日本財団補助金による
1997 年度日中医学協力事業助成報告書
－在留中国人研究者研究助成－

98 年 3 月 24 日

財団法人　日中医学協会

理事長　　中島章殿

Ⅰ．研究者氏名　李　航

研究機関　京都府立医科大学薬理学教室　研究指導者　矢部　千尋　職名　教授

所在地　〒602 京都市上京区河原町通広小路上ル梶井町465　電話　075-251-5332　内線　5333

Ⅱ．過去の研究歴

1992.4－1996.3　名古屋大学医学研究科 大学院　卒業

1996.4－1997.3　国立小児病院.小児医療研究センター　HS研究員

Ⅲ．過去の研究実績

1).Hang Li, et al. "Intranuclear localization of a multifunctional protein,collagenase inhibitor in proliferating human fibroblasts in culture" Acta Anat Nippon. Vol.69, No.4, 508.1994.

2).Hang Li, et al. "Cell cycle-dependent localization of tissue inhibitor of metalloproteinases-1 immunoreactivity in cultured human gingival fibroblasts" Nagoya Journal of Medical Science. Vol.58, No.3, 1995.

3) Hang Li "Depletion of Gelatinolytic Activity in Dental Pulp in Response to the Administration of Lead in Mice" Aichi-Gakuin Dent Sci. No.9, 1-4.1996.

Ⅳ．本年度の研究業績

(1) 学会、研究会等においての口頭発表（学会名・内容）

第71回日本薬理学会年会

Characterization of the genomic regions regulating expression of mouse aldose reductase gene.

第41回日本糖尿病学会年会

マウスアルドース還元酵素の遺伝子構造と遺伝子発現調節領域の解析（予定）

(2) 学会誌等に発表した論文　　無・(有)　（雑誌名・論文名）

THE JAPANESE JOURNAL OF PHARMACOLOGY Vol.76 supplement 46P 1998

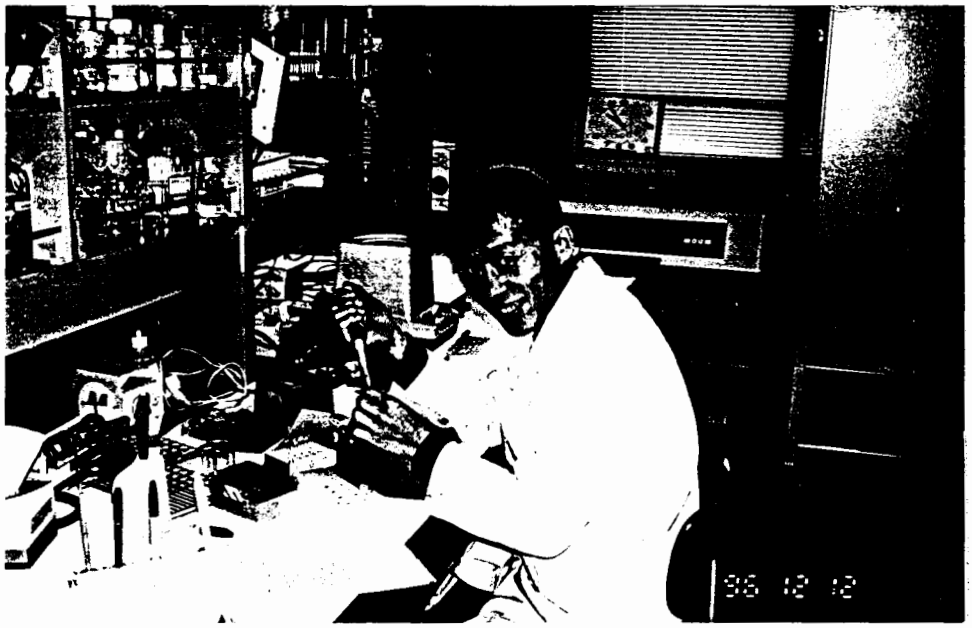
Characterization of the genomic regions regulating expression of mouse aldose reductase gene.

Ⅴ．今後の研究計画及び希望

なるべく早くこの研究テーマ(マウスアルドース還元酵素の遺伝子構造と遺伝子発現調節領域の解析)を完成する。

Ⅵ. 研　究　報　告（日本語、又は英語で書いて下さい。 4,000字以上で記載して下さい。別紙可）

別紙



Ⅶ. 指導教官の意見

李博士は貴財団の助成のもとに15 Kb にわたるマウス Aldose Reductase 遺伝子の構造と遺伝子上流領域の塩基配列を決定し、さらに最新の分子生物学的・細胞生物的手法を用いて本酵素の発現調節領域の解析に着手することが出来ました。

彼は次々と新しい実験技術を習得し、きわめて勤勉に実験を進め、今年度の教室の研究の発展に大きく貢献してくれました。この１年間、貴財団からの助成を受けて李博士が研究に専念できたことを指導教官として深く感謝している次第です。

笠郡千尋

研究報告書

京都府立医科大学薬理学教室　李　航

マウスアルドース還元酵素の遺伝子構造と遺伝子発現調節領域の解析

アルドース還元酵素はNADPHを補酵素とするアルド・ケト還元酵素の一員であり、グルコースをソルビトールに変換する酵素である。この酵素はソルビトールをフルクトースに変換するソルビトール脱水素酵素とともにグルコース代謝の副経路であるポリオールを形成している。

糖尿病合併症の発症に関わるアルドース還元酵素は、高浸透圧負荷により遺伝子上流領域に位置するosmotic response elementを介して発現上昇をきたし、腎髄質の浸透圧調節物質の一つであるソルビトールを産生すると考えられている。しかし腎以外の組織におけるAldose Reductaseの生理的意義は未だ明らかではなく、長期のAldose Reductase阻害剤投与による副作用は予測出来ない。そこで我々は遺伝子改変マウスを用いて本酵素の生理的役割の全貌を解明するべく、今回はマウスアルドースリダクターゼの遺伝子構造とその生理的条件下での発現調節領域について解析を進めた。

まずMouse Aldose ReductaseのcDNAをprobeとしてラムダファージマウスゲノムライブラリーをスクリーニングし、3個のオーバーラップしたクローンを得た。制限酵素地図並びにエクソンーイントロン構造とその接合部の塩基配列を決定した。続いてAldose Reductaseの遺伝子上流領域約3.4 kbをルシフェラーゼレポータープラスミドに挿入し、プロモーター領域のserial deletion mutantを作製すると同時に遺伝子上流領域の塩基配列を決定した。これらのキメラプラスミドをNIH3T3、CV1、CHOの各細胞株にリン酸カルシウム法にて遺伝子導入後、ルシフェラーゼ活性を測定することにより転写活性に重要な領域を同定した。

Aldose Reductaseの発現量はマウスの各組織間において、また各種の培養細胞株において大きく異なることがわかった。即ちAldose Reductaseはほとんどすべての組織(座骨神経、網膜、水晶体、卵巣、 睾丸輸精管、脳、腎臓、肝、骨格筋)に発現していたが、その発現量は各組織において大きく異なっていた。また異なった組織由来の培養細胞株NIH3T3, Y1, NG108, L6, CHO, Hela, MG63, NEC8, CV1) での発現量にも大きな違いがあることがわかった。Mouse Aldose Reductase遺伝子は全長約15 Kbで、10コのExonよりなり、Exon 1とExon 2の間は約4.3 Kbであったが、Exon 2からExon 8までは3.6 Kbの間に局在していた。またMouse Aldose Reductaseプロモーターは、TATA boxと二つのCCAAT boxを持つことが確認され、上流約1.1KbにOsmotic Response Elementと呼ばれる領域とAP1結合コンサンサス配列があった。

