Title
Biochemical characterization of G protein-gated inwardly rectifying potassium channels in mammalian brain

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Biochemical Characterization of G Protein-Gated Inwardly Rectifying Potassium Channels in Mammalian Brain

by

Yaping Joyce Liao

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience Graduate Program

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

To Lily

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Acknowledgment

Many believe that fairytales do not exist in today's world. We have no fireball-spitting, man-eating, bigger-than-the-world dragons. Princesses are hard to find, and Princeses, well, chivalry is mostly dead. How lucky for me that I have a modern fairytale to tell. My story does not involve finding my prince-charming, although I did, nor has its type ever existed in the storybooks. My fairytale is about getting a Doctor of Philosophy in Neuroscience. It involves creatures as fierce as menacing dragons, challenges as insurmountable as slaying a cyclop, and a happy ending that wraps up all good tales. At UCSF, I went through a training process in which a graduate student learns how to think about scientific problems and, more importantly, carries out a successful project of her choosing. To earn my rite-of-passage, I did not slay any monsters, but I managed to publish despite nasty reviewers who stomped on my hardwork. I encountered competitors both in and outside the lab that got me stressing, working, and maturing. During these years of spending many nights and weekends at lab working like a powertool, I was watched over by many angels and Santa's helpers who made sure I took babysteps toward my goals. My mission here is to let these heavenly creatures know how much I have learned from them and will always appreciate having had them as part of my life.

I want to thank Lily for her guidance, teaching, and constant encouragement. I was extremely lucky to have met her and worked in her laboratory. When she received the first set of reviewers' comments for my paper, Lily kept emphasizing how good they were, fearing that my young ego was too delicate for the often harsh tone of such comments. Although she is seemingly sweet and complaisant, Lily also acts as a gentle tyrant who makes sure good science is done in the lab and people are treated fairly. Since she is so resourceful, my project took many more exciting turns than I ever imagined possible. I dedicate my thesis to her, and hope we will always be colleagues.

I also want to thank faculty and administrators of the Neuroscience Graduate Program and the Medical Scientist Training Program for the wonderful academic experience I have had. I especially want to thank my thesis committee or, as Lily called it, my "Fan Club" Lou Reichardt, Cori Bargmann, David Bredt, Brian Kobilka, and unofficial member Yuh Nung Jan for taking the time to have scientific and personal discussions with me. I thank them, especially Lou, for telling me that I have done enough to graduate, since I really had no idea what constitutes a Ph. D. thesis. I want to thank Bill Mobley, my advisor in the Neuroscience Program and role model for pediatric neurology, for always making sure I am happy and doing well in the lab. I am grateful to Patricia Arrandale for organizing program activities and for many fun chats. I especially want to thank Tris Parslow and Jana Toutolmin from the Medical Scientist Training Program for constantly cheering me on. Without their advice and superb organization, I could not have survived my MSTP experience.

I especially enjoyed my rotation projects with Morgan Sheng (Lily Jan), Monica Vetter (Mike Bishop), and Liqun Luo (Yuh Nung Jan). In particular, I want to thank Monica for being the first to tell me that I have done enough for my Ph. D. I want to thank Mike Bishop for helping me realize that I was really interested in neuroscience and should join the Neuroscience Graduate Program. I want to thank some people for collaborations and for providing research materials that made my projects possible: Paul Slesinger, Nila Patil, Markus Stoffel, Stefano Signorini, Jay Brenman, David Bredt, Isabel Fariñas, and Lou Reichardt.

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Wang, Erica Wolff, Jian Yang, Sunny Yee, Susan Younger, Jennifer Yu, Mei Yu, Biao Zhao, Chaoyang Zeng, and Weimin Zhong. Thanks for long conversations about science, life, and the pursuit of happiness. Thanks for all the advice during group meetings and technical help. Thanks for helping me organize an amazing wedding. I also want to thank people in the Gitcher lab for adopting me and letting me use their lunchroom. If I ever have a lab in the future, I would fill it with wonderful people like them. I have especially enjoyed late nights with fellow graduate-student/slave Dave Byrd. Best luck with river kayaking and Belgium.

Through activities and lab, I have befriended many people in the UCSF community. I want to thank my medical school friends, especially Priscilla Hsue, Shelley Chu, and Joe Chou, for loads of fun and meetings over dessert, where we commiserate on working too hard and being "MSPTed-up." I want to thank students in the Neuroscience Program, especially Takao Hensch, Julia Walbridge, Steve Gomperts, and my classmates, for a lot of great memories. I regret not spending more time in graduate school just so I can get to know them better.

I want to thank my prince-charming Tom for the joy he brings me everyday. I never imagined that sharing a life with someone could be so utterly amazing. Thanks for making sure that I eat, exercise, and have fun.

I want to thank my parents and family for being my best friends and cheerleaders. My mom, in particular, has suffered extreme mental anguish due to my scientific pursuits. In high school, Mom used to go to lab with me on weekends because she did not feel safe leaving me alone at lab. She also thought reseach was doing some real damage to my mental balance because I sleepwalked looking for my "enzyme" (I was trying to purify the ADP-ribosyl transferase at the time). In college, she was afraid to call me too often at night, fearing that she would find me at lab instead of safely in my dorm. In graduate school, she simply stopped calling me at lab. My parents have sacrificed greatly to bring me to the United States for education. All my accomplishments and honors are really theirs.

San Francisco, September 20, 1996

Chapter 1

The text of this chapter is a reprint of the material as it appears in *The Journal of Neuroscience in press*. The coauthors listed in this publication Lily Jan and Yuh Nung Jan directed and supervised the research which forms the basis for the dissertation.

Chapter 2

The work presented was done in collaboration with Stefano Signorini in Markus Stoffel's laboratory in the Laboratories of Metabolic Diseases at Rockefeller University. The transgenic mice as well as DNA and RNA data were generated by S. S. Y. J. L. showed that the mice are functional GIRK2 knockout mice and generated all the biochemical analyses including Figures 2A and 3. Stephen A. Duncan contributed to the intellectual designs of the paper.

Appendix

The text of this chapter is a reprint of the material as it appears in *Nature* 365:72-75, 1993. This work was done in collaboration with Morgan Sheng in Lily Jan's laboratory. Y. J. L. and M. S. contributed equally to the scientific design of the experiments. Y. J. L. conducted preliminary experiments using brain membrane biotinylation and subsequent immunoprecipitation with antibodies against Kv1.1-1.5 and Kv4.2 that identified Kv1.2 and Kv1.4 as subunits that associate *in vivo*. Y. J. L. also generated Figures 2 and 3. M. S. generated Figure 1 and the SDS part of Figure 3 and collaborated with Y. J. L. on Figure 2. Lily Jan and Yuh Nung Jan directed and supervised the research.

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List of Abbreviations

A_1: adenosine receptor subtype

ABC: ATP-binding cassette

ACh: acetylcholine

ad: anterodorsal nucleus of the thalamus

ATP: adenosine triphosphate

Ba^{2+}: barium

CA1-3: regions of hippocampus

CB: cerebellum

cRNA: complementary RNA

C-terminus: carboxyl-terminus of protein

CP: caudate-putamen

CTX: cortex

D_1 or D_2: dopamine receptor subtypes

DA: dopamine

dcn: deep cerebellar nuclei

DM, VM, Tu Cin: dorsomedial, ventromedial and tuber cinereum areas of the hypothalamus

dpc: days postcoitus

EEG: electroencephalogram

EGL: external granule cell layer of cerebellum

g: granule cell layer of dentate gyrus or cerebellum

GABA: γ-aminobutyric acid

GIRK: G protein-gated inwardly rectifying potassium channel

GIRK2wv: GIRK2 gene with the G156S *weaver* mutation

GDP: guanosine diphosphate

GTP: guanosine triphosphate

H5: pore-lining region of potassium channels

HP: hippocampus

5-HT: serotonin

IGL: internal granule cell layer of cerebellum

I_{KACh}: acetylcholine-activated potassium current

K^+: potassium

K_{ATP}: ATP-sensitive potassium channels

Kir: inwardly rectifying potassium channels

KCNJ7: human GIRK2

m: molecular layer of dentate gyrus or cerebellum

M1 and M2: transmembrane regions of inward rectifier potassium channels

mmb: *minibrain*

MPTP: N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

N-terminus: amino-terminus of protein

$NMDG^+$: N-methyl-D-glucamine

OB: olfactory bulb

OT: optic tract

P: Purkinje cell layer of the cerebellum

PD: Parkinson's disease

P_K and P_{Na}: permeability for potassium and sodium, respectively

P_K/P_{Na}: permeability ratio of potassium over sodium.

PND: postnatal day

Rb^+: rubidium

S1-S6: transmembrane regions of voltage-gated potassium channels

sl: stratum lucidum of the hippocampus; region where the mossy fiber tract lies

slm: stratum lacunosum moleculare of the hippocampus

SN: substantia nigra, including pars compacta and reticulata

SNc: substantia nigra pars compacta

SNr: substantia nigra pars reticulata

so: stratum oriens of the hippocampus

SOM: somatostatin

SP: substance P

sr: stratum radiatum of the hippocampus

SUR: sulfonylurea receptor

Th: thalamus

VTA: ventral tegmental area

wv: *weaver*

Experimental Goals

G protein-gated inwardly rectifying K^+ channels (GIRK) are known effectors of seven-transmembrane transmitter receptors such as receptors for dopamine, acetylcholine, somatostatin, and opioid peptides. The GIRK2 channel subunit has been identified as the gene whose mutation causes the *weaver* mouse phenotype of ataxia, dopamine deficienty, seizures, and infertility, implicating the role of neuronal excitability in developement. This study examines the distribution and association of GIRK2 and related channel subunits in the rat and mouse brains, their defects in mice with gain-of-function (the *weaver* mice) or loss-of-function mutation of GIRK2, and potential functions of GIRK channels in different brain regions.

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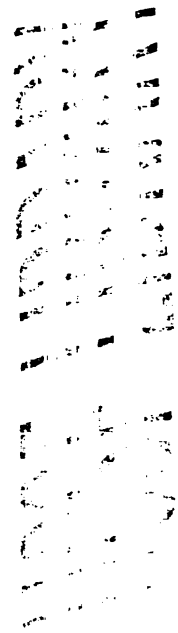
Chapter 1: Introduction



Introduction Outline

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Inwardly Rectifying Potassium Channels

Electrophysiological recordings of Kir currents in different cell types

Inwardly rectifying potassium currents (Kir) have been recorded in simple as well as complex organisms including four kingdoms prokaryotes, yeast, plants, and animals (Jan and Jan, 1996). Kir currents maintain the membrane potential near the equilibrium potential of K^+, participate in the modulation of action potential waveforms and neurotransmitter release, as well as buffer K^+ in the extracellular space. In mammals, they are involved in many important functions such as the control of cardiac function during parasympathetic activation and metabolic blockade (Noma, 1983; Isenberg et al., 1983; Trube and Hescheler, 1984; Vleugels et al., 1990; Nichols and Lederer, 1991) and regulation of insulin secretion in the pancreas (Ashcroft et al., 1984; Cook and Hales, 1984). Kir currents are also found in skeletal muscle (Katz, 1949) and smooth muscle (Standen et al., 1989; Daut et al., 1990; Nelson et al., 1990; Dart and Standen, 1993; Zhang et al., 1994). In the central nervous system, Kir currents are important in EEG arousal (Rainnie et al., 1994) and regulation of dopamine (Kim et al., 1995) and growth hormone release (Bernardi et al., 1993). They are also found in neurons in the olfactory cortex (Constanti and Galvan, 1983), cerebral cortex (Ohno-Shosaku and Yamamoto, 1992), hypothalamus (Pennefather et al., 1988; Ashford et al., 1990; Sims et al., 1991; Bernardi et al., 1993; Barros et al., 1996), dorsal raphe nucleus (Williams et al., 1988; Penington et al., 1993), spinal cord (Nelson and Frank, 1967), and other brain regions (see G protein-gated Kir currents downstream of seven transmembrane receptors) as well as in glia (review Sontheimer, 1994; Newman, 1985, 1986; Ransom and Sontheimer, 1994; Karschin and Wischmeyer, 1995).

Kir currents can be distinguished by several criteria. First, they exhibit inward rectification on the current-voltage plot. Inwardly rectifying channels allow more ions to enter than to exit the cell, probably due to the blockage of the channel pore by cations such as magnesium and polyamines (Matsuda et al., 1987; Vandenberg, 1987; Ficker et al., 1994;

Lopatin et al., Fakler et al., 1995). At more hyperpolarized membrane potentials, the channels are activated, and ions pass through the channel pore into the cell. At more depolarized membrane potentials, the channel can be strongly or weakly inhibited and passes no or little ions out of the cell. Second, the current reverses near the K^+ equilibrium potential and is sensitive to the extracellular concentration or the electrochemical driving force of K^+. Third, the channels are selective for K^+ ion. When external K^+ is replaced by other ions such as Na^+ or N-methyl-D-glucamine ($NMDG^+$), very little current can be conducted through the channels.

