Characterization of the mice deficient in G-substrate, a PKG substrate.

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Introduction

NO(nitric oxide)-cGMP-PKG(cGMP-dependent protein kinase) pathway plays important role in the central nervous system. However, the restricted distribution of the pathway components and the lack of information on the substrate for the PKG have hindered research to examine the physiological roles of the cGMP pathway.

We have characterized G-substrate as an excellent PKG substrate and as a potent protein phosphatase inhibitor localized in cerebellar Purkinje cells. Further, we characterized the structure of mouse G-substrate gene and generated the G-substrate gene-deficient mice. The homozygote G-substrate knockout (KO) mice didn't show ataxia nor morphological abnormality in cerebellar cells. However, the homozygote mice showed reduced cerebellar LTD (long-term depression) temporarily around postnatal week (PW) 6 compared with wild-type (WT) mice. General behaviors of the mice are normal, however, the long-term adaptation optokinetic response (OKR) of eye movement was impaired without any affect on short-term adaptation of OKR.

PKG 基質G-substrate欠損マウスの特徴付け

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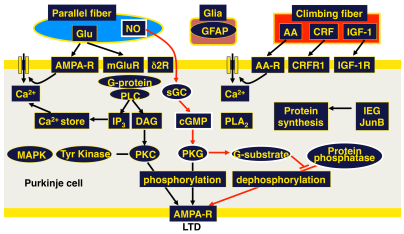
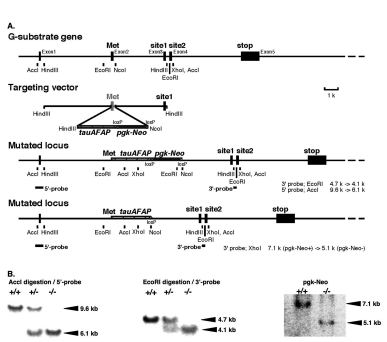
はじめに

NO(一酸化窒素)-cGMP-PKG(cGMP-依存性 kinase) 系は中枢神経系で重要な役割を果たしている。しかし、PKG基質の未同定、PKGが限られた細胞に発現しているなどの理由から、cGMP系の生理機能解析はcAMPに比較しておくれている。

我々は小脳プルキンエ細胞に存在するG-substrateを単離し、強力なタンパク質ホスファターゼ阻害機能を明らかにした。さらに、G-substrate遺伝子欠損マウスを作出して、その生理機能をさらに解析した。遺伝子欠損マウス(-/-)では小脳やプルキンエ細胞等に明らかな形態変化は観察されなかったが、発達段階で一時的に小脳LTDが減弱していた。さらに、小脳依存性学習である視機性眼球応答(OKR)の短期順応は正常であったが、長期順応が著しく障害されていた。NO系が特異的に長期記憶に関わる可能性を示唆している。

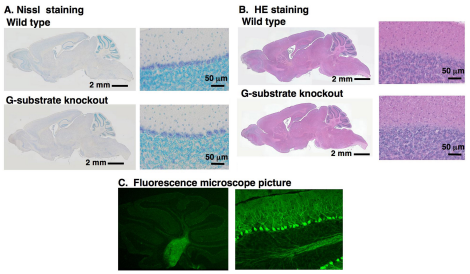


Generation of G-substrate KO mice.



(A) Mouse G-substrate gene structure and targeting vector for the generation of G-substrate KO mice.
(B) Restriction-enzyme-digested genomic DNA was subjected to Southern blot hybridization analysis. The band shift due to the homologous recombination was confirmed by probing the blot with ^{32}P-labeled 3'- and 5'-probes indicated in the figure. The removal of the selection marker, pgk-Neo cassette, was also confirmed by Southern blot analysis using the ^{32}P-labeled 3'-probe.