トランスフェクションアッセイの結果、NIH3T3細胞において転写活性が大きく変わる二つの領域を認めた。一つは上流約-1.1 kbから-0.85 Kbの領域で、この領域には、これまでに報告されたTonEというOsmotic Response Elementが存在し、もう一つは-0.6 Kbから-0.24 Kbの領域で、この領域をけずることにより転写活性は2.0倍低下することが分

かった。一方 CHO 細胞においては、 -0.6 Kb から-0.24 Kb の領域を除くと NIH3T3 細胞同様に、転写活性は 3.5 倍低下したが、 -1.1 kb から -0.85 Kb の領域をけずってもプロモーター活性に大きな違いは認められなかった。

本年度得られた結果をまとめると、

1． Mouse Aldose Reductase 遺伝子は全長約 15 Kb の大きさで、10コの Exon よりなる。
2． Mouse Aldose Reductase の発現量は各組織間において、また各種の培養細胞間において大きく異なる。
3． Mouse Aldose Reductase のプロモーター領域には TATA box と二つの CCAAT box が存在し、 Aldose Reductase プロモーター活性は転写開始点より上流約 1.1 kb を含むフラグメントでほぼ最大活性を示す。
4． NIH3T3 細胞において転写活性が大きく変わる二つの領域がある。一つは上流約 -1.1 kb から -0.85 Kb の領域で、この領域には、 Aldose Reductase 発現の浸透圧調節にかかわる Osmotic Response Element が存在する。もう一つは -0.6 Kb から -0.24 Kb の領域で、この領域をけずることにより転写活性は 2.0 倍低下する。
5． CHO 細胞においては、 -0.6 Kb から -0.24 Kb の領域を除くと NIH3T3 細胞同様に、転写活性は 3.5 倍低下するが、 -1.1 kb から -0.85 Kb の領域をけずってもプロモーター活性に大きな違いは認められない。-1.1 kb から -0.85 Kb の領域が、各組織あるいは培養細胞間における Aldose Reductase の発現量の違いにどのような役割を果たしているかについて、今後更に検討する予定である。

以上、貴財団の奨学金により私の研究を一年間ご援助頂きまして、心より感謝致します。

日本財団補助金による
1997 年度日中医学協力事業助成報告書
－在留中国人研究者研究助成－

10 年 3 月 12 日

財団法人　日中医学協会

理事長　　中島章殿

Ⅰ．研究者氏名　郭薇

研究機関 大阪大学医学部形態機能解研究指導者 濱山正彌 職名 教授

所在地 〒565 大阪府吹田市山田丘2-2 電話 06-879-3221 内線

Ⅱ．過去の研究歴

肝線維症と関係する遺伝子の解析

C型肝炎に関する母子感染及び臨床診断

Ⅲ．過去の研究実績

肝線維症で遺伝子発現の上昇するものとして転写因子と翻訳調節因子の両者がクローニングされたのものを発現して GroEL と TCEP の解析をやってます。

Ⅳ．本年度の研究業績

(1) 学会、研究会等においての口頭発表（学会名・内容）

① 日本神経科学学会 ポスター ラット胎期におけるミオイシトール トランスポーターの遺伝子発現

② 日本解剖学会 口演：ラット結腸におけるミオイシトールトランスポーター(SMIT)の高浸透圧負荷による発現変化。③口演：浸透圧応答性の新規RNA helicase のcDNA の単離と高浸透圧

(2) 学会誌等に発表した論文　　無 ・ (有)　(雑誌名・論文名) 負荷時の発現調節機構の解析

① MOLECULAR BRAIN RESEARCH

Developmental regulation of Na^+/myo-inositol cotransporter gene expression

Ⅴ．今後の研究計画及び希望

新規 RNA helicase の機能解析を継続して研究したいと思います。

Ⅵ. 研　究　報　告（日本語、又は英語で書いて下さい。 4,000字以上で記載して下さい。別紙可）

別紙.

Ⅶ. 指導教官の意見

郭薇氏は短期間で留学生という言語や生活体系の違いも克服し、多くの難しい実験手法を習得し、さらに自主的な考えで研究を発展させ、それらの研究成果を、国際誌や学会を通して発表し、教室に多大な貢献をしてまいりました。現在も我々の研究室の主要な研究プロジェクトの中心人物として研究を引っ張っております。申請者の研究に対する厳しい姿勢と最後までやり抜く態度には、常々感心しております。また一方では、性格は温厚で明るく、素直で、後輩の面倒見も良く、研究室の皆に慕われております。

遠山 正彌

論文：

1. **Guo, W.**, Shimada, S., Tajiri, H., Yamauchi, A., Yamashita, T., Okada, S., Tohyama, M. Developmental regulation of Na+/myo-inositol cotransporter gene expression
Mol Brain Res 51(1997) 91-96

2. Yamashita,T., Shimada, S., Yamaguchi,A., **Guo,W.**, Kohmura, E., Hayakawa, T., and Tohyam, M. Induction of Na+/myo-inositol contransporter mRNA after rat cryogenic injury.
Mol Brain Res 56(1997) 128-134

3. Yamashita,T., Shimada, S., **Guo,W.**, Kohmura, E., Hayakawa, T., Takagi, T. and Tohyam, M. Cloning and functional expression of a brain peptide/histidine transporter.
J Biol Chem 272, 10205-10211, 1997.

4. Tada, K., Tajiri, H., Kozaiwa, K., Sawada, A., **Guo, W.**, and Okada, S. Role of screen for hepatitis Cvirs in children with malignabt disease and who undergo bone marrow transplantation
Transfusion Volume 37, June 1997.

5. Etani, Y., **Guo, W.**, Tajiri, H., Okada, S., Shimada, S., Tohyama, M. Analysis of glucose transporter gene in a case of fructose malabsorption with chronic Diarrhea
Digestion & Absorption 1995, 18, 40-4(Japanese)

6. Tada, K., Tajiri, H., Sawada, A., **Guo, W.**, Etani, Y., Kozaiwa, K., Ozaki, Y., Okada, S. HCV Infection in Children with Primary Malignant Diseases
The Journal of the Japan Pediatric Society (Japanese)

7.**Guo, W.**, Shimada, S., Mushiage, S., Tajiri, H., Yamauchi, A., Yamashita, T., Okada, S., Tohyama, M.
Differential localization and induction of Na+/myo-inositol cotransporter mRNA in the large intestine.
投稿中

8. **Guo, W.**, Shimada, S., Yamashita, T., Yamauchi, A., Okada, S., Tohyama, M.
Purification and Characterization of a new member of DEAD box of RNA helicase
投稿中