G protein- and receptor-activated Kir currents *in vivo*

One type of inwardly rectifying K^+ current is regulated by heterotrimeric G proteins and serves as effectors of seven-transmembrane receptors such as receptors for acetylcholine (ACh), dopamine (DA), somatostatin (SOM), substance P (SP), and opioid peptides. One such Kir current in the heart is activated by acetylcholine, the first known chemical transmitter (Loewi, 1921). Binding of acetylcholine to the m2 muscarinic acetylcholine receptor in the cardiac atrium activates an inwardly rectifying K^+ current (Trautwein and Dudel, 1958; Noma et al., 1979). Opening of the K^+ channel hyperpolarizes membrane of the pacemaker cells to produce the parasympathetic reduction of heart rate and contractility. The Kir channel opening is mediated via activation of heterotrimeric G proteins (Pfaffinger et al., 1985; Breitwieser and Szabo, 1985; Logothetis et al., 1987; Yatani et al., 1987, 1988; Brown and Birnbaumer, 1990; Kurachi et al., 1992; Wickman and Clapham, 1995a,b) and is "membrane-delimited" since no soluble second messenger is required (Sakmann et al., 1983; Soejima and Noma, 1984).

Many examples of G protein-regulated Kir currents downstream of receptors have been found in different brain regions such as the substantia nigra, locus coeruleus, and nucleus basalis of Meynert. The substantia nigra pars compacta (SNc) in the ventral midbrain is one of the major brain nuclei that contain dopaminergic neurons, and the DA

neurons in SNc projects axons to the striatum and dendrites to the substantia nigra pars reticulata (SNr). The SNc DA neurons are known to release DA not only from the axon terminal in the striatum but also from the cell bodies and dendrites in the SN (Cheramy et al., 1981). The somatodendritically released DA binds to D_2-like autoreceptors on SNc DA cell bodies and dendrites and inhibits the activity of these cells (Aghajanian and Bunney, 1977; Lacey et al., 1987; Napier et al., 1986; Pinnock, 1984). The somatodendritic autoreceptors are important in the self-regulation of DA neurons, control of neurotransmitter release, and control of the activity of non-DA cells in the SN. The inhibitory effects of the D_2 autoreceptors are carried out via different second messenger including inhibition of adenyl cyclase (Stoof and Kebabian, 1984) and activation of an inwardly rectifying potassium current (Lacey et al., 1987; Kim et al., 1995). Activation of Kir current occurs following application of DA or quinpirole (a D_2 receptor agonist) to highly purified primary cultures of SNc neurons, and can be blocked by S(-)-sulpiride (a D_2 receptor antagonist). This DA effect is blocked by treatment with pertussis toxin (Kim et al., 1995), which is known to block the autoreceptor-mediated inhibition of SNc neurons (Innis and Aghajanian, 1987). Intracellular application of non-hydrolysable GTP analogue, guanosine 5'-O-(3-thiotriphosphate), can produce an initial spontaneous increase in an inwardly rectifying potassium current, and in a cell that is loaded with a poorly metabolized GDP analogue, guanosine 5'-O-(2-thiodiphosphate), the response to quinpirole is reduced (Kim et al., 1995).

The locus coeruleus consists of tightly packed noradrenergic neurons and is the major supplier of noradrenaline in the brain. Substance P, an excitatory neurotransmitter, inhibits an inwardly rectifying K^+ current (Koyano et al., 1991, 1993; Shen and North, 1992), and somatostatin, an inhibitory neurotransmitter, increases an Kir current (North et al., 1987; Inoue et al., 1988). The SP inhibition of Kir current occurs via a pertussis toxin-insensitive G protein (Nakajima et al., 1988, 1991a), while the SOM activation of Kir occurs via a pertussis toxin-sensitive G protein (North et al., 1987; Inoue et al., 1988; Grigg et al., 1996; Nakajima

et al., 1996). Activation of Kir by SOM can be suppressed by SP, suggesting that the action of the two neurotransmitters converge in the same effector (Velimirovic et al., 1995).

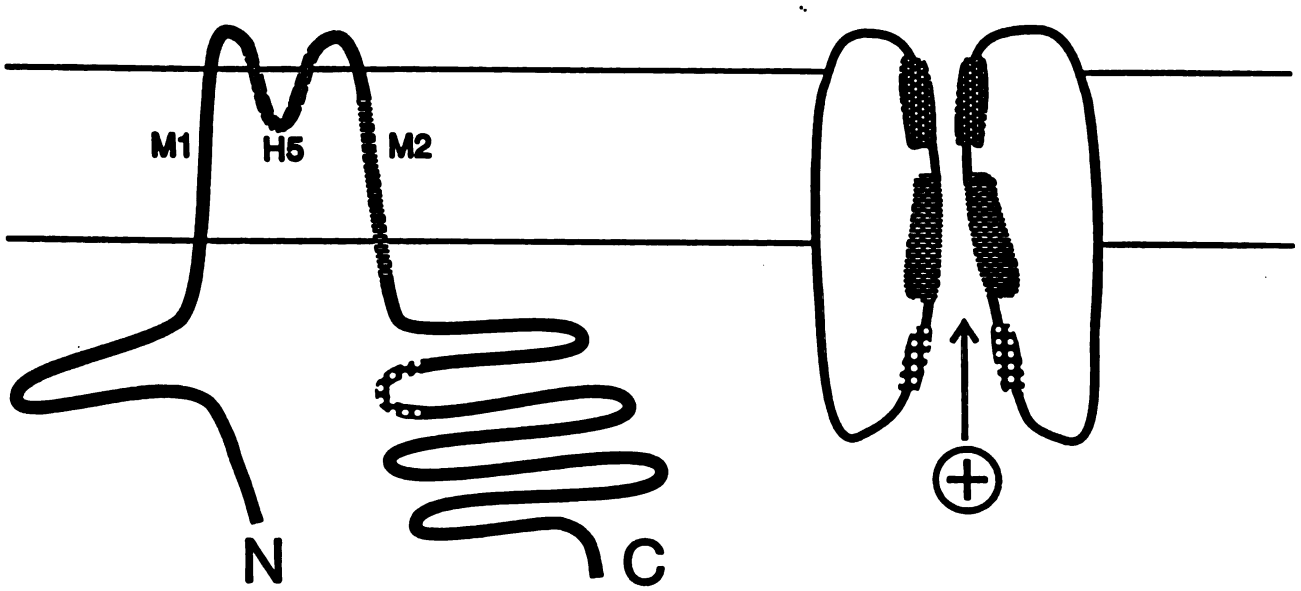
In the nucleus basalis of Meynert, a group of cholinergic neurons in the basal forebrain, application of SP also gives rise to membrane excitation with an associated decrease in membrane conductance, especially if the cells are hyperpolarized. This membrane conductance inhibited by SP is an inwardly rectifying K^+ current (Stanfield et al., 1985; Yamaguchi et al., 1990), and the inhibition occurs via a pertussis toxin-insensitive G protein (Nakajima et al., 1988, 1991b). Since SP levels in the basal forebrain (Dietl et al., 1986) and cerebral cortex (Beal and Mazurek, 1987; Crystal and Davies, 1982) are reduced in Alzheimer patients, it is possible that the lack of SP inhibition of Kir currents may have contributed to the observed pathologies.

Cloning of Kir channels

While Kir currents have been identified since the 1970's, genes that encode for this family of channel subunits were not cloned until recently. The expression cloning of ROMK1 (Ho et al., 1993) and IRK1 (Kubo et al., 1993) was followed by homology screening resulting in cloning of at least 14 channel subunits that are divided into 6 subfamilies based on sequence homology (Chandy and Gutman, 1995; Doupnik et al., 1995).

Structure-function studies

All Kir channel subunits cloned so far possess two transmembrane regions (M1, M2), a pore-lining H5 region, and intracellular N- and C-termini (Doupnik et al., 1995; Jan and Jan, 1996). They are homologous to the second half (S5-H5-S6) of the six transmembrane voltage-gated K^+ channel family (Kubo et al., 1993). It has been postulated that voltage-gated K^+ channel subunits evolved from the Kir subunits by the addition of the voltage sensor and other transmembrane regions (Jan and Jan, 1994). Indeed, a voltage-gated K^+ channel Kv1.1 can be converted from a depolarization-activated channel with outward


M1
H5
M2
N
C

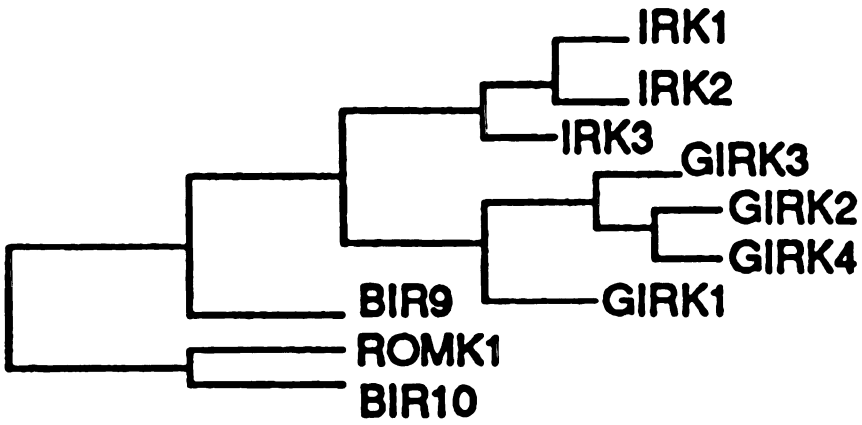

IRK1
IRK2
IRK3
GIRK3
GIRK2
GIRK4
GIRK1
BIR9
ROMK1
BIR10

Figure 1. A. Structural elements of inwardly rectifying K^+ channel include the H5 and M2 segments, and part of the C-terminal hydrophilic domain as part of the permeation pathway. From Jan and Jan, 1996. **B.** Familiy tree of inward rectifier K^+ channels based on sequence homology.

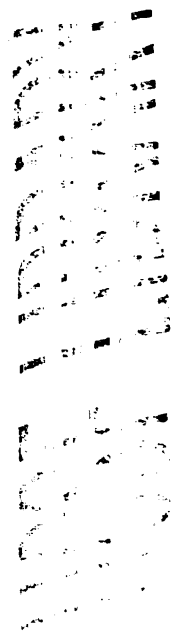


Table 1: Cloned Kir channel subunits

Kir1 or ROMK

Kir1.1 Ho et al., 1993

Kir1.2 Zhou et al., 1994

Kir2 or IRK

Kir2.1 Kubo et al., 1993; Morishige et al., 1993; Ishihara and Hiraoka, 1994; Ishii et al., 1994; Raab-Graham et al., 1994; Ashen et al., 1995

Kir2.2 Koyama et al., 1994; Takahashi et al., 1994

Kir2.3 Lesage et al., 1994; Morishige et al., 1994; Bond et al., 1994; Perier et al., 1994; Tang and Yang, 1994; Makhina et al., 1994; Falk et al., 1995; Collins et al., 1996

Kir3 or GIRK

Kir3.1 Kubo et al., 1993; Dascal et al., 1993; Kobayashi et al., 1995

Kir3.2 Lesage et al., 1994, 1995; Tsaur et al., 1995; Bond et al., 1995; Isomoto et al., 1996

Kir3.3 Lesage et al., 1994

Kir3.4 or CIR Ashford et al., 1994; Krapivinsky et al., 1995

Kir3.5 Hedin et al., 1996

Kir4 or BIRK1

Bond et al., 1994; Bredt et al., 1995

Kir5

Bond et al., 1994

Kir6 or uKATP

Kir6.1 Inagaki et al., 1995a

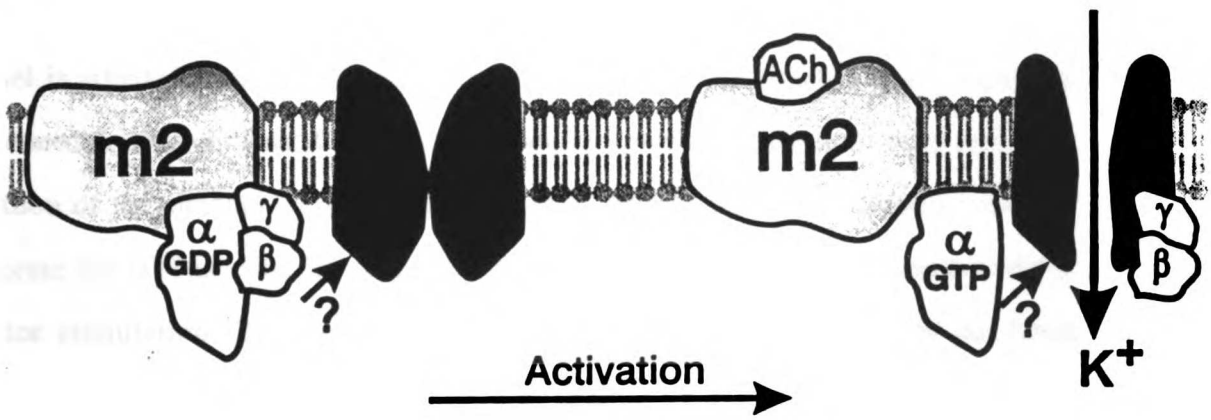
Kir6.2 Inagaki et al., 1995b

rectification to a hyperpolarization-activated channel with inward rectification by deletion of the S1-S4 domain (Tytgat et al., 1994).

