1	Rpl13A	0.356	1	YWHAZ	0.34
1	CycA	0.356	2	Pgk1	0.376
3	Pgk1	0.506	3	Tbp	0.445
4	YWHAZ	0.733	4	ActB	0.507
5	Tbp	0.921	5	CycA	0.539
6	HPRT	1.059	6	Rpl13A	0.559
7	ActB	1.168	7	HPRT	0.627
8	GAPDH	1.252	8	GAPDH	0.7
9	18S	1.356	9	18S	0.864
10	HMBS	1.652	10	HMBS	0.919

Table 3 Candidate reference genes for normalization in primary neonatal rat OLG during LXR agonist treatment ranked according to their expression stability by geNorm and NormFinder

geNorm			NormFinder		
Ranking order	Gene name	Average M value	Ranking order	Gene name	Stability value
1	Pgk1	0.535	1	Pgk1	0.294
1	CycA	0.535	2	CycA	0.338
3	Rpl13A	0.579	3	Rpl13A	0.341
4	HPRT	0.689	4	HPRT	0.419
5	YWHAZ	0.898	5	YWHAZ	0.556
6	ActB	0.974	6	ActB	0.676
7	Tbp	1.346	7	Tbp	0.741
8	18S	1.705	8	18S	0.946
9	GAPDH	2.045	9	GAPDH	1.265

Figure legends

Figure 1 Evaluation of reference genes using geNorm in different glial cell cultures.

(A) Average expression stability measure (M) of 10 reference genes, during stepwise exclusion of the least stable control gene. Lower M value of average expression stability indicates more stable expression. (B) Determination of the optimal number of control genes for normalization on the basis of a pair-wise variation ($V_{n/n+1}$) analysis. Every bar represents the change in normalization accuracy when stepwise adding more endogenous controls according to ranking in fig 1A.

Figure 2 Evaluation of reference genes using geNorm in primary neonatal rat OLGs during LXR agonist treatment.

(A) Average expression stability measure (M) of 10 reference genes, during stepwise exclusion of the least stable control gene. Lower M value of average expression stability indicates more stable expression. (B) Determination of the optimal number of control genes for normalization on the basis of a pair-wise variation ($V_{n/n+1}$) analysis. Every bar represents change in normalization accuracy when stepwise adding more endogenous controls according to ranking in fig 2A.

Figure 3 Determination of the optimal number of reference genes for normalization based on the calculation of the Acc. SD. (A) in different glial cell cultures and (B) during LXR agonist treatment. The lowest value for the Acc. SD. in panel A was found when 5 reference genes were used, in panel B when 3 reference genes were used. Data analysis was performed by NormFinder.

Figure 4 qPCR data vary depending on choice of reference genes.

The cholesterol related gene was normalized with the geometric mean (GM) of the three best genes of geNorm and NormFinder (CycA, Pgk1, Rpl13A) (A) and the GM of the most unstable genes GAPDH and 18S according to both programs (B). Results are presented as mean ± SEM of five independent preparations (n=5). Statistics: Kruskall Wallis test (post test: Dunns, compares all pairs of columns). $P \leq 0.05$ is considered statistically significant.