郭薇

研 究 報 告 書

私は小児の消化器系の疾患に興味を持ち、最初に、肝線維症で同一患者の正常な肝組織と線維症の起こっている肝組織の間で differential display 法を用いることにより、プロトオンコジーンのRETと結合するGrb10と、RNA結合蛋白であるiron-responsive element-binding protein (IRE-BP)が肝線維症で遺伝子発現が上昇していることを明らかにした。肝線維症で遺伝子発現の上昇するものとして転写調節因子と翻訳調節因子の両者がクローニングされたのは興味深い。この二つの遺伝子については症例を増やし、肝線維症との関係を検討中である。このようなヒトの剖検組織を用いた疾患関連遺伝子の検索を進める一方、基礎的な研究として、消化管での浸透圧変化に対する細胞保護機構を解析している。グリセロール注腸動物や脱水モデル動物を用いて、大腸の吸収上皮細胞や杯細胞でミオイノシトールトランスポーターのmRNAが管腔内の浸透圧が上昇した際や、血清浸透圧が上昇した際に顕著に上昇することを明らかにし、大腸の上皮細胞が細胞外の浸透圧変化から細胞を保護するためにミオイノシトールの取り込みを増加させていることを示した。さらに、ミオイノシトールトランスポーターの発達段階での遺伝子発現及び蛋白発現の変化を解析し、中枢神経系で胎生期に一過性の非常に強い発現が神経系に認められることを明かとした。最近、Differential Display法により浸透圧調整に関係する新規のRNAhelicaseのcDNAを単離して、現在その機能解析を行っています。

消化管は管腔内に様々な摂取物、水分、分泌液が存在し、これらの濃度や量が吸収や分泌によって複雑に調整されている。このことは消化管上皮が絶えず浸透圧の変化に曝されていることを意味する。さらに大腸上皮では浸透圧勾配を利用し、水分の吸収を行うために、この部位の細胞には極端に高い浸透圧負荷が、常時かかっている。またこれらの浸透圧変化及び調節に異常を来すと下痢等の様々な消化器症状を来す。細胞外の浸透圧変化は細胞内の体積や電解質組成に、大きな影響を与えるため、浸透圧変化から細胞を保護する機構が存在すると考えられる。このような浸透圧変化からの細胞保護機構の研究は主に腎臓で行われてきた。浸透圧物質（オスモライト）のトラ

ンスポーターは細胞外の高浸透圧に対抗して、オスモライトを細胞内に蓄積することによって、細胞内外の浸透圧バランスを保つという有効浸透圧作用と、同時に細胞内の蛋白質や核酸の高次構造が高浸透圧により変化するのを防ぐ細胞機能保護作用を示すと考えられている。私どものグループはオスモライトトランスポーターの発現調節について、多くの解析を進めてきた。しかし、浸透圧の変化する刺激からオスモライトトランスポーターの発現誘導までの経路はほとんど明らかにされていない。そこで本研究では大腸上皮由来のCaco-2細胞を用いて、Differential Display 法によって、浸透圧負荷時に必要となる新規の遺伝子を解析する。

消化器系において高浸透圧刺激からオスモライトのトランスポーターの遺伝子発現誘導がおこるまでの経路に関与している遺伝子を検討するため、CACO-2培養細胞を、高張、等張、低張の培養液下で、培養しmRNA抽出後、differential display法を用いて浸透圧負荷時に新たに発現する遺伝子を同定する。これらのin vitro系を用いてdifferential display法で得られた浸透圧応答候補遺伝子を、ノーザンブロット解析により実際にCACO-2培養細胞で浸透圧負荷時に、発現誘導がかかるかどうかスクリーニングする。浸透圧負荷時に発現が上昇する遺伝子については、蛍光プライマー法を用いて塩基配列を決定する。その中で新規遺伝子についてはそのcDNAの全長を獲得するために、高浸透圧下で培養したCACO-2細胞から、cDNAライブラリーを作製し、スクリーニングする。このスクリーニングでも全長が得られない場合は、5'や3'race法を用いてcDNAを延長する。

これらの浸透圧応答遺伝子は、その相同性から機能が推測できるものに関しては、cDNAを適切な発現ベクターに組み替えて、HEK細胞発現系やアフリカツメガエル卵母細胞発現系を用いて適切な解析を行い、浸透圧変化時におけるこの遺伝子の役割を検討する。また大腸菌の発現系を用いて、浸透圧応答遺伝子の蛋白を単離し、抗体を作製し、結合蛋白を作製しました。

これらの機能解析により浸透圧応答遺伝子の特性を明らかにするとともに、in

vivoでの浸透圧負荷モデル、虚血、浮腫等の動物モデルを用いてその発現調節を解析する。さらにCACO-2細胞を用いて、antisense法により、浸透圧負荷時にこれらの浸透圧遺伝子の発現が抑制されると℃のような細胞傷害が起こるか検討する。

最近、私はこの実験計画をスタートし、Differential Display法の系から、浸透圧負荷により遺伝子発現が上昇するRNAhelicase遺伝子ファミリーに属する新しい遺伝子を単離しました。現在大腸菌BL21細胞を用いて、融合蛋白を合成し、２本鎖のDNAやRNAのhelicase活性があるかどうか検討中です。

郭 筱

98. 3. 11.

Reprinted from

MOLECULAR BRAIN RESEARCH

Molecular Brain Research 51 (1997) 91–96

Research report

Developmental regulation of Na^+/*myo*-inositol cotransporter gene expression

Wei Guo [a,b,*], Shoichi Shimada [b], Hitoshi Tajiri [a], Atsushi Yamauchi [c], Toshihide Yamashita [b,d], Shintaro Okada [a], Masaya Tohyama [b]

[a] *Department of Pediatrics, Osaka University School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565, Japan*
[b] *Department of Anatomy and Neuroscience, Osaka University School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565, Japan*
[c] *First Department of Medicine, Osaka University School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565, Japan*
[d] *Department of Neurosurgery, Osaka University School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565, Japan*

ELSEVIER

BRAIN RESEARCH

SCOPE AND PURPOSE

MOLECULAR BRAIN RESEARCH is a special section of **Brain Research** which provides a medium for prompt publication of studies on molecular mechanisms of neuronal, synaptic and related processes that underlie the structure and function of the brain. Emphasis is placed on the molecular biology of fundamental neural operations relevant to the integrative of the nervous system.

TYPES OF PAPERS

1. **Interactive Reports** are papers describing original, high quality, fundamental research in any area of Neuroscience. These will first appear electronically on the WWW (**http:/www.elsevier.com/locate/bres** or **http://www.elsevier.nl/locate/bres**) and published soon after in the relevant section of *Brain Research*. The on-line version may include additional data sets, 3-D/confocal images, animations, etc., and be linked to relevant on-line databases. Comments from readers may be appended later in a linked ***Discussion Forum*** at the discretion of an appointed Moderator.

2. **Research Reports** reporting results of original fundamental research in any branch in the brain sciences. It is expected that these papers will be published about six months after acceptance.

3. **Short Communications** reporting on research which has progressed to the stage when it is considered that the results should be made known quickly to other workers in the field. The maximum length allowed will be 1500 words or equivalent space in tables and illustrations. It is expected that Short Communications will be published about three months after acceptance.

4. **Protocols:** full-length protocols in any area of Neuroscience, to be published in the journal section *Brain Research Protocols*. Updates on published protocols submitted by the authors thereof, describing new developments which are of sufficient interest to the neuroscience community, but which do not warrant a completely new submission, will be published as **Protocol Updates**. The maximum length allowed will be 1500 words or equivalent space in tables and illustrations. Comments on published Protocols describing useful hints and "tricks" related to any aspect of the Protocol, such as timing, equipment, chemicals, troubleshooting, etc., will be published in the on-line version of the journal as linked **Technical Tips** at the discretion of the Editor.