The Kir channel subunits diverge in the first half of the N-terminus and the second half of the C-terminus. The second half of the N-terminus, two transmembrane regions, H5 region, and first half of the C-terminus are relatively well conserved among all family members. The functions of the conserved regions in the N- and C-terminus are not clear. They may mediate interaction with each other or with other proteins that are important in the structure and function of Kir channel subunits. There is evidence that the N-terminus (Fink et al., 1996), the M2 transmembrane region, and the first half of the C-terminus (Tinker et al., *submitted*) are important for both homo- and heteromultimerization of channel subunits. The N- and the C-terminus have also been shown to be important for interaction with G proteins (Kunkel and Peralta, 1996; Huang et al., 1996)

Site-directed mutagenesis of cloned channels has faciliated the structure-function studies of Kir channels. Mutation of a conserved glycine in the H5 region of GIRK2 abolishes channel selectivity (Slesinger et al., 1996; Kofuji et al., 1996; Navarro et al., 1996), similar to mutation of the analogous residue in voltage-gated K^+ channels (Heginbotham et al., 1994). A residue in the second transmembrane segment seems to be important for K^+ selectivity and channel blockade by cytoplasmic cations such as magnesium and polyamines (Stanfield et al., 1994; Lu and MacKinnon, 1994; Wible et al., 1994; Reuveny et al., 1996). Since a residue in the C-terminus is also important for K^+ permeation and inward rectification, it is possible that part of the C-terminus may contribute to the channel pore (Taglialatela et al., 1994, 1995; Yang et al., 1995a). This residue in the C-terminus is acidic (aspartate) in strongly rectifying K^+ channels such as Kir2 and Kir3, and the analogous residue is neutral (asparagine) in weakly rectifying K^+ channels such as Kir1 and Kir6, indicating that this residue is important in shaping the rectification profile determined by affinity for blocking cations like magnesium and polyamines.

A



B

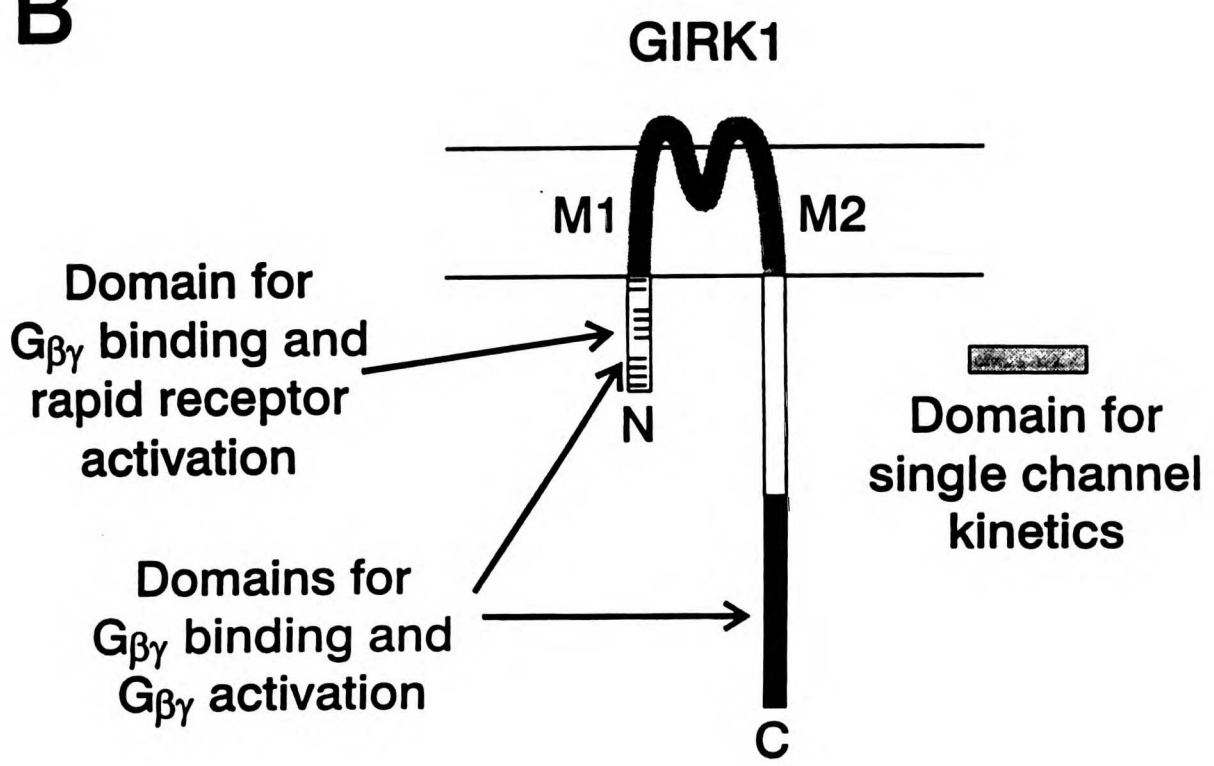


Figure 2. Proposed mechanism of G protein activation of muscarinic K^+ channel.

A. Schematic representation of the working model on the activation of the GIRK1 channel following muscarinic stimulation. At rest, the muscarinic receptor, inactive G protein heterotrimer (Gαβγ), and the GIRK1 channel may form a local complex that is specified, in part, by the binding interaction of the G protein with the GIRK1 channel. Because the channel is situated near the receptor-G protein complex, the channel is exposed to a high local concentration of "free" Gβγ subunits upon receptor stimulation, thereby enabling rapid activation of the channel. The interaction of the channel with the activated Gα-GTP may accelerate the GTPase activity of the Gα-GTP, promoting rapid deactivation at the end of receptor stimulation (Breitwieser and Szabo, 1988). **B.** A summary of the functional domains implicated in G protein activation of GIRK1. The hydrophobic domain (M1-H5-M2) of GIRK1 contains a gate governing the single-channel openings. The N- and C-terminal hydrophilic domains of GIRK1, on the other hand, contain regions important for the Gβγ binding (Huang et al., 1995) and Gβγ activation of the GIRK1 channel. The N-terminal domain of GIRK1 also facilitates the rapid activation following receptor stimulation and binds the Gαβγ heterotrimer (Huang et al., 1995). From Slesinger et al., 1995.

Reconstitution of K_{ATP}

Expression of different channel subunits in heterologous systems gives rise to inwardly rectifying K^+ currents that resemble various Kir currents observed *in vivo*. Some channel subfamilies give rise to ATP-inhibitable K^+ currents (K_{ATP}) that may correspond to Kir currents important in K^+ excretion in the kidney (Kir1), insulin-secretion in pancreatic β cells (Kir6), and cellular protection of neurons in hypoxic or low oxygen states (Kir6? Kir4? Kir2?). The molecular equivalent of K_{ATP} *in vivo* may consist of at least two different subunits: the channel pore-forming α subunit and the β subunit which are members of the ATP-binding cassette (ABC) superfamily of twelve-transmembrane receptors (Higgins, 1995). Kir6.2 has been shown to interact with the sulfonylurea receptor (SUR), a member of the ABC superfamily, to give rise to a Kir current that is very sensitive to ATP (μM range) and can be inhibited by sulfonylurea drugs such as glibenclamide and activated by K^+ channel openers such as pinacidil (Inagaki et al., 1995b). Since sulfonylurea drugs are commonly used to treat non-insulin-dependent diabetes mellitus (adult-onset, type II), identification and characterization of the molecular basis for the sulfonylurea-sensitive K_{ATP} current is crucial for rational drug design and has tremendous clinical applications. In addition to Kir6.2, a strongly rectifying potassium channel IRK3 or Kir2.3 can also be inhibited by higher concentration of ATP (mM range), but its function is not clear (Collins et al., 1996).

G protein- and receptor-regulation of cloned Kir channels

Kir3 or GIRK channel subunits may form the molecular basis for Kir currents that are gated by heterotrimeric G proteins downstream of seven transmembrane receptors. *In vivo*, receptors that activate Kir channels to hyperpolarize the cell membrane include those for acetylcholine (m_2), adenosine (A_1), ATP (P_2), dopamine (D_2), γ-aminobutyric acid ($GABA_B$), opioid (μ, δ, κ), serotonin (5-HT_1; 5-HT_2), norepinephrine (α2), and somatostatin (reviews North et al., 1987; North, 1989; Brown, 1990; Nicoll et al., 1990; Inoue et al.,

1992). Other receptors such as those for substance P, neurotensin, thyrotropin-releasing hormone, and angiotensin II inhibit K_{ir} channels to depolarize the cell membrane (reviews listed above; Stanfield et al., 1985; Yamaguchi et al., 1990; Takano et al., 1995). Receptor-mediated G-protein regulation of different Kir currents have been described in both pre- and postsynaptic membranes (North et al., 1987; North, 1989; Nicoll et al., 1990; Brown, 1990; Inoue et al., 1992), so these Kir currents may be involved in the regulation of transmitter release as well as neuronal excitability.

GIRK channel have been shown to be activated by G proteins and seven transmembrane receptors (Kubo et al., 1993; Dascal et al., 1993; Lester and Dascal, 1993; Reuveny et al., 1994; Wickman et al., 1994; Takao et al., 1994; Slesinger et al., 1995; Kunkel and Peralta, 1995). Both N- and C-termini of GIRK channel interact with both Gα and Gβγ subunits (Kunkel and Peralta, 1995; Huang et al., 1995; Slesinger et al., 1995). When GIRK subunits are co-expressed with a seven transmembrane receptor, G protein-activated Kir currents can be activated by agonist application. GIRK1 channels can be activated by m_2 ACh receptor (Kubo et al., 1993b; Dascal et al., 1993; Gadbut et al., 1994), β_2-adrenergic receptor (Lim et al., 1995), δ opioid receptor (Dascal et al., 1993), μ opioid receptor (Chen and Yu, 1994; Kovoor et al., 1995), κ opioid receptor (Ma et al., 1995; Henry et al., 1995), and 5-HT_{1A} receptor (Dascal et al., 1993; Kovoor et al., 1995). GIRK2 can be activated by δ opioid receptor (Lesage et al., 1994; Kofuji et al. 1995) and m_2 receptor (Slesinger et al., 1996), and GIRK4 by m_2 receptor (Krapivinsky et al., 1995). While *in vitro* evidence shows that many neurotransmitter receptors have the potential to regulate Kir channels composed of GIRK subunits, it is not known just which receptors and GIRK channels are coupled to mediate the neurotransmitter effects on cell excitability in the brain.

Heteromultimerization of GIRK channel subunits

Kir channel subunits are homologous to the carboxyl-terminal half of voltage-gated K^+ channels, which are known to assemble into homo- as well as hetero-tetrameric channels

(Jan and Jan, 1992, 1994; Green and Millar, 1995; MacKinnon, 1991; Liman et al., 1992; Sheng et al., 1993 [appendix 2]; Wang et al., 1993). There is recent experimental evidence that four subunits of IRK1 form a functional Kir channel (Inanobe et al., 1995; Yang et al., 1995). *In vitro* co-expression experiments have shown that GIRK channel subunits can form homo- or heteromeric channels that are activated by neurotransmitter receptors and G-proteins. Co-expression of GIRKs 1 and 2 or 1 and 4 in heterologous systems gives rise to more robust currents than the sum of currents due to expression of either subunits alone, suggesting that heteromeric channels can form (Kofuji et al., 1995; Duprat et al., 1995; Krapivinsky et al., 1995; Lesage et al., 1995).

In the heart, GIRK1 has been shown to co-immunoprecipitate with GIRK4 (Krapivinsky et al., 1995) and the GIRK1/GIRK4 heteromeric channel may form the basis for the muscarinic ACh receptor-activated K^+ current that slows down the heart rate (Kurachi, 1994). GIRK1 and GIRK2 have been shown to co-immunoprecipitate when co-expressed in TSA201 cells (Lesage et al., 1995) or COS cells (Navarro et al., 1996). It is not known whether G protein-regulated Kir channels of different compositions exist in various brain regions and in different subcellular compartments.

