Disruption of type 5 adenylyl cyclase negates the developmental increase in Gαolf expression in the striatum

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Abstract The two stimulatory G protein α subunits, Gαs and Gαolf, activate adenylyl cyclase in a similar way. We examined whether type 5 adenylyl cyclase knockout, the major striatal isoform, can differentially and/or developmentally change the expression of these G proteins in the striatum. Gαs and Gαolf expressions at birth were unaffected in knockouts, which, however, demonstrated a blunted developmental increase in Gαolf, but not Gαs. Adenylyl cyclase activity was unaffected at birth, but subsequently became lower in knockouts. These findings suggest that type 5 adenylyl cyclase does not contribute to striatal cAMP signaling at birth. However, it may play an important role in developmental changes in the expression of Gαolf, but not Gαs.

Key words: Adenylyl cyclase; Golf; Gs; Knockout; Striatum; Development

1. Introduction

Adenylyl cyclase (AC) is a membrane-bound enzyme that catalyzes the conversion of ATP to cAMP upon activation by G proteins, leading to a cascade of phosphorylation reactions within the cell [1,2]. There are at least nine isoforms of AC within the family, which show distinct biochemical properties and tissue distribution. Heterotrimeric G proteins consist of multiple subtypes, which are subdivided into at least four subfamilies. The stimulatory G protein subfamily is characterized by the presence of two G protein α subunits, Gαs and Gαolf, both of which can directly activate AC regardless of its isoform, at least in vitro. Gαs, a ubiquitous subtype, and Gαolf, a major olfactory subtype, share more than 80% amino acid identity, and it is thus believed that both have similar biochemical properties in vitro [3,4]. It remains poorly understood, however, whether the expression of both G protein species (Gαs and Gαolf) is similarly regulated by cAMP signal in intact animals.

Several studies have demonstrated that the adult striatum expresses Gαolf and type 5 AC (AC5) as the dominant subtype [5–7]. We were interested in whether changes at the level of AC can regulate the expression of G proteins, and if so, whether either or both AC and G proteins are developmentally regulated in the striatum. A very recent study from our laboratory has demonstrated that the expression of Gαs-like protein in the striatum was decreased in mice with a disrupted AC5 gene (AC5KO) without changes in the expression of other G protein species such as Gi [8]. AC5KO mice exhibited motor dysfunction that mimicked Parkinson's disease; this dysfunction was apparent in adults, but not in younger mice. In this regard, mice with disrupted D1 or A2a receptor genes demonstrated that the expression of Gαolf, but not Gαs, was affected [6], suggesting that Gαolf and Gαs are differentially regulated in vivo, although both have similar biochemical properties in vitro [9].

2. Materials and methods

2.1. Generation of knockout mice

The AC5 gene was disrupted by the homologous recombination technique in the exon with the first translation initiation site as described previously [8,10]. All mice were 129/SvJ-C57BL/6 mixed-background littermates from F1 heterozygote crosses. This study was approved by the Animal Care and Use Committee at both Yokohama City University School of Medicine and New Jersey Medical School.

2.2. Sample preparation for immunoblotting and AC assays

Mice were killed at postnatal day (P) 0, 7, 14, or 21. The brains were rapidly removed and the striatal tissues were dissected in ice-cold phosphate-buffered saline under a microscope, quickly frozen in liquid nitrogen, and stored at −80°C until use. After thawing, the tissues were homogenized in a buffer containing 50 mM Tris–HCl (pH 8.0), 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 200 mM sucrose, and a protease inhibitor mixture containing 20 μg/ml 1-chloro-3-tosylamido-7-amino-L-2-heptanone, 10 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, 50 U/ml egg white trypsin inhibitor, and 2 μg/ml aprotinin (buffer A) by using a glass homogenizer on ice. The homogenates were centrifuged at 500×*g* for 10 min at 4°C. The supernatants were retained and further centrifuged at 100 000×*g* for 30 min at 4°C. Crude membrane preparations were made by resuspending the pellet in the same buffer without EGTA (buffer B).

2.3. Immunoblot analysis

Western blotting for various G proteins and AC isoforms was conducted using the membrane preparation of striatal tissues at each developmental stage. Immunodetection was performed using the Chemiluminescent system (Amersham Pharmacia Biotech, Piscat-

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Fig. 1. Immunodetection of Gαolf and Gαs in the cortex, striatum, and heart. A commercial Gαs antibody (A), a Gαs-specific antibody (004/49SP) (B), and a Gαolf-specific antibody (186/49SP) were compared. Detection of Gαolf (Golf) and the short (Gs short) and long forms (Gs long) of Gαs is shown in wild type (WT) and AC5KO (KO) among the three tissues (10 μg from cortex, striatum, and heart).

away, NJ, USA). Antibodies to the above molecules were obtained from Upstate Biotechnology (Lake Placid, NY, USA), NEN Life Science Products (Boston, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). The specific antibodies for Gαs (004/49SP) and Gαolf (186/49SP) were developed by a member of the present team as previously described [6,11].