SUBMISSION OF MANUSCRIPTS

Submission of a paper to ***Molecular Brain Research*** is understood to imply that it deals with original material not previously published (except in abstract form), and that it is not being considered for publication elsewhere. Manuscripts submitted under multiple authorship are reviewed on the assumption that all listed authors concur with the submission and that a copy of the final manuscript has been approved by all authors and tacitly or explicitly by the responsible authorities in the laboratories where the work was carried out. If accepted, the article shall not be published elsewhere in the same form, in either the same or another language, without the consent of the Editors and Publisher. The Publisher and Editor regret that they are unable to return copies of submitted articles except in the case of rejected articles, where only one set of manuscript plus figures will be returned to the author.

Manuscripts in English should be organised according to the ***Brain Research*** *Guidelines for the Submission of Manuscripts* and sent to the appropriate address shown below. Authors should state clearly the section of the journal for which the article/Interactive Report should be considered.

Research Reports &
Short Communications:
Professor D.P. Purpura
Brain Research, Office of the Dean
Albert Einstein College of Medicine
Jack and Pearl Resnick Campus
1300 Morris Park Avenue
Bronx, NY 10461, USA
Tel.: (1) (718) 430-2387
Fax: (1) (718) 430-8980
E-mail: brain@aecom.yu.edu

Interactive Reports:

Professor F.E. Bloom
Brain Research Interactive
Dept. of Neuropharmacology
The Scripps Research Institute
10666 N. Torrey Pines Road
La Jolla, CA 92037, USA
Fax: (1) (619) 784-8851
E-mail: smart@scripps.edu
Website: http://smart.scripps.edu

Protocols &
Protocol Updates:
Dr. Floris G. Wouterlood
Department of Anatomy
Faculty of Medicine
Free University
van der Boechorststraat 7
1081 BT Amsterdam
The Netherlands
Fax: (31) (20) 444-8054
E-mail: fg.wouterlood.anat@med.vu.nl

*Correspondence regarding **accepted** manuscripts relating to proofs, publication and reprints should be sent to:*
Brain Research, Elsevier Science B.V., P.O. Box 2759, 1000 CT Amsterdam, The Netherlands. Tel. (31) (20) 485-3474; Fax: (31) (20) 485-3271; E-mail: a.prukar@elsevier.nl.

EDITORIAL BOARD

GUIDELINES FOR THE SUBMISSION OF MANUSCRIPTS

These can be found in the "front matter" of the last issue of each volume of the journal.
The preferred medium for *final* submission is on disk with accompanying manuscript (see "Electronic manuscripts").

ELSEVIER

Molecular Brain Research 51 (1997) 91–96

MOLECULAR BRAIN RESEARCH

Research report

Developmental regulation of Na^+/*myo*-inositol cotransporter gene expression

Wei Guo [a,b,*], Shoichi Shimada [b], Hitoshi Tajiri [a], Atsushi Yamauchi [c], Toshihide Yamashita [b,d], Shintaro Okada [a], Masaya Tohyama [b]

[a] *Department of Pediatrics, Osaka University School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565, Japan*
[b] *Department of Anatomy and Neuroscience, Osaka University School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565, Japan*
[c] *First Department of Medicine, Osaka University School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565, Japan*
[d] *Department of Neurosurgery, Osaka University School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565, Japan*

Accepted 17 June 1997

Abstract

myo-Inositol plays a role in many important aspects of cellular regulation including membrane structure, signal transduction and osmoregulation. It is taken up into the cells by the Na^+/*myo*-inositol cotransporter (SMIT). We investigated developmental changes in the expression of SMIT mRNA and protein in the rat. In the fetal rat brain, SMIT mRNA was abundantly and diffusely expressed throughout the whole brain and the spinal cord. Positive signals were expressed in neuronal and non-neuronal cells in these regions. SMIT is gradually down-regulated nearer birth, but intense signals were still detected in the brain at postnatal day one. In the adult rat brain, very weak hybridization signals were detected throughout whole brain except for the choroid plexus where SMIT mRNA expression remained high. In contrast, the pattern of developmental regulation of SMIT gene expression in the kidney was opposite to that seen in the brain. Signals in the kidney were very weak during embryonic stages, whereas SMIT expression increased significantly after birth. These results suggest that *myo*-inositol and its transporter play an important role in the CNS developmental stage. © 1997 Elsevier Science B.V.

Keywords: Osmolyte; Blood–brain barrier; Na^+/*myo*-inositol cotransporter

1. Introduction

myo-Inositol and its various biochemical derivatives are widely distributed in mammalian tissues and cells [10]. The levels of *myo*-inositol in most mammalian cells or tissues are much higher than those in plasma and interstitial fluids [5,13]. Plasma concentrations in adult mammals have been reported to be 10–200 μM [10], whereas tissue *myo*-inositol levels are usually greater than 1 mM. Sodium-dependent *myo*-inositol transporter (SMIT) appears to be responsible for the maintenance of these concentration gradients. Interestingly, *myo*-inositol concentrations in fetal mammalian serum may be elevated with levels which are sometimes greater than 1 mM, depending on the time of gestation [4,9]. Similarly, while the range of *myo*-inositol in adult cerebrospinal fluid (CSF) is 100–500 μM [20,22], the concentration in fetal CSF may reach 3 mM [1].

The reasons for the elevated *myo*-inositol levels in fetuses are unknown. Fruen and Lester have shown more recently that sodium-dependent *myo*-inositol uptake in fetal brain cells in vitro is also increased when compared to adult cells [6]. They postulated that disruption of *myo*-inositol homeostasis in Down syndrome (trisomy 21) may affect the developing brain and thus contribute to the pathogenesis of mental retardation, the most consistent and debilitating feature of the syndrome. There is some supportive evidence for their hypothesis. When polyol species were examined in CSF, a significant increase in the level of *myo*-inositol alone was observed in Down syndrome compared with controls [21]. Furthermore, recent mapping of a human SMIT gene onto the long arm of chromosome 21 [3] suggested that the altered *myo*-inositol homeostasis

* Corresponding author. Fax: +81 (6) 879-3229.

PII S0169-328X(97)00220-9

involving the CSF may result from increased transport. Despite these studies, no information is available about expression of SMIT during fetal development.

To gain insight into the expression of SMIT during time of gestation, we determined regional distributions of SMIT mRNA in rat fetuses using in situ hybridization. The results obtained demonstrated that SMIT is highly expressed in the fetal brain and spinal cord, whereas there were only weak signals in the kidney. The significance of SMIT in development of the CNS will be discussed.

2. Materials and methods

2.1. In situ hybridization

Pregnant Wistar rats were anesthetized with pentobarbital (65 mg/kg intraperitoneally) and fetuses were removed at embryonic day (E) 14, E16, and E18. Animals were also examined at postnatal day (P) 1 ($n = 5$ for each age). Fetuses and P1 rats were sacrificed by decapitation and their whole bodies were immediately frozen at −80°C. Adult rats (postnatal week 3, $n = 5$) were treated in the same manner and their brains and kidneys were removed. Serial coronal sections (5 μm thick) were obtained from the frozen tissues and the whole bodies with a cryostat and stored in a tightly closed case at −80°C. The antisense probe for SMIT was synthesized from a 490 bp rat SMIT cDNA (bases 808–1297) insert cloned into the Novagen T-vector. The sense probe for SMIT was synthesized from a 490 bp rat SMIT cDNA insert cloned in the vector pSPORT 1. To synthesize hybridization riboprobes by in vitro transcription, this sequence was first linearized by digestion with restriction endonucleases of *Eco*RI for both antisense and sense RNA synthesis. The linearized cDNA was then incubated at 37°C for 60 min with a mixture of reagents. This mixture consisted of 2 μl of transcription buffer (×5), 0.5 μl of 100 mM dithiothreitol, 0.5 μl of RNase inhibitor, 0.5 μl of 10 mM ATP, CTP and GTP, 5 μl of [^{35}S]UTP (NEG-039H, New England Nuclear), 0.5 μl of DNA template (1 mg/ml) with 1 μl of appropriate RNA polymerase (T7 RNA polymerase for antisense; SP6 RNA polymerase for sense probe). DNA was digested by addition of 2 μl of DNase (1 U/μl) and incubation at 37°C for 10 min. Efficacy of labeling was estimated by counting radioactivity of the synthesized probes.