The *weaver* mouse

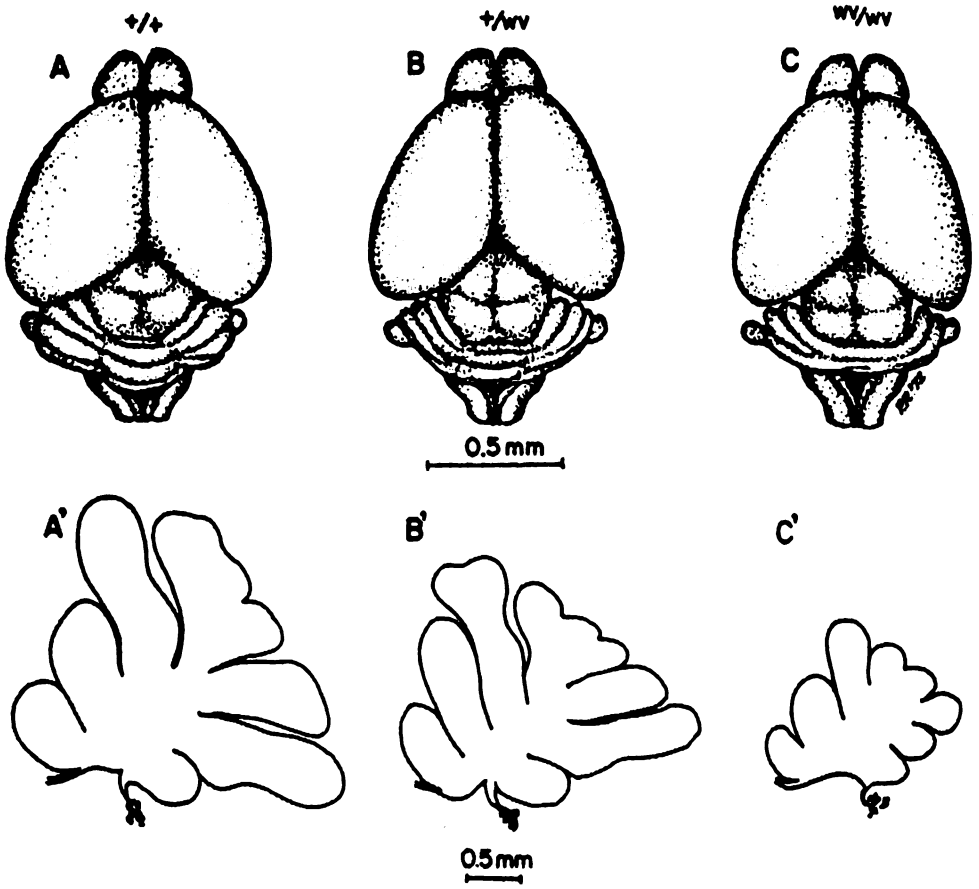
The *weaver* (*wv*) mouse arose spontaneously in the Jackson Laboratories and was identified in 1964 due to its apparent neurological problems including "instability of gait and posture, hypotonia, tremor, " dopamine deficiency, and spontaneous seizures (Lane et al., 1964; Sidman et al., 1965). Identification and characterization of the *weaver* gene have sustained almost 40 years of intense, and often controversial, scientific investigation. There is strong evidence that the uncoordinated movement or ataxia is due to the degeneration of cerebellar neurons (Rakic and Sidman, 1973b,c; Sotelo and Changeux, 1974; Hatten et al., 1984), and that the dopamine deficiency is due to death of midbrain dopaminergic neurons (Lane et al., 1977; Roffler-Tarlov and Graybiel, 1984; Gupta et al., 1987; Bayer et al., 1995). Other phenotypes including the propensity to undergo tonic-clonic seizures (Eisenberg and Messer, 1989) and male sterility due to hypospermatogenesis and degeneration of testicular support cells (Vogelweid et al., 1993; Harrison and Roffler-Tarlov, 1994) are still not well understood. While ataxia, dopamine deficiency, and male sterility are more pronounced or only evident in the homozygous *weaver* mice, spontaneous seizures are more common in the heterozygous *weaver* mice in at least four independent *wv* mouse colonies (Seyfried, 1982; Goldowitz and Koch, 1986; Eisenberg and Messer, 1989; Triarhou and Ghetti, 1989). Seizures can be induced in the *wv/wv* mice by subjecting them to behavioral tests (Goldowitz and Koch, 1986). It has been hypothesized that the seizures observed in both *wv/+* and *wv/wv* mice may be partly due to the reduction of SNc DA dendrites in the midbrain of *wv/+* mice (Triarhou and Ghetti, 1989). While other brain areas have not been identified to be seizure-genic in the *wv* mice, there is one report of disorganization of pyramidal neurons in the CA3 area of the hippocampus, an area that is well-known to be seizure-prone (Sekeguchi et al., 1995). In the rest of the introduction, I will focus on the pathology found in the cerebellum and midbrain in *wv/wv* and *wv/+* mice. In these two brain regions, the *wv* gene product has been postulated to play a role in differentiation, neuronal migration, as well as

survival, and there is evidence that *wv* function is be cell-autonomous (Goldowitz, 1989; Goldowitz and Mullen, 1982) or non-autonomous (Gao et al., 1992; Gao and Hatten, 1993;.

Cerebellum

Given that granule cells, Purkinje cells, and Bergmann glia exhibit structural and physiological abnormalities in the *weaver* cerebellum, I should first briefly describe their development in normal mice (Rakic and Sidman, 1973b,c; Hatten et al., 1984, 1986; Willinger and Margolis, 1985). Granule cells are born at the rhombic lip near the ventricular zone and migrate to the external granule cell layer. They undergo mitosis in the germinal zone of the external granule cell layer (EGL) to give rise to 6-8 rows of granule cells that span about 50 microns. After exit from mitosis, they assume a bipolar shape to send out processes parallel to the pial surface (the parallel fibers). Upon contact with processes of the Bergmann glia, granule cells migrate perpendicular to and away from the pial surface to form the internal granule cell layer (IGL). The movement of the granule cell soma to the IGL depends on the actively exploring club-shaped growth cone and elongation of the leading neurite, which occurs in a rapid and discontinuous fashion. There is a small amount of membrane rippling of the neurite shaft which ceases at the termination of growth. The soma does not move and the cell body membrane is quiescent during elongation. The nucleus and surrounding organelles then translocate into the cytoplasm of the leading neurite, leaving a lagging neurite behind. In this fashion, the granule cells migrate along the radial processes of the Bergmann glia, pass the developing Purkinje cell layer, to form the IGL. The EGL virtually disappears by postnatal day (PND) 15, leaving the molecular layer composed of parallel fibers and some interneurons.

As early as PND 5 by light microscopy and PND 10 by gross examination, differences in the size of the cerebella of +/+, *wv*/+, and *wv*/*wv* mice can be found, while the number and relative size of the lobules appear the same (Rakic ant Sidman, 1973b,c). The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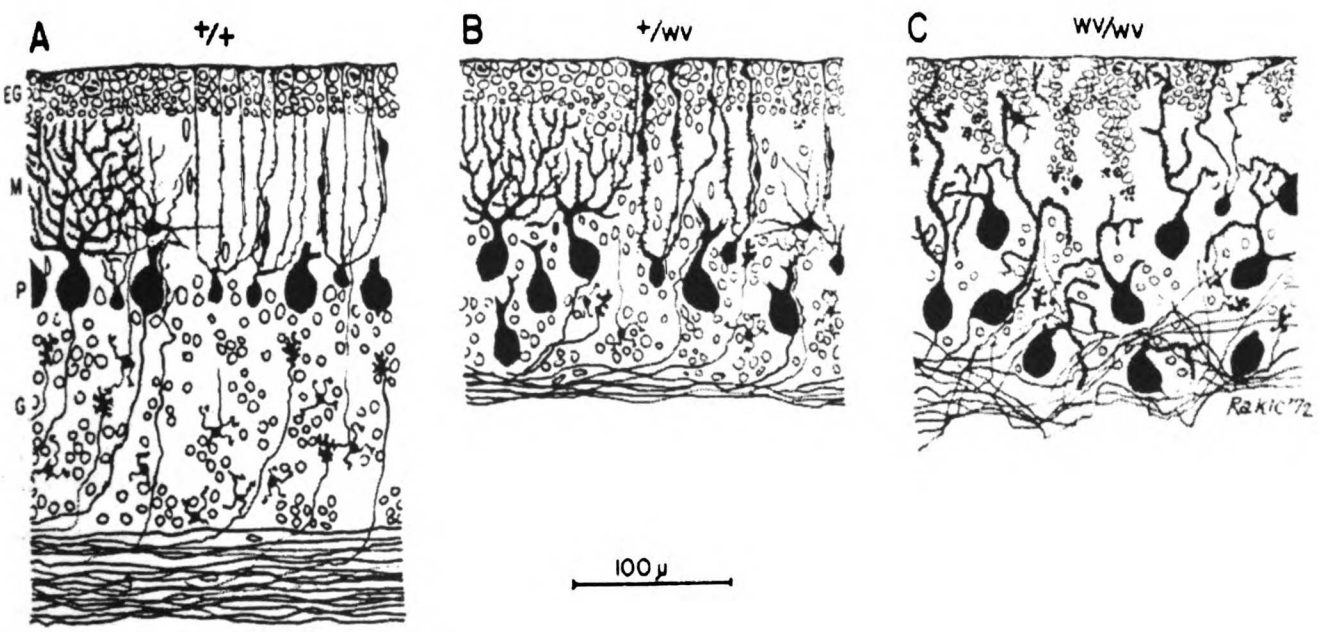
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Figure 3.

A. Gross anatomy of brains from wildtype, *+/wv*, and *wv/wv* mice. From Rakic and Sidman, 1973a. B. Histology of cerebellum from wildtype, *+/wv*, and *wv/wv* mice. From Rakic and Sidman, 1973b.

size reduction in the cerebella of *wv/+* and *wv/wv* mice has been attributed to the apoptotic death of granule cells, since granule cells comprise the largest neuronal population in the cerebellum (Rakic and Sidman, 1973a,b; Smeyne and Goldowitz, 1989; Gillardon et al., 1995; Migheli et al., 1995). The *wv/wv* granule cells have been carefully studied in many aspects of their development: mitosis, neurite outgrowth, interaction with Bergmann glia, and migrational behavior. These studies include analysis of these neurons *in vivo* as well as in primary cell cultures. The granule cell precursors proliferate normally in the germinal zone of the external granule cell layer (Rezai and Yoon, 1972), and they exit from the mitotic zone to the premigratory zone (Rakic and Sidman, 1973b). At the premigratory zone of *wv/wv* cerebellum, there is a prominent layer of pyknotic cells, and the majority of the surviving granule cells does not assume the bipolar shape (Rakic and Sidman, 1973b,c). No parallel fibers are observed in the developing molecular layer, probably because there is little granule cell neurite formation in the *wv/wv* cerebellum. Neurite formation has been studied in more detail by time-lapse microcinematography of developing *wv/wv* granule cells in culture. While the rate of neurite initiation of the *wv/wv* granule cells is greater than that of the wildtype cells, the average neurite length as well as rate of neurite extension of *wv/wv* granule cells are much less, probably because *wv/wv* granule cells tend to retract and re-initiate their neurites (Willinger and Margolis 1985a,b). Interestingly, the *wv/wv* granule cells exhibit constant movement of the cell body, neurite surface, and growth cones, and the cell body of some *wv/wv* granule cells migrates rapidly back and forth along the neurite, instead of in one direction. The activity level is especially elevated right before cell death, in contrast to the normally quiescent behavior of cells targeted for apoptosis. Due to abnormalities in neurite extension and migration, most granule cells arrest in differentiation and eventually die in the external granule cell layer, close to the site of their genesis (Rakic and Sidman, 1973b,c).

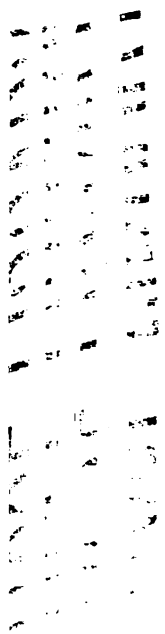
Abnormalities in not only granule cells but also Purkinje cells and Bergmann glia can be observed in the developing *wv/wv* cerebellum. In the absence of external and internal

granule cell layers, the Purkinje cells in *wv/wv* cerebellum are not organized into a discrete layer, and their dendrites often orient randomly. An elevated number of dendritic spines is present on Purkinje dendrites, although most of them do not form functional synapses (Rakic and Sidman, 1973b). The Bergmann glial fibers are abnormally thick and less elaborate. The early *wv* papers also suggest that abnormalities in the Bergmann glia observed early in development, perhaps due to cell-autonomous effects of the *wv* gene, could have contributed to the aberrant neuronal phenotype in the *wv/wv* cerebellum (Rakic and Sidman, 1973a,b,c; Hatten et al., 1984). Because the development of Bergmann glia depends on the normal development of the granule cells (Hatten et al., 1984; Willinger and Margolis, 1985a,b), and that wildtype granule cells can migrate on *wv/wv* glia (Hatten et al., 1986), it is more likely that the defect in Bergmann glia is secondary to the granule cell abnormalities.