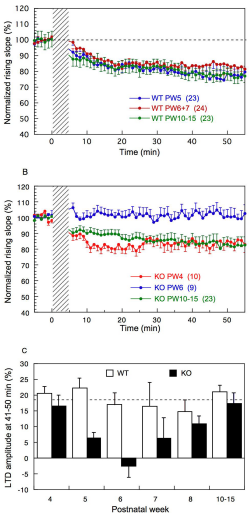
Histochemical examinations



Cerebellar structures of WT and KO mice.

(A)Nissl staining of cerebellar slices obtained from WT and KO mice. The thin sections (30 μm) were obtained from paraformaldehyde-fixed brain using a cryostat and were subjected to Nissl staining.
(B) HE (hematoxylin and eosin) staining of brain slices obtained from WT and KO mice.
(C) Fluorescence images of slice obtained from G-substrate KO mice. The frozen thin sections (20 μm) were observed under a fluorescence microscope. AFAP, a GFP derivative, was expressed under the control of the G-substrate promoter.

Electrophysiological examinations



Age-dependent expression of cerebellar LTD in slice preparation from G-substrate KO mice.

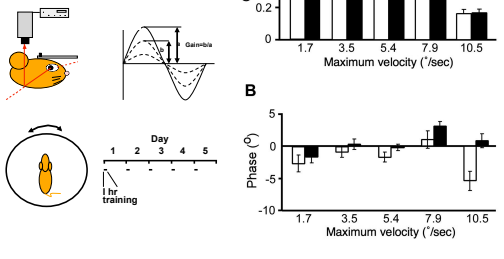
(A) Averaged time profile of 3 groups of WT Purkinje cells, shown with different colors. Ordinate, rising slope of PF-EPSPs relative to the average measured 5 min before conjunction. The shaded band indicates the application of conjunction of PF stimulation and depolarizing pulses. In brackets, number of Purkinje cells tested. Vertical bars, standard errors.
(B) Similar to (A) but for G-substrate-deficient Purkinje cells.
(C) Histograms showing LTD amplitude at 41-50 min for different PW (postnatal week) in WT and G-substrate-deficient Purkinje cells. Filled columns are G-substrate KO mice, and empty columns are WT littermates. Vertical bars, standard errors.
The numbers of cells used for the experiments are as follows; for WT mice PW4 (12), PW5 (23), PW6 (14), PW7 (10), PW8 (14), PW10-15 (23); for KO mice PW4 (10), PW5 (9), PW6 (9), PW7 (6), PW8 (11), PW10-15 (29).

Comparison of basic electrophysiological properties of Purkinje cells in the G-substrate gene knockout and wild-type mice.*