In situ hybridization techniques for SMIT mRNA (RNA probe) were based on those of Wilkinson et al. [23] with some modifications. Briefly, sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 20 min. After washing with PBS, the sections were treated with 10 mg/ml of proteinase K in 50 mM Tris–HCl and 5 mM EDTA (pH 8.0) for 5 min at room temperature. They were fixed again in the same fixative, then acetylated with acetic anhydride in 0.1 M triethanolamine, rinsed with PBS, dehydrated and air-dried. The ^{35}S-labeled RNA probes (sense and antisense) were diluted in hybridization buffer, applied to the sections and covered with siliconized coverslips. Hybridization was performed overnight in a humid chamber at 55°C. The hybridization buffer consisted of 50% deionized formamide, 0.3 M NaCl, 20 mM Tris–HCl, 5 mM EDTA, 10 mM PB, 10% dextran sulfate, 1 × Denhardt's solution, 0.2% sarcosyl, 500 mg/ml yeast tRNA, and 200 mg/ml herring sperm DNA (pH 8.0). The probe concentration was 5×10^5 cpm/150 μl per slide. After hybridization, the slides were soaked in 5 × SSC at 55°C, and the coverslips were allowed to slough off. The sections were then incubated at 65°C in 50% deionized formamide with 2 × SSC for 30 min. After rinsing with RNase buffer (0.5 M NaCl, 10 mM Tris–HCl, 5 mM EDTA (pH 8.0)) four times for 10 min each time at 37°C, the sections were treated with 1 mg/ml of RNase A in RNase buffer for 30 min at 37°C. After an additional washing in RNase buffer, the slides were incubated in 50% formamide with 2 × SSC for 30 min at 65°C, rinsed with 2 × SSC and 0.1 × SSC for 10 min each at room temperature, dehydrated through an ascending alcohol series and air-dried.

X-ray film was placed on uncoated sections for 3 days. The slides were then coated with Ilford K-5 emulsion diluted in distilled water containing 2% glycerine (1:1). The slides were exposed for 3 weeks in a tightly sealed dark box at 4°C, developed in Kodak D-19, fixed with photographic fixer, stained with thionine and coverslipped. The tissue sections were examined under a light microscope. For quantitative assessment of SMIT mRNA expression on the macroautoradiograms, an optical density of a target region was measured, and optical density ratio (ODR) was calculated in comparison with the film background density. Statistical analysis was performed using non-parametric analysis of the Mann–Whitney *U*-test with two-tailed probability.

2.2. Western blotting

Anti-SMIT antibody was raised against a synthetic peptide, CTPPPTKEQ, corresponding to amino acids 533–540 of SMIT [12]. The peptide was coupled with KLH and used to immunize rabbits.

Extracts were prepared from E16, E18 and P1 and P21 rat brain. Aliquots of 20 μg of protein were electrophoresed in 10% SDS–polyacrylamide gels. Proteins were transferred from the gels onto nitrocellulose membranes in a modified Towbin transfer buffer (25 mM Tris, 192 mM glycine, pH 8.4, containing 0.05% 2-mercaptoethanol). The nitrocellulose was blocked with blot buffer (20 mM Tris (pH 7.6), 150 mM NaCl, 0.05% Tween-20, 0.05% NaN_3) containing 3% BSA for 1 day at 4°C. The filters were probed at room temperature for 2 h with anti-SMIT rabbit antiserum diluted in blot buffer, washed

with blot buffer, and then incubated for 2 h with anti-rabbit IgG HRP antibody diluted 1:500 in blot buffer containing 1% BSA. After washing with blot buffer, immunoreactivity was visualized using the ECL system (Amersham, Braunschweig, Germany).

3. Results

Throughout the present study, adjacent sections were hybridized with both sense and antisense probes to confirm the specificity of SMIT mRNA hybridization signals. Signals were observed only in sections hybridized with the antisense probes. Fig. 1 and Fig. 2 show developmental changes in the expression of SMIT mRNA; since significant expression was observed in the fetal brain and the kidney after birth, we focused on these two organs.

3.1. SMIT mRNA expression in the brain

The most intense SMIT mRNA hybridization signals were constantly found in the choroid plexus of the lateral ventricle and fourth ventricle constantly from E14 to P21. In contrast, other regions of the brain showed intense hybridization signals until P1, while these hybridization signals were markedly decreased and reached low levels at P21.

Detailed observations revealed low to moderate levels of SMIT mRNA in the neocortical neuroepithelium, septum, rhinencephalon, pallidal subventricular zone, pal-