The fairly normal histology of adult *wv/+* granule cells in the cerebellum explains the apparently recessive effect of the *wv* mutation on cerebellar function. *wv/wv*-like abnormalities in the developing *wv/+* cerebellum, however, constitute strong evidence that the *wv* mutation functions in a semi-dominant, dosage-dependent manner. During development of *wv/+* cerebellum, there is an elevated number of degenerating granule cells and Bergmann glial cells, a broadening and slightly irregular organization of the Purkinje cell layer, and a decrease in the size of the molecular layer (Rezai and Yoon, 1992; Rakic and Sidman, 1973b,c). In primary cell culture, *wv/+* granule cells do elaborate extensive neurites but exhibit slower growth cone advancement, increased frequency of neurite retraction, and increased membrane movements of the neurite and soma during and after elongation, resulting in slower migration rate (Willinger and Margolis, 1985a,b). In many respects, the developmental abnormalities in *wv/+* cerebellum appear intermediate to those of wildtype and *wv/wv* cerebella.

Midbrain

Dopaminergic neurons in the midbrain are found in the A8 (retrorubral area), A9 (substantia nigra), and A10 (ventral tegmental area) cell groups. The A9 DA neurons are found mostly in the substantia nigra pars compacta (SNc) region, which is several cell layers thick and forms a medioventral to laterodorsal diagonal line in the ventral midbrain. The dendrites of the SNc DA cells project and synapse with neurons in the substantia nigra pars reticulata (SNr), which is composed of mostly GABAergic and a few DA neurons. The axons of the SNc DA cells project to the striatum to form the nigro-striatal pathway that regulates motor behavior. The A10 DA cells are found medial to the A9 DA cells and project to the ventral striatum (e.g. nucleus accumbens) to form the mesolimbic pathway that is important for emotion and addiction. Both A8 and A10 DA cells also project to various brain regions, including the cerebral cortex. The number of DA neurons in the A8-10 cell groups can vary in a strain-and species-specific manner (Smith et al., 1990).



Other than the obvious cerebellar defects, the level of dopamine is about 50% decreased in the forebrain of adult *wv/wv* mice (Lane et al., 1977), while there is no major difference between wildtype and *wv/+* (Simon et al., 1994). The dopamine deficiency seems region specific (see Table below) and can be explained by the degeneration of specific DA cell groups.

At PND 7, the number and spatial distribution of tyrosine hydroxylase (TH)-positive mesencephalic neurons in *+/+*, *wv/+*, and *wv/wv* midbrain are similar, so early developmental steps are normal in the *wv/wv* midbrain. As early as PND 20, however, the dendrites of the SNc DA neurons that extend into the SNr are severely reduced in the *wv/+* mice, and the remaining dendrites are disorganized and thin. The dendrites of the *wv/+* DA neurons in the SNc are reduced by 60% while the number of DA neurons in the midbrain, DA content in the neostriatum, and pattern of synaptic connectivity of nigrostriatal axon terminals are normal (Triarhou and Ghetti, 1989; Roffler-Tarlov et al., 1996). In the PND 20 midbrain of *wv/wv* mice, however, there is a 42% decrease of SNc DA cells as well as dramatic loss of

Table 2: Dopamine levels in different *weaver* brain regions

Dorsal striatum	30%
Ventral striatum*	100%
Midbrain	70%
Olfactory tubercle#	73%

*includes nucleus accumbens and ventral caudate-putamen

#receive projections from non-nigral midbrain DA neurons, A8 and A10 groups

(Schmidt et al., 1981, 1982; Roffler-Tarlov and Graybiel, 1984; Richter et al., 1992)

Table 3: Decrease of midbrain dopaminergic neurons in *wv/wv* mice

	PND 20	PND90
A8 (retrorubral field)	0%	56%
A9 (subst. nigra)	42%	69%
A10 (ventral tegm area)	0%	26%

(Triarhou et al., 1986, 1988; Gupta et al., 1987)

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dendrites. Upon close examination, the greatest loss of TH-positive cells occurs in the ventral portion of the SNc (Roffler-Tarlov and Graybiel, 1984; Smith et al., 1990). This cell population is believed to provide the majority of inputs to the striatal patches (Gerfen et al., 1987). The loss of the ventral SNc DA neurons, along with the observed loss of TH-positive axon terminal in the dorsal striatum, explains the dramatic loss of DA and TH levels in both of these brain areas. There is also loss of cells in the mid-SNc region, the retrorubral area, as well as in the ventral tegmental area that may be responsible for the loss of TH-positive terminals in the striatal matrix and cerebral cortex (Graybiel et al., 1988).

Consistent with the DA deficiency and cell loss in the *wv/wv* mice, there are alterations in the proteins that are important in different aspects of DA metabolism in the DA neurons. There seems to be a decrease in the activity of tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis (Schmidt et al., 1982; Richter et al., 1992; Simon et al., 1994). Due to decreased DA levels, there is also a reduction in the DA transporter activity that is responsible for the recovery of DA after its release into the synaptic cleft. There is 50% decrease at PND 3 and 70% decrease at older ages in the DA uptake in the striatum (Pullara and Marshall, 1989; Richter et al., 1992; Simon et al., 1994). There is also increased D_2 receptor binding sites (^{3}H-spiperidol) in dorsal but not ventral striatum and decreased D_1 receptor binding (^{3}H-SCH23390) throughout the striatum (Pullara and Marshall, 1989). As a result of the above changes, the *wv/wv* mice exhibit behavioral sensitivity to DA receptor agonists, apomorphine and pergolide (Schmidt et al., 1982)

Several factors have been identified that are associated with the increased vulnerability of particular DA neurons in the *weaver* mouse, including absence of calbindin-D28K and late-generation during development. In the *wv/wv* mice, calbindin-positive neurons in SNr and VTA are spared as compared to the dramatic cell loss found in the calbindin-negative SNc neurons (Gaspar et al., 1994). Calbindin is a calcium-binding protein, and calcium-buffering may contribute to resistance to metabolic insults. Differences in the time of origin between wildtype and *wv/wv* DA neurons have also been found. Most

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wv/wv DA neurons are generated early from E10-12, while more wildtype neurons are generated slightly later (Bayer et al., 1995a,b).

The pattern of cell loss and DA deficiency in the *weaver* mice closely resembles those of the Parkinson's Disease (PD) (Roffler-Tarlov and Graybiel, 1984). It is possible that the fine tremor in *weaver* mouse (Triarhou and Getti, 1989) and the resting tremor seen in PD patients are both due to death of dopaminergic cells in the substantia nigra. The calbindin-negative dopaminergic neurons (SNc; the nigrostriatal pathway) are also more vulnerable in idiopathic PD or in N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD, while the calbindin-positive neurons such as those in the ventral tegmental area (the mesolimbic pathway) are relatively spared (Yamada et al., 1990; Gaspar et al., 1994). While the similarity in cell loss between PD and *weaver* mice is striking, such pattern of cell loss is also found in other conditions such as nigrostriatal degeneration (Fearnley and Lees, 1991; Yamada et al., 1990) or in experimentally induced nigral cell death (Iacobino et al., 1992; Okamura et al., 1995). Bilateral transplantation to the striatum of mesencephalic cell suspensions is an experimental procedure used to treat PD in humans, and *weaver* mice grafted with DA cells perform slightly better in several tests of motor skills such as the rotor-rod equilibrium test as well as in their locomotor coordination and activity (Triarhou et al., 1995).

There are some major differences between PD and *weaver* mice. Behaviorally, the *weaver* mice exhibit ataxia due to profound loss of cerebellar granule cells; the PD patients exhibit akinesia and rigidity. Some brain regions that are affected in PD such as the nucleus raphe dorsalis and locus coeruleus (Gibb, 1989) are not known to be defective in the *weaver* mice (Gupta et al., 1987; Lane et al., 1977). The major damage to the mesocortical DA projections in PD is not observed in the *weaver* mice (Gaspar et al., 1994). The H5 region of GIRK2, location of the *weaver* mutation, was analyzed in 50 cases of both idiopathic and familial PD patients (Bandmann et al., 1996). The H5 region of human GIRK2 (KCNJ7) only differs in two nucleotides from that of mouse GIRK2. Sequencing of blood or brain

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DNA finds no difference between these 50 patients and controls. While it is unlikely that the *wv* mutation causes human PD, the striking similarity in the degeneration of SNc DA cells may indicate that this neuronal population is more vulnerable to certain types of insults. The *wv* mice is still one of the best animal models for PD and can be used to screen treatment options for PD patients.

Identification and distribution of the *weaver* gene

The *weaver* locus has been mapped to chromosome 16 in the mouse, which corresponds to the Down's syndrome regions of human chromosome 21 (Reeves et al., 1989). Utilization of the detailed maps in both human and mouse chromosomes in this region facilitated the identification of two candidate *weaver* genes: a previously cloned G protein-gated inwardly rectifying potassium channel (*girk2*) and a gene that is important in neurodevelopment in the fly (*minibrain* or *mmb*) (Patil et al., 1995). Sequencing of both genes in the *wv* mice reveals no change in *mmb* but a missense mutation in a residue (G156S) (Patil et al., 1995) that is believed to line the ion permeation pathway of potassium channels (Heginbotham et al., 1994).

Consistent with its identification as the *wv* gene, GIRK2 mRNA is found in brain regions known to be affected by the *wv* mutation such as the cerebellar granule cells and substantia nigra (Kobayashi et al., 1995; Patil et al., 1995; Karschin et al., 1996; Kofuji et al., 1996; Navarro et al., 1996), and both GIRK1 and GIRK2 proteins are expressed in cerebellar granule cells and Purkinje cells during development (Slesinger et al., 1996). Based on in situ hybridization studies, GIRK1, GIRK2, GIRK3, but not GIRK4 mRNAs can be found at 14.5 days postcoitus (dpc) in the ventricular zone of the cerebellar anlage including the rhombic lip, the area from which granule cell precursors originate (Kofuji et al., 1996; Mjaatvedt et al., 1995). GIRK1, GIRK2, and GIRK3 transcripts can be seen in the external and developing internal granule cell layers between 17.5 dpc and PND 6 (Patil et al., 1995; Mjaatvedt et al., 1995; Kofuji et al., 1996; Navarro et al., 1996). GIRK2 mRNA distribution

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in the cerebellum exhibits no gradient or temporal differences in development (Kofuji et al., 1996).

Electrophysiological properties of GIRK2*wv* channels in *Xenopus* oocytes

The *wv* mutant form of GIRK2 (G156S) causes the homomeric GIRK2 channels to be non-selective and conduct sodium as well as potassium ions. Expression of GIRK2 and m2 muscarinic acetylcholine receptor cRNAs in *Xenopus* oocytes gives rise to a carbachol-induced, fast-activating potassium current that exhibits inward rectification. This current can be constitutively activated by GTPγS or co-expression of Gβγ cRNAs, and exhibits strict potassium selectivity (P_K/P_{Na} = 20) (Slesinger et al., 1996; Kofuji et al., 1996; Navarro et al., 1996; Surmeier et al., 1996). The expression of low amount of GIRK2G156S (from now on referred to as GIRK2*wv*) and m2 receptor cRNAs gives rise to elevated basal current and a reduced carbachol-inducible current. The expression of high amount of GIRK2*wv* cRNA with m2 receptor gives rise to high basal current that cannot be further induced with agonist application. GIRK2*wv* channels exhibit reduced selectivity for potassium and allow more Na^+ ions to pass through (P_K/P_{Na} = 2; Kofuji et al., 1996); and they show inward rectification whether K^+ or Na^+ is carrying the basal current. Oocytes expressing GIRK2*wv* channels die quickly, probably due to the elevated basal current and gain-of-function Na^+ current (Slesinger et al., 1996; Navarro et al., 1996)

GIRK1 and GIRK2 are believed to form heteromeric channel complexes *in vitro*. Co-expression of GIRK2, GIRK1, and m2 receptor cRNAs yields greater currents than the sum of currents in oocytes expressing either GIRK1 or GIRK2 together with the m2 receptor. Since GIRK1 currents activate more slowly than GIRK2 currents, the heteromeric channels can be distinguished by an intermediate rate of channel activation (Slesinger et al., 1996). Expression of GIRK2*wv*, GIRK1, and m2 receptor cRNAs gives rise to potassium channels with decreased basal as well as carbachol-induced currents which activate at a similar rate to the GIRK2/GIRK1 currents. In addition, one can also find a carbachol-induced Na^+ current

that is associated with the presence of GIRK2*wv* homomeric channels. It appears that channels of different composition may exist in these oocytes, and the effects of the *wv* mutation may vary with the type of GIRK channel subunits expressed by the neuron.