2.4. AC assay

AC assays were conducted in a reaction mixture containing 80 mM Tris malate (pH 7.4), 0.4 mM EGTA, 2 mM $MgSO_4$, 1 mM 3-isobutylmethylxanthine, 5 mM phosphocreatine, 50 U/ml creatine phosphokinase, 0.02% ascorbic acid, 0.1 mM ATP ($2–3\times10^6$ cpm of [^{32}P]ATP), and 40 μg of membrane protein in a final assay volume of 200 μl [12]. Assays were conducted at 30°C for 20 min. The reaction was terminated by the addition of 100 μl of 50 mM Tris–HCl containing 45 mM ATP and 2% sodium dodecyl sulfate. AC activity was linear within the incubation time up to 30 min.

2.5. Statistics

All data are given as means ± S.E.M. Statistical significance was determined using either Student's *t*-test or analysis of variance. $P < 0.05$ was taken as a minimal level of significance.

3. Results

3.1. Changes in G proteins

A commercially available Gαs antibody detected both 52- and 45-kDa bands in all three tissues (cortex, striatum, and heart) (Fig. 1A). The intensity of these two bands was similar between AC5KO and WT in the cortex and heart, but a difference was observed in the striatum. It should be noted that AC5 is abundantly expressed in both the striatum and heart, but the expression of G protein was different only in the striatum. In the striatum of AC5KO, the expression of the 52-kDa band remained unchanged from that in WT, but the expression of the 45-kDa band was decreased; this was in agreement with our previous findings [8]. The commercially available Gαs antibody may cross-hybridize to Gαolf because of their high amino acid sequence similarity, and thus we were interested in examining the expression of Gαolf in the striatum of AC5KO. We were also interested in developmental changes in G protein expression in AC5KO because they may be related to the development of motor dysfunction in AC5KO.

We examined the expression of Gαolf and Gαs using specific antibodies that can differentially detect each G protein species [6,11] (Fig. 1B,C). We found that the expression of Gαs, both the short and long forms, was similar between AC5KO and WT in all tissues (Fig. 1B). In contrast, the expression of Gαolf, which is most abundant in the striatum among the three tissues, was decreased in AC5KO. These findings suggested that AC5KO mice underwent a specific decrease in the striatal expression of Gαolf, but not Gαs.

We then examined the developmental changes of these G proteins in the striatum. It should be noted that motor dysfunction in AC5KO was apparent in adults, but not in early developmental stages [8], suggesting that the defect in striatal cAMP signaling occurs only in adults. In both WT and AC5KO, the expression of the long form of Gαs decreased and that of the short form of Gαs increased with development (P0 through P21), which was in agreement with previous reports [13,14] (Fig. 2). Importantly, the same changes occurred in AC5KO and WT. Developmental changes in the expression of Gαolf were similar to those of the short form of Gαs in WT; Gαolf levels increased dramatically with development, reaching a plateau after P14 (Fig. 3). This developmental increase, however, was blunted in AC5KO; the expression of Gαolf was similar until P7, but significantly lower in AC5KO than in WT after P14.

These findings suggested that the amount of G proteins (Gαolf and both forms of Gαs) was similar at birth, but the amount of Gαolf differed thereafter in AC5KO. We also examined changes in other G protein species, Gβ and Gαi, but found no difference between AC5KO and WT (data not shown). Because Gαolf is the dominant stimulatory Gα protein in the striatum, the impact of blunted developmental increase on cAMP signaling in the striatum of AC5KO is likely