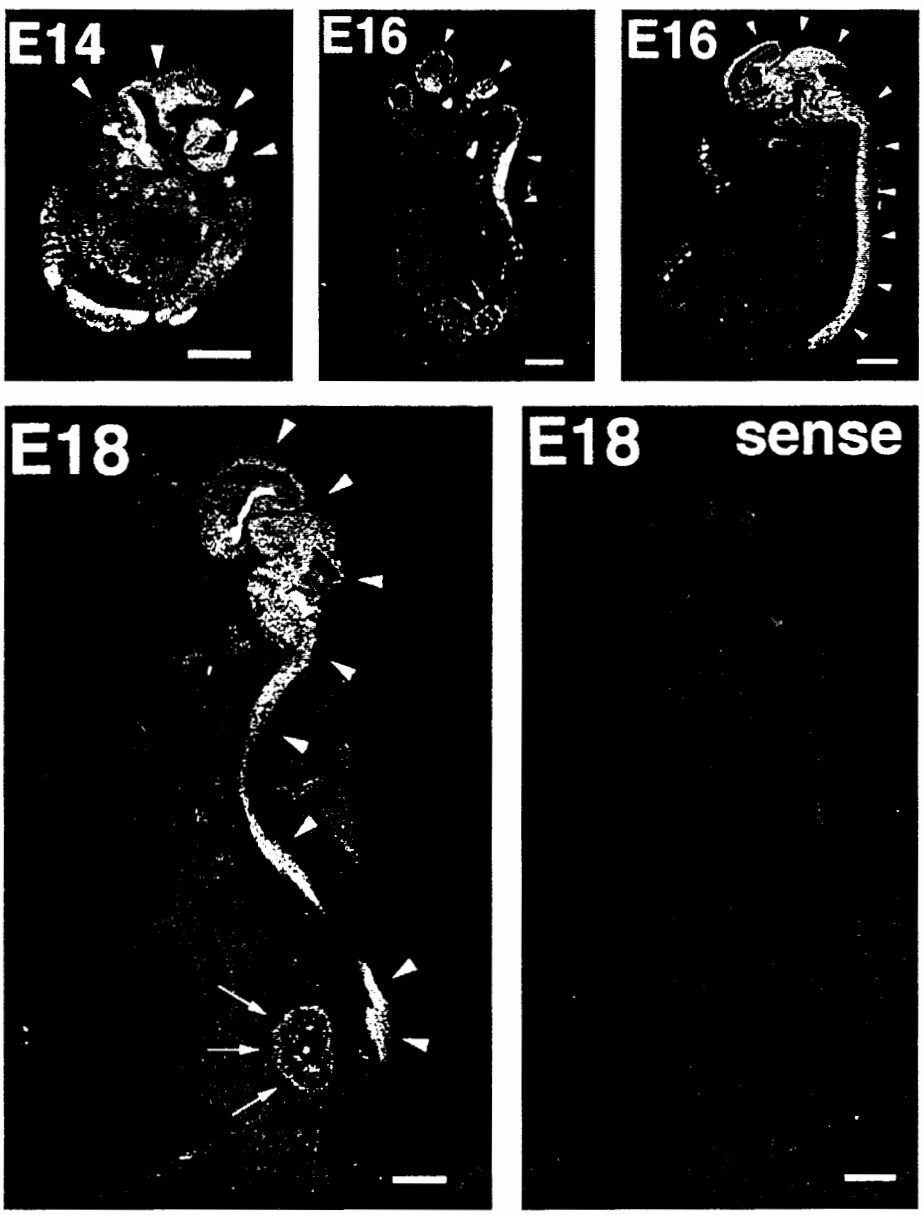


Fig. 1. SMIT mRNA hybridization signals were widely distributed throughout the fetal brain (E14, E16, E18) with the most intense signals in the choroid plexus. In contrast, SMIT mRNA levels were low in the fetal kidney. No specific hybridization signals were found using a sense SMIT probe. Scale bar = 2 mm.

lidum and thalamus at E14, while intense hybridization signals were seen in the hypothalamus, pretectum, tegmentum, anterior pons, posterior pons, medulla and spinal cord. At E16, intense hybridization signals were observed in the cortical plate and neocortical neuroepithelium, whereas weak signals were detected in the intermediate zone in the cerebral cortex. Most of the other areas in the brain showed moderate to intense hybridization signals with less intense signals in the pallidal subventricular zone. Moderate to intense hybridization signals were also seen in the neural layer of the retina at E16. At E18, almost all the regions in the brain and neural layer of the retina showed moderate to intense SMIT mRNA signals with less intense signals in the intermediate zone in the cerebral cortex, striatal subventricular zone and pallidal subventricular zone. At P1, moderate to intense hybridization signals were found throughout whole brain. Particularly high expression of SMIT mRNA was detected in the subiculum, CA1–3 fields in the hippocampus, hypothalamus and amygdaloid complex. At P21, very low signals were widely distributed throughout the whole brain with slightly higher intensity in the olfactory bulb, hippocampus and cerebe lum. No signals were found with the sense probe.

3.2. SMIT mRNA expression in the kidney

In contrast, SMIT mRNA levels were low in the kidne during fetal stages, whereas the hybridization signals wei markedly increased after birth.

Detailed observation revealed weak SMIT mRNA sig nals in the nephrogenic zone, and low-level signals wei scattered in the medulla at E16. Moderate hybridizatio signals were sporadically spread over cortical an medullary regions with higher levels in the nephrogeni zone at E18. At P1, intense hybridization signals wer concentrated in the medulla, whereas weak signals wer scattered in the cortex. At P21, SMIT mRNA was in tensely expressed in the outer medulla. SMIT mRNA signals showed a gradient of concentration along the corti comedullary axis from the inner medulla to the papillar tip, with the most abundant transcript levels in the papil lary tip. These signals in the papillary tip were as intens

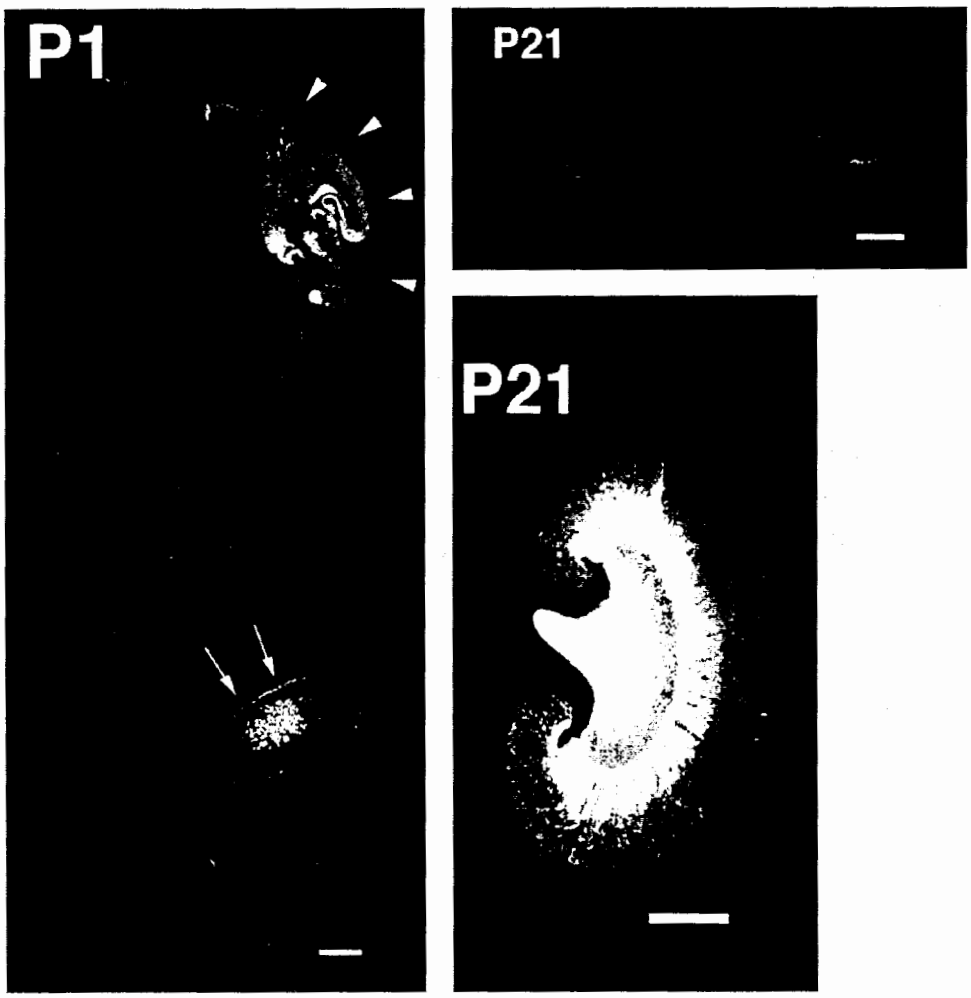


Fig. 2. Postnatal day 1 (P1) rat, and the brain and kidney of postnatal day 21 (P21) adult rat. The brain showed intense hybridization signals at P1, while these hybridization signals were markedly decreased and reached low levels at P21. In contrast, the hybridization signals in the kidney were markedly increased after birth, especially in the medulla.

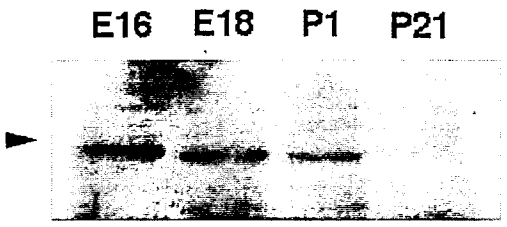


Fig. 3. On Western blotting analysis, polyclonal antiserum against SMIT recognized a single protein with M_r of 80000. The levels of SMIT protein in the brain were decreased from embryonic day 16 (E16) to postnatal day 21 (P21). Arrowhead indicates 83 kDa marker.

as those in the outer medulla. Moderate hybridization signals were scattered in the cortex.