G protein-activated potassium currents in *weaver* cerebellar granule cells

Dissociated cerebellar granule cells from wildtype and *weaver* mice have been studied for differences in their electrophysiological properties. It has been shown that cerebellar granule cells from young (PND 4-10) mice can be purified and maintained in culture (Hatten et al., 1984; Willinger and Margolis, 1985a). Like granule cells *in vivo*, they extend neurites, migrate on astroglia, and express certain developmental markers. In a voltage ramp from depolarized to hyperpolarized potentials, wildtype cerebellar granule cells exhibit inwardly rectifying potassium current upon activation of $GABA_B$ receptor or somatostatin receptor, which is known to open Kir channels through G-protein activation. The current properties are very similar to those of the GIRK channels. This current can also be elicited by GTPγS but not GTP and is inhibited by Ba^{2+}. In the *weaver* cerebellar granule cells, little inwardly rectifying K^+ currents can be found with baclofen, somatostatin, or GTPγS application, and there is little Ba^{2+}-sensitive current present (Kofuji et al., 1996; Surmeier et al., 1996; P. A. Slesinger, unpublished data). The *wv* granule cells also appear to be permeable to Na^+ but not to large cation N-methyl-D-glucamine ($NMDG^+$), like the GIRK2*wv* channels expressed in *Xenopus* oocytes (Kofuji et al., 1996 but see Surmeier et al., 1996). As a control, a large outwardly rectifying, voltage-sensitive K^+ current (Steward et al., 1995) is the same in wildtype and *weaver* cerebellar granule cells (Kofuji et al., 1996; Surmeier et al. 1996).

This thesis examines the distribution and association of GIRK2 and related channel subunits in the rat and mouse brains using in situ hybridization, immunohistochemistry, immunoblots, and immunoprecipitation techniques. Behavioral differences and alterations in

the protein expression of GIRK2 and GIRK1 exist in mice with gain-of-function (the *weaver* mice) or loss-of-function mutation of GIRK2. Studies of normal and mutant mice suggest that G protein-gated Kir channels composed of GIRK2 and GIRK1 subunits serve important functions in the developing and adult nervous system.

Chapter 2:

Heteromultimerization of G protein-Gated Inwardly Rectifying K+ Channel Proteins GIRK1 and GIRK2 and Their Altered Expression in the *weaver* Brain

Abstract

The *weaver* gene (GIRK2) is a member of the G protein-gated inwardly rectifying potassium channel family, known effectors in the signal transduction pathway of neurotransmitters such as acetylcholine, dopamine, opioid peptides, and substance P in modulation of neurotransmitter release and neuronal excitability. GIRK2 immunoreactivity is found in but not limited to brain regions known to be affected in *weaver* mice, such as the cerebellar granule cells and dopaminergic neurons in the substantia nigra pars compacta. It is also observed in the ventral tegmental area, hippocampus, cerebral cortex, and thalamus. GIRK2 and GIRK1, a related family member, have overlapping though distinct distributions in rat and mouse brains. In regions where both channel proteins are expressed such as the cerebral cortex, hippocampus, and cerebellum, they can be co-immunoprecipitated, indicating that they interact to form heteromeric channels *in vivo*. In the brain of the *weaver* mouse, GIRK2 expression is dramatically decreased. In regions where both GIRK1 and GIRK2 distribution overlap, both GIRK1 and GIRK2 expressions are severely disrupted in *weaver* mutant mice, probably due to their co-assembly. The expression patterns of these GIRK channel subunits provide a basis for consideration of the machinery for neuronal signaling as well as the differential effects of the *weaver* mutation in various neurons.

Key Words: *weaver* mouse, G protein, inwardly rectifying potassium channel, dopamine, hippocampus, substantia nigra, cerebellum, heteromultimerization, GIRK

Introduction

The *weaver* (
et al. 1965; Rakic an
al., 1977; Schmidt et
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Tarlov, 1994). Recen
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the *wv* gene (Patil et a
for review see Goldo
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Introduction

The *weaver* (*wv*) mouse exhibits a diverse range of defects including ataxia (Sidman et al, 1965; Rakic and Sidman, 1973a,b; Hatten et al., 1984), dopamine deficiency (Lane et al., 1977; Schmidt et al., 1982; Roffler-Tarlov and Graybiel, 1984), seizures (Eisenberg and Messer, 1989), and hypospermatogenesis (Vogelweid et al., 1993; Harrison and Roffler-Tarlov, 1994). Recently, a G protein-activated inwardly rectifying potassium channel $K_{ir}3.2$ or GIRK2 (Lesage et al., 1994; Tsaur et al., 1995; Bond et al., 1995) has been identified as the *wv* gene (Patil et al., 1995; Slesinger et al., 1996; Kofuji et al., 1996; Navarro et al., 1996; for review see Goldowitz and Smeyne, 1995; Hess, 1996). GIRK channels are membrane proteins that conduct K^+ currents at or near the resting membrane potential and are important in controlling cell excitability (Hille, 1992; Kubo, 1994; Doupnik et al., 1995; Jan and Jan, 1994, Wickman and Clapham, 1995a,b). They are regulated by G proteins and have been shown to mediate the actions of G protein-coupled receptors for transmitters (Breitwieser and Szabo, 1985; Pfaffinger et al., 1985; North, 1989; Brown, 1990; Brown and Birnbaumer, 1990; Nicoll et al., 1990). GIRK2 mRNA is found in brain regions known to be affected by the *wv* mutation such as the cerebellar granule cells and substantia nigra (Kobayashi et al., 1995; Karschin et al., 1996), and both GIRK1 and GIRK2 proteins are expressed in the cerebellar granule cells and Purkinje cells during development (Patil et al., 1995; Slesinger et al., 1996; Kofuji et al., 1996; Navarro et al., 1996).

K_{ir} channels are tetramers (Yang et al., 1995) and hence could exist as homo- or heteromeric complexes. In heterologous expression systems, GIRK1 (Dascal et al., 1993; Kubo et al., 1993), unlike GIRK2, does not seem to form functional homomeric channels and may require either GIRK2 or GIRK4 to form functional channels (Kofuji et al., 1995; Duprat et al., 1995; Krapivinsky et al., 1995a,b; Lesage et al., 1995; Hedin et al., 1996). Co-expression of GIRKs 1 and 2 (or 1 and 4) in heterologous systems most likely leads to the formation of both homomeric GIRK2 (or GIRK4) channels and heteromeric GIRK1/2 (or 1/4) channels (Kofuji et al., 1995; Duprat et al., 1995; Krapivinsky et al., 1995a,b; Lesage et

al., 1995; Slesinger et al., 1996; Velimirovic et al., 1996; Spauschus et al., 1996). Interestingly, the *wv* mutant form of GIRK2 (G156S) causes the homomeric GIRK2 channels to be non-selective and conduct sodium as well as potassium ions, whereas the function of the heteromeric GIRK1/GIRK2 channels is greatly reduced by the *wv* mutation (Slesinger et al., 1996; Kofuji et al., 1996; Navarro et al., 1996; but see Surmeier et al., 1996). Thus, the effects of the *wv* mutation may vary with the type of GIRK channel subunits expressed by the neuron.

To study the GIRK channels *in vivo*, we used western blotting, in situ hybridization, and immunohistochemistry to determine the distribution of GIRK1 and GIRK2 in wildtype rat and mouse brains. Co-immunoprecipitation of GIRK1 and GIRK2 from wildtype brain regions and the drastic decrease in expression of both channel proteins in the *wv* mouse brain indicate that heteromultimers of GIRK1 and GIRK2 exist as a major component of GIRK channels in the mammalian brain.

Materials and Methods

In vitro expression of channel proteins

GIRK1, GIRK2, GIRK4, and IRK1 mRNAs were synthesized *in vitro* following manufacturer's instructions (Ambion T7 kit) and injected into *Xenopus* oocytes. Oocytes were processed after two days for western analysis of channel proteins. The oocytes were lysed by pipeting and washed in 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, and protease inhibitors (see Brain Membrane Preparation). Residual membrane was solubilized in 2% SDS sample buffer (includes 5% β-mercaptoethanol), vortexed with acid-washed glass beads, heated to 75°C for 45 min., and analyzed by western blotting. Presence of proteins was shown by recording specific inward rectifier K^+ currents from oocytes or by probing the blots with channel-specific antibodies. GIRK1, GIRK2, GIRK4, and IRK1 proteins were also synthesized via *in vitro* translation in the presence of rabbit reticulocyte lysate (Promega) and analyzed similarly. The presence of proteins was assayed by ^{35}S-methionine incorporation and exposure to autoradiographic film as well as by probing the blots with channel-specific antibodies.

Brain Membrane Preparation

Adult Sprague-Dawley male rats were anesthetized by brief exposure to halothane (Sigma), decapitated, and quickly dissected for cerebral cortex, hippocampus, cerebellum, spinal cord, and liver. For the cortex, care was taken to remove as much white matter as possible. Tissues were chopped up in ice cold 0.32 M sucrose, 5 mM Tris pH 7.4, 50 µg/ml pA-PMSF, 1 µg/ml leupeptin, 2 µg/ml Aprotinin A, 1 µg/ml pepstatin (Boehringer Mannheim) and dounce homogenized. Membrane isolation was carried out via differential centrifugation steps and stored in -80°C as aliquots in 20 mM Tris pH 7.4, 1 mM EDTA, and protease inhibitors until use. Protein concentration was assayed using the Bio-Rad kit using BSA as standard.

Antibody Production and Purification

The peptides for GIRK1, GIRK2, and IRK1 were synthesized by Dr. C. Turck at HHMI, UCSF. The peptide sequences are as follows: GIRK1N (residues 6-42) RKFGDDYQVVTTSSGSGLQPQGPGQGPQQQLVPKKKC; GIRK1C (residues 346-375) CHATFEVPTPPYSVKEQEEMLLMSSPLIAPA; GIRK2N (residues 20-51) DQDVESPVAIHQPKLPKQARDDLPRHISRDRTC (1 and 9 identical and 2 and 2 amino acids conserved to GIRK3 and GIRK4, respectively); GIRK2C (residues 403-422) CEKNPEEQTERNGDVANLENE (0 and 3 identical and 1 and 3 conserved amino acids to GIRK3, and GIRK4, respectively) (based on the sequence of GIRK2 or GIRK2A; Tsaur et al., 1995; Lesage et al., 1995). Peptide coupling and generation of polyclonal antibodies in rabbits were done by Caltag Corporation, South San Francisco, CA. Antibodies were affinity purified with appropriate peptide columns, which were generated by coupling peptides to Sulfolink coupling gel (Pierce), following the manufacturer's protocol.

Western Blotting

Protein samples were prepared in sample buffer (125 mM Tris pH 6.8, 20% glycerol, 1-2% SDS, 5% β-mercaptoethanol), heated at 75°C for 30 min., and analyzed by 10% SDS-PAGE gels. Western blots were blocked with Superblock (Pierce) or 5% non-fat dried milk in TBST buffer (150 mM NaCl, 10 mM Tris pH 8.0, 0.1% Tween-20) for 10 min. or 1 hr., respectively. Primary antibody (1 μg/ml), primary antibody in the presence of competitive peptides (10 μg/ml), and secondary antibody (donkey anti-rabbit antibody from Amersham) were diluted in 2% normal goat serum and 0.5% BSA in TBST. Blots were incubated in primary antibodies for 1 hr. at room temperature or overnight at 4°C, washed in TBST, incubated in secondary antibody for 30 min., developed by the ECL method, and briefly exposed to Hyperfilm-ECL (Amersham).

Immunohistochemistry

Animals used included adult Sprague-Dawley rats (5-6 weeks old) and *weaver* as well as wildtype C57BL/6 mice. *weaver* mice were purchased from Jackson Labs and bred by N. Patil as well as Y.J.L. Mice were genotyped by sequencing tail genomic DNA for the presence of the G to A mutation. All animals were treated in accordance with the policy on the use of animals in neuroscience research. Animals were briefly exposed to halothane (Sigma), injected intraperitoneally with pentobarbitol, and perfused with 4% formaldehyde (Polysciences, Inc.) and 0.1% glutaraldehyde in PBS. The brain and spinal cord were dissected and post-fixed for 30 minutes to overnight. Fifty μm vibratome sections were collected in 0.1 M Tris pH 7.6; blocked with 1-3% H_2O_2, 0.1 M Tris pH 7.6; washed with 50 mM Tris, 100 mM NaCl and 50 mM Tris pH 7.6, 100 mM NaCl, 0.1% Triton X-100; and then blocked in 3-10% normal goat serum and 0.1-3% BSA. Rabbit polyclonal antibodies were used at 1 μg/ml. Monoclonal antibodies against calbindin (Sigma), parvalbumin (Sigma), P65 (courtesy of L. F. Reichardt and I. Fariñas), and tyrosine hydroxylase (courtesy of L. F. Reichardt and I. Fariñas; Pel-Freeze) were used at 1:1000 or 1:5000 dilution. Biotinylated donkey anti-rabbit or anti-mouse IgG Fab (Jackson Labs) were used at 1:200 dilution as secondary antibodies. Sections were developed with Vectastain ABC kit (Vector Labs, Burlingame, CA) and diaminobenzidine and mounted in Permount (Fisher Scientific). Different brain areas were identified based on comparison with rat brain atlas (Paxinos and Watson, 1986). Antibodies specific for sequences in the N- and C-terminal domains of the same channel subunit yielded the same staining patterns, although antibodies against N-terminal sequences in general gave stronger staining. Little or no staining was detected in the absence of GIRK1 or GIRK2 antibody, in the presence of pre-immune sera instead of the primary antibodies, or when the primary antibody was incubated with the antigenic peptide (Fig. 3C; data not shown).