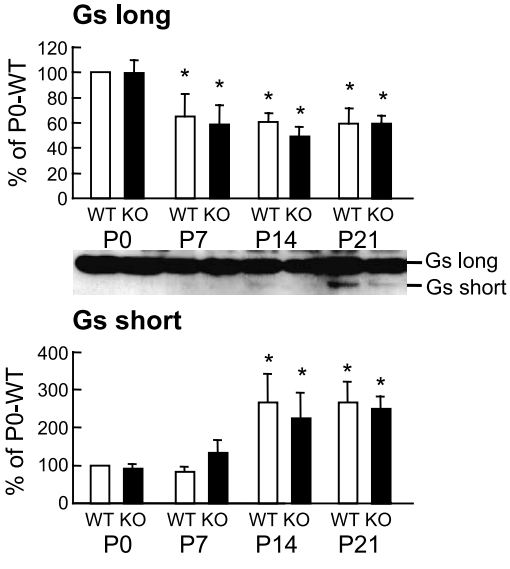

Fig. 2. Developmental changes in the expression of Gαs. Striatal membranes were prepared from wild type (open bars, WT) and AC5KO (closed bars, KO) at P0, P7, P14, and P21, followed by immunoblotting for Gαs. Representative photos of immunoblotting, using 10 μg of tissue protein per lane, are also shown (insets). Values are shown as a percentage of WT at P0. Means ± S.E.M. are shown. *$P < 0.05$ relative to P0, $n = 5$.

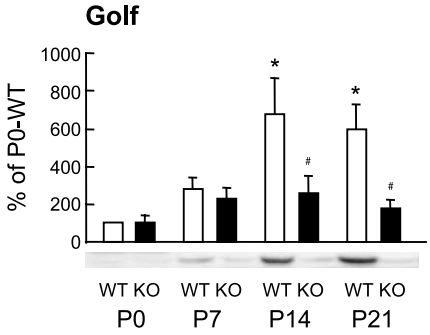

Fig. 3. Developmental changes in the expression of Gαolf. Striatal membranes were prepared from wild type (open bars, WT) and AC5KO (closed bars, KO) at P0, P7, P14, and P21, followed by immunoblotting for Gαolf (Golf). Representative photos of immunoblotting, using 10 μg of protein per lane, are also shown (insets). Values are shown as a percentage of WT at P0. Means ± S.E.M. are shown. $^{*}P < 0.05$ relative to P0, $^{\#}P < 0.05$ relative to WT, $n = 5$.

to be significant. Further, it is tempting to speculate that Gαolf and Gαs, both of which activate AC in a similar way in vitro, may be regulated differentially in the striatum.

3.2. Changes in AC

The above findings suggested a specific defect in the developmental increase of Gαolf in AC5KO. We thus examined whether similar changes occurred at the level of AC during development. AC activity was measured using striatal membrane preparations at different developmental stages (Fig. 4). Total AC activities, both basal and stimulated (NaF and forskolin), were markedly increased with development in WT. In AC5KO, however, total AC activities were increased, but reached a plateau from P7 to P14, which is reminiscent of changes in Gαolf (Fig. 3). AC activity at birth was not different between AC5KO and WT. The difference in AC activity between WT and AC5KO observed at P14 and P21 was greater upon forskolin stimulation than in the presence of NaF, suggesting changes in the composition of AC isoforms.

We thus examined whether changes in the expression of specific AC isoforms played a role in such alterations in AC activity. Among AC isoforms, AC3 and AC5 were readily detected in the striatum by the use of commercially available antibodies; however, other AC antibodies, including AC1 or AC2, did not readily detect signals in the striatum (data not shown). In WT animals, we found that the expression of AC5

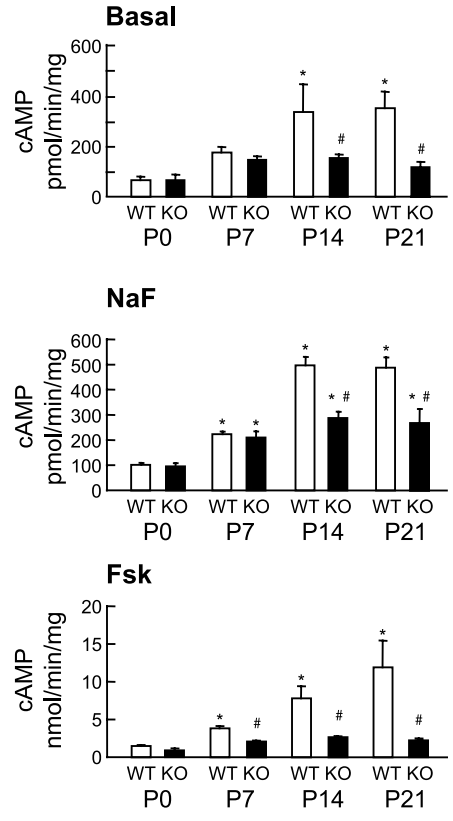

Fig. 4. Developmental changes in AC activity. Striatal membranes were prepared from wild type (open bars, WT) and AC5KO (closed bars, KO) at P0, P7, P14, and P21, followed by AC assays. AC assays were conducted in the absence (Basal) or presence of 10 mM NaF or 100 μM forskolin (Fsk). Values are shown as a percentage of WT at P0. Means ± S.E.M. are shown. $^{*}P < 0.05$ relative to P0, $^{\#}P < 0.05$ relative to WT, $n = 4$.