Electrophysiological properties	Age	Mice lines: Knockout mean ± SE (n)	Wild-type mean ± SE (n)
Membrane potential (mV)	PW4	-70.1 ± 1.9 (10)	-72.9 ± 0.5 (7)
	PW5	-62.7 ± 1.3 (22)	-62.7 ± 0.8 (22)
	PW6	-60.3 ± 1.4 (13)	-59.9 ± 1.0 (17)
	PW7	-59.8 ± 1.3 (9)	-61.6 ± 0.8 (21)
	PW8	-59.8 ± 0.4 (11)	-61.0 ± 1.4 (19)
	PW10-15	-60.3 ± 0.6 (56)	-60.9 ± 1.8 (16)
Membrane resistance (MΩ)	PW4	33.8 ± 0.8 (8)	33.1 ± 0.3 (9)
	PW5	33.7 ± 0.5 (20)	33.4 ± 0.7 (22)
	PW6	33.2 ± 0.9 (12)	30.0 ± 0.9 (17)
	PW7	30.3 ± 1.0 (9)	30.6 ± 0.8 (21)
	PW8	30.1 ± 0.7 (13)	30.1 ± 0.8 (19)
	PW10-15	30.1 ± 0.4 (59)	31.5 ± 0.9 (16)
PF-EPSP peak size (mV)	PW4	8.8 ± 0.3 (10)	8.3 ± 0.3 (10)
	PW5	8.6 ± 0.3 (23)	8.4 ± 0.3 (24)
	PW6	8.1 ± 0.3 (13)	8.9 ± 0.4 (19)
	PW7	8.3 ± 0.8 (9)	8.6 ± 0.4 (21)
	PW8	8.1 ± 0.7 (14)	8.2 ± 0.5 (19)
	PW10-15	8.1 ± 0.2 (57)	8.4 ± 0.2 (16)
PF-EPSP rise time (10-90%) (msec)	PW4	5.5 ± 0.5 (10)	5.6 ± 0.4 (10)
	PW5	5.9 ± 0.3 (23)	5.1 ± 0.2 (24)
	PW6	6.0 ± 0.5 (14)	4.6 ± 0.2 (19)
	PW7	4.1 ± 0.3 (9)	4.2 ± 0.3 (21)
	PW8	4.3 ± 2.4 (14)	5.0 ± 0.3 (19)
	PW10-15	4.4 ± 0.1 (37)	4.6 ± 0.3 (16)
PF-EPSP half width (msec)	PW4	26.3 ± 1.4 (10)	25.5 ± 0.9 (10)
	PW5	28.6 ± 0.8 (23)	25.2 ± 0.6 (24)
	PW6	28.3 ± 1.4 (14)	28.5 ± 1.2 (19)
	PW7	24.6 ± 1.4 (9)	23.5 ± 0.8 (21)
	PW8	23.7 ± 1.0 (14)	25.4 ± 1.0 (19)
	PW10-15	24.0 ± 0.5 (57)	24.3 ± 0.8 (16)
PF-EPSP paired-pulse facilitation (before/after conjunction)	PW4	1.34 ± 0.04(10)/ 1.31 ± 0.04(10)	1.42 ± 0.06 (9)/ 1.42 ± 0.09 (8)
	PW5	1.31 ± 0.04(13)/ 1.30 ± 0.04(11)	1.46 ± 0.07 (5)/ 1.46 ± 0.09 (5)
	PW6	1.37 ± 0.06(7)/ 1.34 ± 0.07 (8)	1.42 ± 0.03 (9)/ 1.35 ± 0.01 (8)
	PW7	1.34 ± 0.02(44)/ 1.31 ± 0.02(35)	1.39 ± 0.03 (32)/ 1.35 ± 0.02 (22)
	PW10-15	1.39 ± 0.02 (34)/ 1.41 ± 0.02 (34)	1.36 ± 0.04 (9)/ 1.31 ± 0.05 (6)
Climbing fiber responses Initial peak (mV)	PW6+7, PW10-15	86.1± 1.6 (5) 83.5± 3.3 (4)	80.0 ± 1.4 (10)
Duration (msec)	PW6+7 PW10-15	88.1 ± 9.0 (4) 81.8 ± 4.3 (4)	80.0 ± 4.1 (10)

*These data were analyzed statistically with factorial ANOVA. Genotype-dependent differences were observed only in paired pulse facilitation, which was slightly smaller in G substrate-deficient Purkinje cells than wild-type both before (P=0.017) and after (P=0.042) conjunction, but no other significant difference was observed in any of other listed items. Age-dependent variations were observed in membrane potential, membrane resistance, PF-EPSP rise time, and PF-EPSP half width (P<0.001), but not in others (P>0.05). The observed differences, however, were rather small, and their implications in LTD induction or OKR adaptation are unclear. CS, conjunctive stimulation.

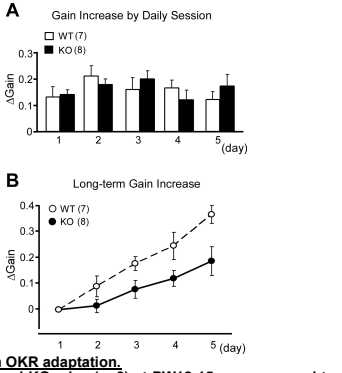
Behavioral examinations



OKR dynamics of WT and G-substrate KO mice.