In Situ Hybridization

Antisense and sense oligonucleotides (45-mers) were designed for the hamster GIRK2 sequence and end-labeled with α-^{33}P-dATP or α-^{35}S-dATP (Amersham). The in situ hybridization protocol was carried out as published (Wisden and Morris, 1994). End-labeling with α-^{33}P-dATP gave better signal-to-noise ratio than with α-^{35}S-dATP. The antisense oligonucleotides contained the sequences gaccaggacgtggaaagcccagtggccattcaccag ccaaagttgcct (N terminus), ctggctaacagggcagagctgcccctgagttggtctgtgtccagc (C-terminus), and gagaagaacccggaagagcagacggagaggaatggtgacgtggc (C-terminus control; with 5 nucleotides different from the corresponding rat sequence). Complementary sense oligonucleotides to the above sequences were also synthesized and used as controls. The C-terminus control and sense oligonucleotide showed background level of signal (data not shown).

Immunoprecipitation

For immunoprecipitation experiments, equal amount of rat brain membranes (50-200 μg) from cortex, hippocampus, and cerebellum was lysed in non-denaturing buffer containing 1-2% Triton X-100, 150-500 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, and protease inhibitor cocktail (see Brain Membrane Preparation). Our previous experience with immunoprecipitation of voltage-gated K^+ channels showed that this detergent and salt concentration is strong enough to solubilize rat brain membranes and to prevent non-specific interactions, but not strong enough to abolish channel subunit interactions (Sheng et al., 1993). Membranes were also solubilized in 1-2% CHAPS or 60 mM n-octyl glucoside, and preliminary evidence indicated that use of different detergents did not alter the results of the immunoprecipitation experiments. The solubilized supernatant was precleared with some or all of the following items: a non-specific, purified rabbit antibody, purified recombinant protein A-sepharose beads (Pierce), and avidin and biotin beads (Pierce). After preclearing, the solubilized membrane was incubated first with antibodies for 2 hrs. to overnight in 4°C and then with recombinant protein A-sepharose beads for 2 hrs., washed extensively (4-7

times) in 1 ml lysis buffer/protease inhibitors, and analyzed on 10% SDS-PAGE and by western blotting with biotinylated antibodies. No specific signals were detected when we omitted the primary antibodies during immunoprecipitation (data not shown).

Since GIRK1 (62 kD) and GIRK2 (48-50 kD) are both about the size of the immunoglobulin heavy (IgH) chain (55 kD), the strong IgH band obscured the relevant portion of the blot, thereby rendering interpretation of immunoprecipitation experiments nearly impossible. Different approaches to covalently couple purified antibodies to beads either abolished the ability of the antibodies to immunoprecipitate or failed to consistently remove all IgH from the blots (data not shown). We therefore chose to avoid the use of secondary antibodies which would mark the IgH band. Instead, we biotinylated antibodies against GIRK1 N- or C-termini or GIRK2 N-terminus after affinity purification and used streptavidin-HRP for detection. Affinity-purified antibodies were biotinylated with NHS-LC-Biotin (Pierce) following manufacturer's directions and purified using swift desalting columns (Pierce). Antibodies were washed with PBS and concentrated with Centriprep columns (Amicon).

To ensure that the biotinylated antibodies did not react with IgH chain, we denatured 1 μg of immunopure rabbit IgG (Pierce) and analyzed with unlabeled vs. biotinylated antibodies (data not shown). Biotinylated antibodies did not react with denatured Ig heavy and light chains, but they did give rise to two kinds of undesirable background bands. On blots probed with biotinylated antibodies, there were strong 85-120 kD bands, which could not be competed off with 10 μg competitive peptides (Fig. 4C). The other kind of background bands was a result of the antibodies' cross-reactivity with protein A, which ran as 40-50 kD molecular weight bands. The presence of this kind of background bands was confirmed when whole protein A-sepharose beads (Pharmacia) or recombinant, purified protein A-sepharose beads (Pierce) was treated with SDS sample buffer, analyzed on western blots, and visualized by different biotinylated antibodies (data not shown). As a result, a lysis buffer control (Fig. 4 BUFFER) was processed in parallel with all immunoprecipitation

Results

Western analysis shows that GIRK1 and GIRK2 are both present in cerebral cortex, hippocampus, and cerebellum.

We generated rabbit polyclonal antibodies for analysis of GIRK2 and GIRK1 channel proteins *in vivo*. To demonstrate antibody specificity, GIRK2 and GIRK1 antibodies were tested on immunoblots containing GIRK1, GIRK2, GIRK4, and IRK1 proteins synthesized *in vitro*. Channel proteins were generated in *Xenopus* oocytes injected with cRNA and by *in vitro* translation in rabbit reticulocyte lysate. The presence of different channel proteins was demonstrated by recording specific inwardly rectifying K^+ currents from the oocytes, probing with antibodies against each channel, or by autoradiography of proteins containing ^{35}S-methionine. Antibody against the N-terminus of GIRK2 recognized a specific 48 kD band in oocytes injected with GIRK2 cRNA but not in oocytes injected with water or other inwardly rectifying K^+ channel cRNAs (Fig. 1A left panel). Antibody against the N-terminus of GIRK1 detected a 55 kD band in the lane containing GIRK1 protein synthesized in rabbit reticulocyte lysate in the absence of pancreatic microsomal membrane, but it did not detect a band in lanes containing GIRK2, GIRK4, or IRK1 proteins (Fig. 1B left panel).

Affinity-purified rabbit polyclonal antibodies against the N- or C-terminus of GIRK2 and GIRK1 were used to identify GIRK2 and GIRK1-immunoreactive bands in membranes prepared from rat cerebral cortex, hippocampus, as well as cerebellum. Antibodies against the N-terminus of GIRK2 detected a broad double band of around 48-50 kD in cerebral cortex, hippocampus, cerebellum, and spinal cord (Fig. 1A middle panel). The size of the GIRK2-immunoreactive band was consistent with that of the *in vitro* translated protein, and the doublet may reflect the presence of alternatively spliced forms of GIRK2 (Lesage et al., 1994, 1995; Tsaur et al., 1995; Isomoto et al., 1996; N. Patil, unpublished observation). The 38 kD band was a cross-reacting protein detected on western blots of rat but not mouse brain membranes. Antibody against the N-terminus of GIRK2 preadsorbed with competitive peptide gave rise to no immunoreactive bands (Fig. 1A right panel). Antibody against the C-

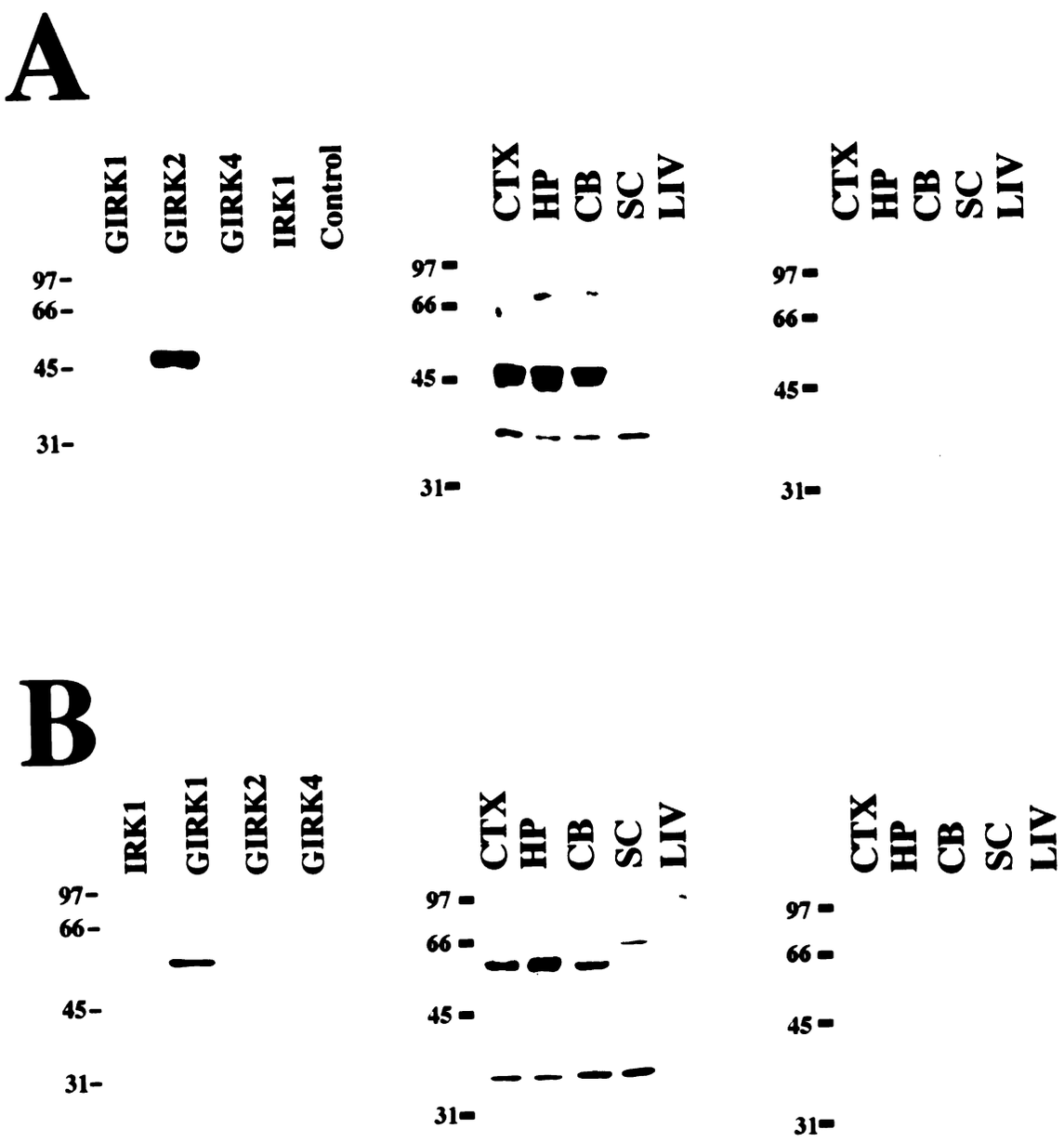
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Figure 1: GIRK1 and GIRK2 are present on membranes from different rat brain regions.

A. and B. left panels Western blots probed with antibody against the N-terminus of GIRK2 or GIRK1, respectively, to demonstrate antibody specificity. In A left panel, western blot of GIRK1, GIRK2, GIRK4, and IRK1 proteins expressed in Xenopus oocytes is probed with antibody against the N-terminus of GIRK2. Control represents oocytes injected with water. In B left panel, western blot of *in vitro* synthesized IRK1, GIRK1, GIRK2, and GIRK4 proteins is probed with antibody against the N-terminus of GIRK1. **A. and B. middle panels** Membranes from rat cerebral cortex, hippocampus, and cerebellum contain GIRK2 (48 kD) and GIRK1 (58-60 kD) proteins, respectively. There are also faint GIRK2 bands in the spinal cord. See Results for details. **A. and B. right panels** Control western blots using antibody against the N-terminus of GIRK1 or GIRK2 in the presence of antigenic peptide. CTX: cerebral cortex, HP: hippocampal formation, CB: cerebellum, SC: spinal cord, LIV: liver.

terminus of GIRK2 could not detect any immunoreactive bands on western blots of brain membranes.

Antibody against the N- or C-terminus of GIRK1 stained an immunoreactive band of around 60-62 kD in cerebral cortex, hippocampus, and cerebellum (Fig. 1B middle panel; data not shown). No GIRK1 protein was detectable in liver, consistent with the observed absence of GIRK1 mRNA based on northern analysis (Kubo et al., 1993). The 35 kD band was recognized by antibody against the N-terminus but not by antibody against the C-terminus of GIRK1. GIRK1 antibodies in the presence of antigenic peptide could not detect any immunoreactive bands (Fig. 1B right panel).

Immunohistochemistry and in situ hybridization reveal overlapping though distinct expression patterns of GIRK1 and GIRK2 channel subunits in rat.