was hardly detectable at birth, but increased with development, reaching a plateau after P14 (Fig. 5). AC3 expression was similar to AC5, increasing with development and reaching a plateau after P14. Importantly, there was no difference in the expression of AC3 between AC5KO and WT, suggesting that there is no compensatory increase of AC3 in AC5KO. The lack of increase in AC activity in AC5KO after P7 most likely reflected a lack of AC5 expression in the striatum while the expression of other AC isoforms, such as AC3, appeared unaffected in AC5KO. More importantly, AC5 was not read-

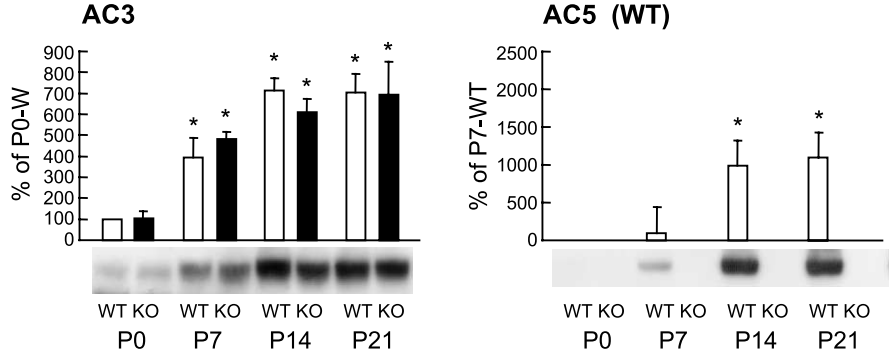

Fig. 5. Developmental changes in the expression of AC3 and AC5. Striatal membranes were prepared from wild type (open bars, WT) and AC5KO (closed bars, KO) at P0, P7, P14, and P21, followed by immunoblotting for AC3 and AC5. Representative photos of immunoblotting, using 25 μg of protein per lane, are also shown (insets). Values are shown as a percentage of WT at P0 (AC3) or P7 (AC5). Means ± S.E.M. are shown. $^{*}P < 0.05$ relative to P0, $^{\#}P < 0.05$ relative to WT, $n = 4$.

ily detectable and AC activity was similar at birth between AC5KO and WT, suggesting that AC5 does not contribute to striatal AC activity at birth, even though it is the major AC isoform in the adult striatum.

The above findings suggested that there was a defect in developmental increase of Gαolf in AC5KO. The magnitude of difference in cAMP signaling, as determined by AC activity, the amount of G proteins and AC isoforms, was insignificant at birth but increased significantly thereafter. This may explain, at least partially, why the apparent motor dysfunction in AC5KO was not obvious at birth. Our findings also suggest that the expression of Gαolf is affected developmentally by the activity of AC. It is interesting to note that the expression of Gαs, which can interact with AC5, was unchanged in AC5KO. Similarly, the expression of AC3, which can interact with Gαolf, was not altered in AC5KO either. Because both AC3 and Gαolf are originally found and dominantly expressed in the olfactory epithelium, no changes were expected in Gαolf expression without changes in AC3 expression. Perhaps Gαolf interacts more closely with AC5 than with AC3 in the striatum. This interaction may play an important role in the regulation of dopaminergic signaling in the basal ganglia [15]. In support of this hypothesis, in Gαolf knockout mice, the locomotor responses to cocaine or amphetamine, which also require dopaminergic signaling and AC [16], were attenuated [15]. To examine the molecular mechanisms underlying the changes of Gαolf, we conducted a series of Northern blot analyses. However, we found no changes in the mRNA expression of Gαolf between AC5KO and WT (data not shown), suggesting that the change in G protein levels occurred post-transcriptionally.

Our results also suggested that AC5 is the dominant AC isoform in the striatum in adults, but not at birth because membrane AC activity was similar between AC5KO and WT. AC5 expression is increased with development in both the striatum and heart [17], where AC5 is the dominant isoform. More importantly, the disruption of AC5 did not alter this developmental increase of another AC isoform, AC3, suggesting the absence of compensatory changes in other AC isoforms. It is tempting to speculate that Gαolf levels were regulated not only by the usage of Gαolf-stimulating receptors [6], but also by the presence of its effector AC isoform in the striatum.

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