3.3. Western blotting analysis

We also analyzed levels of SMIT protein in the rat brain at E16, E18, P1 and P21 by immunoblotting. As s ı in Fig. 3, SMIT was expressed as a single compon with an M_r of 80000. The protein levels of SMIT throughout developmental stages showed a pattern similar to that observed for SMIT mRNA. The brain SMIT protein levels were high during embryonic stages and decreased with development.

4. Discussion

Our results indicated that SMIT mRNA and SMIT protein are highly expressed in the fetal CNS and decrease with development. This result is consistent with in vitro study using cultured brain cells from fetal mice [6] in which Na^+-dependent *myo*-inositol uptake in fetal brain cells was significantly higher than that in adult brain cells. Their results together with our observations suggest that the development of the brain from the fetal to the adult stage is associated with a significant decrease in SMIT a ty as well as in its mRNA and protein levels. A s ar decrease in SMIT activity accompanies differentiation of neuroblastoma cells in culture [19]. Changes in brain SMIT with development may be related to the high ambient concentration of *myo*-inositol. Considering the marked elevation of its concentration in fetal plasma [4,9] and CSF [1], one would expect that intracellular concentration would be extremely high. Since *myo*-inositol levels in most mammalian tissues are 10–100-fold higher than those in plasma [5,13], its concentration in the fetal CNS might be more than 10 mM. This raises the question why the fetal CNS need such a large amount of *myo*-inositol. The rate of synthesis of phosphatidylinositol would not be markedly affected because it is already saturated at much lower concentrations [2]. Low levels of ambient *myo*-inositol seem to be adequate to maintain normal cellular phosphatidylinositol production. Thus, it is possible that *myo*-inositol plays another role in the fetal brain that is distinct from that of a simple substrate for phospholipid biosynthesis.

In addition to its role in membrane structure and signal transduction, *myo*-inositol functions as an osmolyte in the kidney and the brain [7,8]. It is accumulated under hypertonic conditions by increasing SMIT activity [17]. The abundance of SMIT mRNA and the transcription rate of the SMIT gene increased when the cultured kidney cells were cultured in hypertonic medium, suggesting that transcription is the primary step in regulation of *myo*-inositol transport by hypertonicity [12,25]. Similar results were obtained from brain glial cells [18] and lens epithelial cells [26]. We have recently reported the localization and regulation of SMIT mRNA in kidney [24], brain [11,14], eye [16] and ear [15]. These results strongly suggest an important role of SMIT in the CNS regarding cell volume regulation. Although we found intense expression of SMIT in the fetal CNS, it is unknown whether the transporter is also under osmoregulatory control. Berry et al. [2] suggested that *myo*-inositol may be an osmolyte in fetal endothelial cells since they have a high-affinity transport system for *myo*-inositol and accumulate more *myo*-inositol with fetal bovine serum than with adult bovine serum. It is possible that *myo*-inositol functions as an osmolyte in the fetal CNS. We speculate that the intense expression of SMIT might be related to the immature blood–brain barrier in the fetus, which is much more permeable to a variety of solutes than that in adults.

Our results thus suggest that SMIT plays an important role in the fetal CNS but not in the kidney. The hypothesis proposed by Fruen et al. [6] regarding the relationship between Down syndrome and SMIT gene is consistent with our results. In patients with Down syndrome, mental retardation and neurological abnormalities are always present but the kidney is usually normal. Further studies are necessary to clarify the significance of *myo*-inositol and its transporter in this syndrome.

References

[1] F.C. Battaglia, G. Meschia, J.N. Blechner, D.H. Barron, The free *myo*-inositol concentration of adult and fetal tissues of several species, Q. J. Exp. Physiol. 46 (1961) 188–193.
[2] G.T. Berry, R.A. Johanson, J.E. Prantner, B. States, J.R. Yandrastz, *myo*-Inositol transport and metabolism in fetal aortic endothelial cells, Biochem. J. 295 (1993) 863–869.
[3] G.T. Berry, J.J. Mallee, H.M. Kwon, J.S. Rim, W.R. Mulla, M. Muenke, N.B. Spinner, The human osmoregulatory Na^+/*myo*-inositol cotransporter gene (SLC5A3): molecular cloning and localization to chromosome 21, Genomics 25 (1995) 507–513.
[4] J.D. Campling, D.A. Nixon, The inositol content of foetal blood and foetal fluids, J. Physiol. 126 (1954) 71–80.
[5] R.M.C. Dawson, N. Freinkel, The distribution of free *meso*-inositol in mammalian tissues, including some observation on the lactating rat, Biochem. J. 78 (1961) 606–610.
[6] B.R. Fruen, B.R. Lester, High affinity [^{3}H]inositol uptake by dissociated brain cells and cultured fibroblasts from fetal mice, Neurochem. Res. 16 (1991) 913–918.

[7] A. Garcia-Perez, M.B. Burg, Renal medullary organic osmolytes, Physiol. Rev. 71 (1991) 1081–1115.
[8] S.R. Gullans, J.G. Verbalis, Control of brain volume during hyperosmolar and hypoosmolar conditions, Annu. Rev. Med. 44 (1993) 289–301.
[9] M. Hallman, S. Slivka, P. Wozniak, J. Sills, Perinatal development of *myo*-inositol uptake into lung cells: Surfactant phosphatidylglycerol and phosphatidylinositol synthesis in the rabbit, Pediatr. Res. 20 (1986) 179–185.
[10] B.J. Holub, Metabolism and function of *myo*-inositol and inositol phospholipids, Annu. Rev. Nutr. 6 (1986) 563–597.
[11] K. Inoue, S. Shimada, Y. Minami, H. Morimura, A. Miyai, A. Yamauchi, M. Tohyama, Cellular localization of Na^+/*myo*-inositol cotransporter mRNA in the rat brain, Mol. Neurosci. 7 (1996) 1195–1198.
[12] H.M. Kwon, A. Yamauchi, S. Uchida, A.S. Preston, A. Garcia-Perez, M.B. Burgand, J.S. Handler, Cloning of the cDNA for a Na^+/*myo*-inositol cotransporter, a hypertonicity stress protein, J. Biol. Chem. 267 (1992) 6297–6301.
[13] L.M. Lewin, Y. Yannai, S. Sulimovici, P.F. Kraicer, Studies on the metabolic role of *myo*-inositol. Distribution of radioactive *myo*-inositol in the male rat, Biochem. J. 156 (1976) 375–380.
[14] Y. Minami, K. Inoue, S. Shimada, H. Morimura, A. Miyai, A. Yamauchi, T. Matsunaga, M. Tohyama, Rapid and transient up-regulation of Na^+/*myo*-inositol cotransporter transcription in the brain of acute hypernatremic rats, Mol. Brain Res. 40 (1996) 64–70.
[15] Y. Minami, S. Shimada, K. Inoue, H. Morimura, A. Miyai, A. Yamauchi, T. Matsunaga, M. Tohyama, Expression of Na^+/*myo*-inositol cotransporter mRNA in the inner ear of the rat, Mol. Brain Res. 35 (1996) 319–324.
[16] H. Morimura, S. Shimada, Y. Otori, A. Yamauchi, Y. Minami, K. Inoue, A. Miyai, I. Ishimoto, Y. Tanoand, M. Tohyama, Expression of Na^+/*myo*-inositol cotransporter mRNA in normal and hyperton stress rat eyes, Mol. Brain Res. 35 (1996) 333–338.
[17] T. Nakanishi, R.J. Turner, M.B. Burg, Osmorregulatory changes i *myo*-inositol transport by renal cells, Proc. Natl. Acad. Sci. USA 8 (1989) 6002–6006.
[18] A. Paredes, M. MacManus, H.M. Kwon, K. Strange, Osmoregula tion of Na^+-inositol cotransporter activity and mRNA levels in brai glial cells, Am. J. Physiol. 263 (1992) C1282–C1288.
[19] C.P. Reboulleau, Inositol metabolism during neuroblastoma B50 ce differentiation: effects of differentiating agents on inositol uptake, J Neurochem. 55 (1990) 641–650.
[20] C. Servo, J. Palo, E. Pitkanen, Polyols in the cerebrospinal fluid an plasma of neurological, diabetic and uraemic patients, Acta Neurol Scand. 56 (1977) 111–116.
[21] H.U. Shetty, M.B. Schapiro, H.W. Holloway, S.I. Rapoport, Polyo profiles in Down syndrome, J. Clin. Invest. 95 (1995) 542–546.
[22] S.L. Smith, M.V. Novotny, *myo*-Inositol levels in the cerebrospina fluid of infants, J. Chromatogr. 223 (1981) 173–175.
[23] D.G. Wilkinson, G. Peters, C. Dickson, Expression of the FGF-related proto-oncogene int-2 during gastrulation and neurulation in the mouse, EMBO J. 7 (1988) 691–695.
[24] A. Yamauchi, A. Miyai, S. Shimada, Y. Minami, Y. Tohyama, E. Imai, T. Kamada, N. Ueda, Localization and rapid regulation of Na^+/*myo*-inositol cotransporter in rat kidney, J. Clin. Invest. 96 (1995) 1195–1201.
[25] A. Yamauchi, S. Uchida, A.S. Preston, H.M. Kwon, J.S. Handler, Hypertonicity stimulates transcription of the gene for Na^+/*myo*-inositol cotransporter in MDCK cells, Am. J. Physiol. 264 (1993) F20–F23.
[26] C. Zhou, N. Agarwal, P.R. Cammarata, Osmoregulatory alterations in *myo*-inositol uptake by bovine lens epithelial cells, Invest. Ophthalmol. Vis. Sci. 35 (1994) 1236–1242.