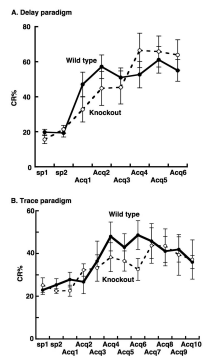
Left - Schematic diagram of OKR measurements. Eye movement induced by oscillating screen was observed through CCD camera. The gain is calculated maximum eye movement (b) divided by screen movement (a).

Right - Mice (12 week old) were subjected to sinusoidal screen oscillation (15° peak-to-peak, 0.11-0.33 Hz) at a maximum velocity of 1.7-10.5 °/s.
(A)Screen velocity-dependency of OKR gains.
(B) OKR phases. Positive and negative values respectively indicate phase delay and advance. Open columns are WT mice and filled columns are KO mice.



Long-term OKR adaptation.

WT (n=7) and KO mice (n=8) at PW12-15 were exposed to 1-hr sustained screen oscillation every day for 5 days. The mice were kept in the dark except during the screen oscillation. (A)Daily OKR adaptation. ΔGain, gain changes obtained after 1-hr oscillation on each day. Filled columns are KO mice, and empty columns are WT littermates.
(B) Cumulative OKR gain change induced through 5-day session. Ordinate, ΔGain increases from the start gains on each day as measured from the start gain on day 1. Open circles are for WT and filled circles for KO mice.



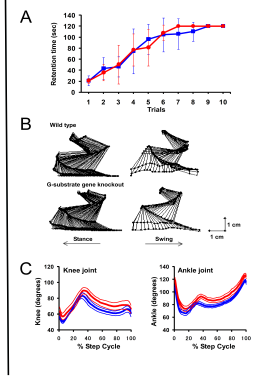
Eyeblink conditioning.

Performance was expressed as an index of CR% (the frequency of the conditioned response in the valid trials).

(A) The WT mice (n=11) and KO mice (n=10) were trained in a delay paradigm. In this paradigm, the conditioned stimuli preceded and co-terminated with the unconditioned stimuli.

(B) The WT (n=8) and KO mice (n=8) were trained in a trace paradigm. In the trace paradigm, a stimulus-free trace interval of 500 ms was interposed between the CS and the US.

Filled circles, WT mice; open circles, KO mice.



Locomotion test- Rotor rod and treadmill.

(A)The rotor rod test was carried out using WT (n=5, blue) and KO (n=6, red) mice at 8 rpm. Fall latency was measured.

(B) Representative stick figures of one complete step cycle (left panel, stance; right panel, swing) of a right hindlimb on the treadmill at a belt speed of 8 m/min.

(C) The joint angles of the knee and the ankle in sagittal plane are shown during a normalized step cycle in WT (blue, n=5) and KO mice (red, n=5). The patterns of the joint angular movements were similar in WT and KO mice. Both joints in KO mice were maintained in a slightly more extended position compared to those in the WT mice.

Conclusions

1) The G-substrate KO mice did not express neither mRNA nor protein of G-substrate and they survived and mate normally.
2) The G-substrate KO mice did not show ataxia and no significant difference was observed between WT and G-substrate KO mice in rotorod test and other general behavior analyses.
3) The temporal reduction of cerebellar LTD around 6-week old G-substrate KO mice may suggest the phosphtase mechanisms underlying cerebellar LTD might undergo the molecular change during development (See Launey et al, PNAS, 2004).
4) Slow long-term OKR adaptation in G-substrate KO mice may indicate the involvement of G-substrate in OKR consolidation.
5) The mice deficient in PKG also showed the impaired long-term adaptation in VOR without any effect on short-term one (Feil et al, 2003). NO pathway may be involved specifically in long-term memory in eye movement.

For further info, please refer to Endo et al, PNAS 106, 3525-3530, 2009.