Elsevier Science B.V.

Fax: (31) (20) 485 3271
Phone: (31)(20) 485 3474

Postal Address:
Brain Research
Elsevier Science B.V.
P.O. Box 2759, 1000 CT Amsterdam
The Netherlands

Courier Service Address:
Brain Research
Elsevier Science B.V.
Molenwerf 1, 1014 AG Amsterdam
The Netherlands

* * *

If you need information about your accepted manuscript, proof, etc. then phone or FAX us at the above numbers, stating the journal name and article code number. We can also FAX this journal's Instructions to Authors to you.

SUBSCRIPTION AND PUBLICATION DATA 1997

Brain Research (including **Molecular Brain Research, Developmental Brain Research, Cognitive Brain Research Brain Research Protocols** and **Brain Research Reviews**) will appear weekly and be contained in 57 volumes (120 issues): **Brain Research, Volumes 743–778** (36 volumes in 72 issues), **Molecular Brain Research, Volumes 41–50** (10 volumes in 20 issues), **Developmental Brain Research, Volumes 98–104** (7 volumes in 14 issues), **Cognitive Brain Research, Volume 5** (1 volume in 4 issues) **Brain Research Protocols, Volume 1** (1 volume in 4 issues) and **Brain Research Reviews, Volumes 24–25** (2 volumes in 6 issues). Please note that **Volumes 41–43** of **Molecular Brain Research, Volume 743** of **Brain Research** and **Volume 5** (issues **no. 1 and 2**) of **Cognitive Brain Research** were published ahead of schedule in 1996, in order to reduce publication time. The volumes remain part of the 1997 subscription year.

Separate subscriptions: Molecular Brain Research, Vols. 41–50, Developmental Brain Research, Vols. 98–104, Cognitive Brain Research, Vol. 5, Brain Research Protocols, Vol. 1 and **Brain Research Reviews, Vols. 24 and 25,** may also be ordered separately. Prices are available from the Publisher upon request. Subscriptions are accepted on a prepaid basis only, unless different terms have been previously agreed upon.

Subscription orders can be entered only by calender year (Jan.-Dec.) and should be sent to Elsevier Science B.V., Order Fulfillment Department, P.O. Box 211, 1000 AE Amsterdam, The Netherlands, Tel.: (31) (20) 485 3642, Fax: (31) (20) 485 3598, or to your usual subscription agent.

Postage and handling charges include surface delivery except to the following countries where air delivery via SAL (Surface Air Lift) mail is ensured: Argentina, Australia, Brazil, Canada, Hong Kong, India, Israel, Japan, Malaysia, Mexico, New Zealand, Pakistan, P.R. China, Singapore, South Africa, South Korea, Taiwan, Thailand and USA. For all other countries airmail rates are available upon request.

Claims for missing issues must be made within six months of our publication (mailing) date, otherwise such claims cannot be honoured free of charge.

Orders, claims, and product enquiries: please contact the Customer Support Department at the Regional Sales Office nearest you: **New York,** Elsevier Science, P.O. Box 945, New York, NY 10159-0945, USA. Tel: (+1) 212-633-3730, [Toll free number for North American customers:1-888-4ES-INFO (437-4636)], Fax: (+1) 212-633-3680, e-mail: usinfo-f@elsevier.com; **Amsterdam,** Elsevier Science, P.O. Box 211, 1000 AE Amsterdam, The Netherlands. Tel: (+31) 20-485-3757, Fax: (+31) 20-485-3432, e-mail: nlinfo-f@elsevier.nl; **Tokyo,** Elsevier Science, 9-15, Higashi-Azabu 1-chome, Minato-ku, Tokyo 106, Japan. Tel: (+81) 3-5561-5033, Fax: (+81) 3-5561-5047, e-mail: kyf04035@niftyserve.or.jp; **Singapore,** Elsevier Science, No. 1 Temasek Avenue, #17-01 Millenia Tower, Singapore 039192. Tel: (+65) 434-3727, Fax: (+65) 337-2230, e-mail: asiainfo@elsevier.com.sg

Advertising Information: Advertising orders and enquiries may be sent to: *International:* Elsevier Science, Advertising Department, The Boulevard, Langford Lane, Kidlington, Oxford, OX5 1GB, UK; Tel.: (+44) (0) 1865 843565; Fax: (+44) (0) 1865 843976. *U.S.A. & Canada:* Weston Media Associates, Dan Lipner, P.O. Box 1110, Greens Farms, CT 06436-1110, USA, Tel.: (203) 261 2500; Fax: (203) 261 0101. *Japan:* Elsevier Science Japan, Marketing Services, 1-9-15 Higashi-Azabu, Minato-ku, Tokyo 106, Japan; Tel.: (+81) 3 5561 5033; Fax: (+81) 3 5561 5047.

ADONIS Identifier. This Journal is in the **ADONIS** Service, whereby copies of individual articles can be printed out from CD-ROM on request. An explanatory leaflet can be obtained by writing to **ADONIS** B.V., P.O. Box 17005, 1001 JA Amsterdam, The Netherlands.

♾ The paper used in this publication meets the requirements of ANSI/NISO Z39.48-1992 (Permanence of Paper).