We used antibodies against the N- and C-terminal cytoplasmic domains of GIRK1 and GIRK2 to study their distribution in rat brain (see Materials and Methods for controls). Antibodies against either the N- or the C-terminus of the same channel subunit gave rise to similar immunoreactive patterns. Immunohistochemical studies using these antibodies revealed that GIRK1 and GIRK2 have overlapping distribution in some brain regions (Fig. 2, Fig. 3, Table 1). Strong GIRK1 and GIRK2 immunoreactivity was observed in cerebral cortex (CTX) (Fig. 2A-B, 3A-B, Table 1), lateral septal nuclei (data not shown), hippocampal formation (HP) (Fig. 2A-B, Fig. 3D-E, Table 1), and cerebellum (Fig. 2D-E, Fig. 3I-L), whereas certain other regions express predominantly GIRK1 or GIRK2. Strong GIRK1 but low or background level of GIRK2 staining was found in the caudate-putamen (CP) and globus pallidus (Fig. 2A-2B), thalamus (Th) (Fig. 2A-B; for a few exceptions see Table 1 and Fig. 2B), oculomotor nucleus (Table 1), red nucleus (data not shown), and mesencephalic nuclei of trigeminal nerve (data not shown). Strong GIRK2 but background level of GIRK1 staining was observed in the substantia nigra (Fig. 3F-H) and ventral tegmental area (data not shown but see Fig. 6A for GIRK2 staining in mouse brain). Low or background level

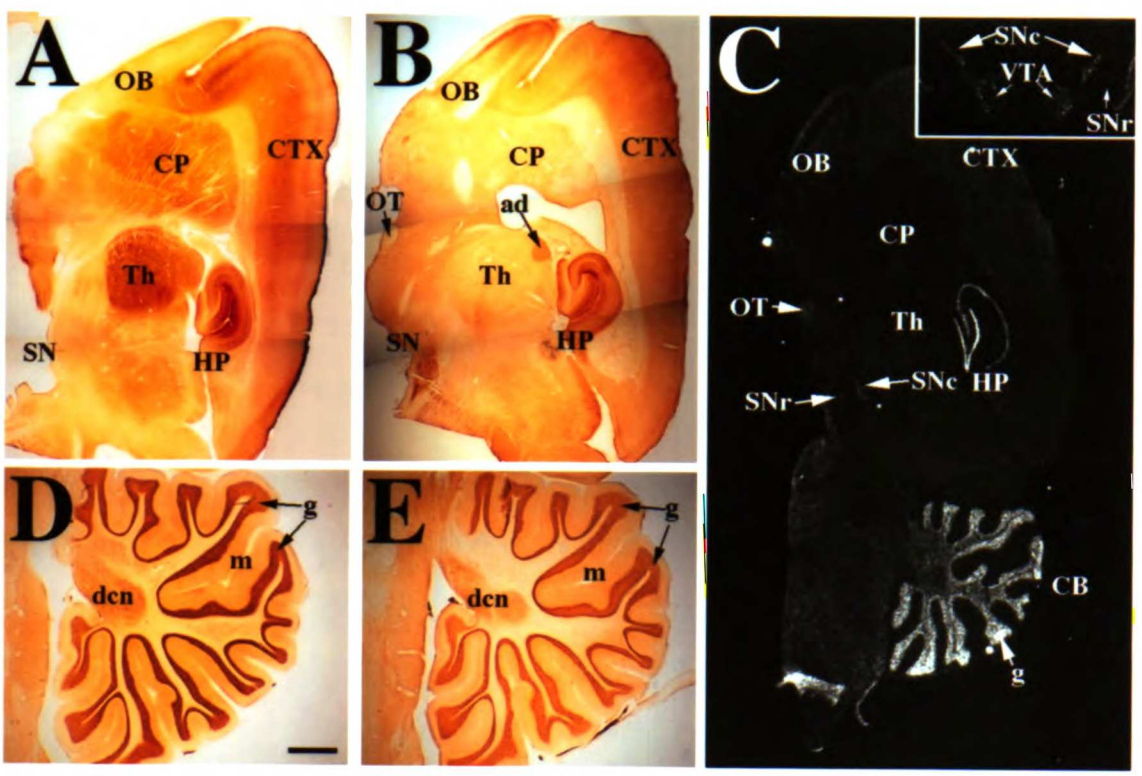
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Figure 2: Overview of GIRK1 and GIRK2 Distribution in the rat brain.

A. and D. Parasagittal view of GIRK1 antibody staining pattern. There is strong staining in the cerebral cortex, caudate-putamen, globus pallidus, thalamus, hippocampus, cerebellum, but not in the substantia nigra and hypothalamus. **B. and E.** Parasagittal view of GIRK2 immunoreactivity. There is strong staining in the cerebral cortex, hippocampus, substantia nigra, anterodorsal thalamic nucleus, and cerebellum but not in many thalamic nuclei, caudate-putamen, and hypothalamus. **C.** Parasagittal view of GIRK2 mRNA distribution. **Inset** is a coronal view of GIRK2 mRNA distribution in the ventral midbrain. Signal can be observed in the hippocampus, cerebellum, substantia nigra pars compacta, and ventral tegmental area but not in corpus callosum, thalamus, olfactory bulb, and substantia nigra pars reticulata. **Scale bar: A-B**: 1 mm, **C and Inset**: 2 mm, **D-E**: 0.8 mm. **Abbreviations: ad**: anterodorsal thalamic nucleus; **CB**: cerebellum; **CP**: caudate-putamen; **CTX**: cerebral cortex; **dcn**: deep cerebellar nuclei; **g**: granule cell layer of the cerebellar cortex; **HP**: hippocampus; **m**: molecular layer of the cerebellar cortex; **OB**: olfactory bulb; **OT**: optic tract; **SN**: substantia nigra; **SNc**: substantia nigra pars compacta; **SNr**: substantia nigra pars reticulata; **Th**: thalamus

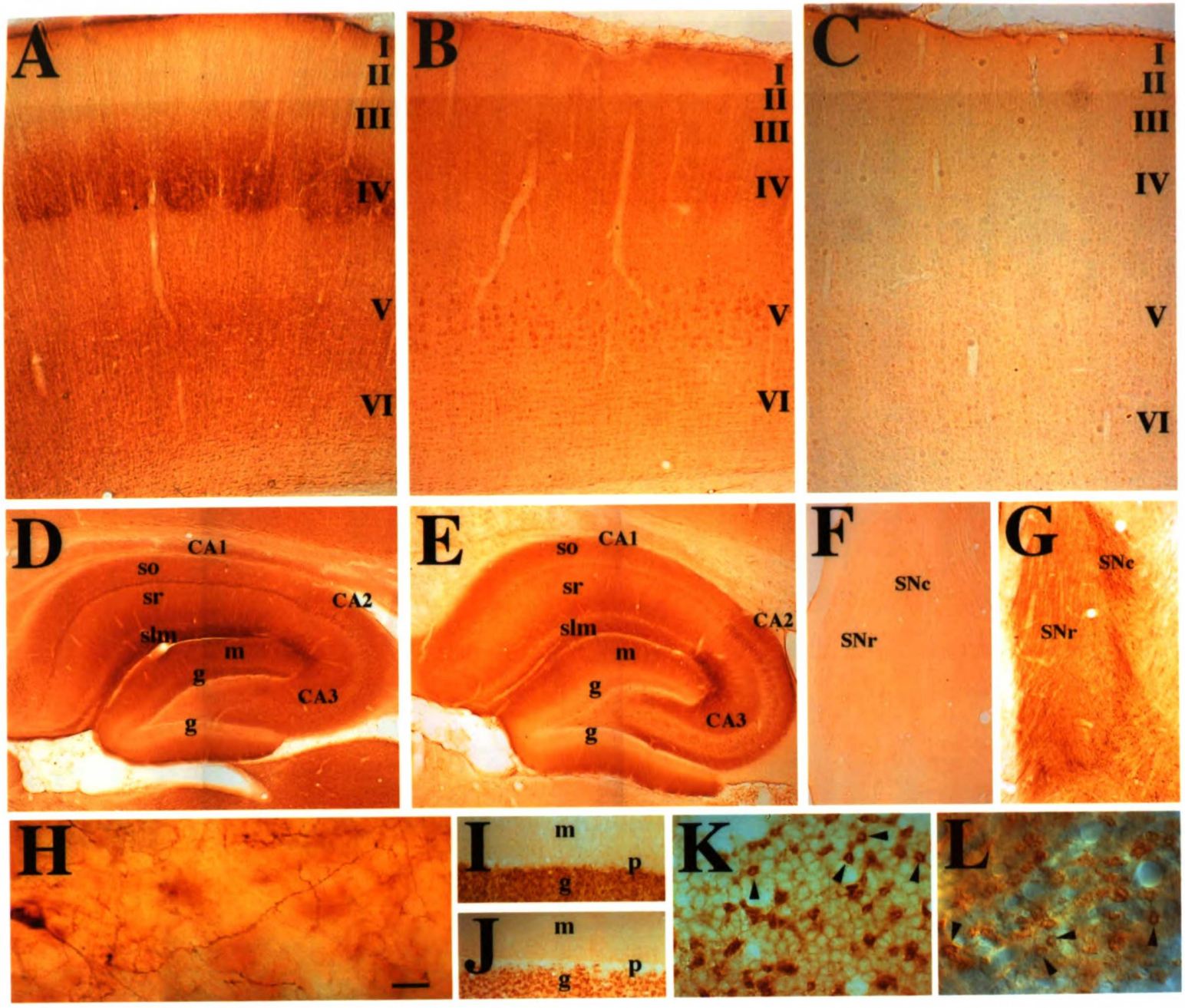
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Figure 3: Higher magnification views of GIRK1 and GIRK2 staining in rat cerebral cortex, hippocampus, substantia nigra, and cerebellum.

A. GIRK1 immunoreactivity in the barrel cortex. There is intense staining of layers IV-VI neurons and neurites. See text for details. I-VI correspond to layers of the cerebral cortex. **B.** GIRK2 immunoreactivity in the hindlimb area of cortex. In the forelimb and hindlimb areas of the cortex, there is more intense staining of all layers. In particular, the layer V large pyramidal cells are the most strongly stained cells in the cortex. **C.** Negative control for cortex with the omission of the primary antibody. **D.** GIRK1 immunoreactivity of the hippocampal formation. The entire molecular layer, which includes terminal fields of the perforant pathway and commissural fibers, is strongly stained. Note that the superior blade of the dentate granule cell is much more stained than the inferior blade despite no differences in mRNA level (DePaoli et al., 1994; Karschin et al., 1994). There is strong staining of the CA3, CA2 and CA1 pyramidal cells (CA1 > CA2 = CA3). Stratum lacunosum moleculare, the terminal field of the perforant pathway, is strongly stained. Other layers that receive commissural fibers [stratum radiatum] and non-hippocampal fibers such as those from the thalamus [stratum oriens] are moderately stained. Stratum lucidum, the terminal field for the mossy fibers, has relatively little immunoreactivity. **E.** GIRK2 immunoreactive pattern in the hippocampus. Strongest staining is in the CA2 pyramidal cells and CA3 pyramidal cells closest to CA2. There is light staining of the CA1 pyramidal cells. The intensity of staining in strata lacunosum moleculare, radiatum, and oriens (ordered strong to weak, slm > so > sr) corresponds to that of the pyramidal cell layer (CA2 > CA3 > CA1). **F. and G.** GIRK1 and GIRK2 staining in the substantia nigra, respectively. There is little GIRK1 staining, while GIRK2 immunoreactivity can be seen in the cell body and dendrites of the substantia nigra pars compacta neurons. **H.** Higher magnification view of a SNc neuron sending dendrite ventrally into the pars reticulata. GIRK1 (**I and K**) and GIRK2 (**J and L**) immunoreactivity in the cerebellum. For both GIRK1 and GIRK2, there is strong staining of the granule cell layer, moderate staining of the deep cerebellar nuclei, and light staining of the molecular

layer. There is little staining of the Purkinje cells. **K. and L.** Higher magnification view of GIRK1 and GIRK2 immunoreactivity of the cerebellar granule cell layer, respectively. Both GIRK1 and GIRK2 antibodies stain the glomeruli (arrowheads) very strongly, while GIRK1 antibodies also highlight the outline of granule cells. **Scale bar: A-C**: 0.2 mm, **D-E**: 0.3 mm, **F-G**: 0.6 mm, **H**: 0.02 mm, **I-J**: 0.04 mm, **K-L**: 0.02 mm. **Abbreviations: g**: granule cell layer in the dentate gyrus of the hippocampus (**D-E**) or granule cell layer of the cerebellum (**I-J**); **CA1-3**: regions of hippocampus proper; **m**: molecular layer of the dentate gyrus of the hippocampus (**D-E**) or of the cerebellum (**I-J**); **p**: Purkinje cell layer; **slm**: stratum lacunosum moleculare; **so**: stratum oriens; **sr**: stratum radiatum. For additional abbreviations see Fig. 2.

Table 1: Protein Distribution of GIRK1 and GIRK2 in Rat Brain and Spinal Cord*

	GIRK1		GIRK2			GIRK1		GIRK2	
	Signal	Type	Signal	Type		Signal	Type	Signal	Type
Telencephalon					Hippocampus				
Olfactory Bulb	+		+	a,d	CA3				
Cerebral Cortex					Stra. Oriens	++	c	++	c
Layer I, II, III	+	d,e	+	c	Stra. Pyramidal	++	a,b,d	++	a,b,d
Layer IV	+++	a,d,e	++	c	Stra. Radiatum	++	d	++	d
Layer V	++	a,d	+++	a,b,d	Stra. Lucidum	+/-	d	+/-	d
Layer VI	++	a,d	+	a,d	Stra. Lacu. Mol.	+++	d	+++	d
Island of Calleja	+/-		++	a,d	CA2				
Caudate-Putamen	+	a,d	+/-	d	Stra. Oriens	++	c	++	c
Globus Pallidus	+	a,b,c	+/-	c	Stra. Pyramidal	++	a,b,d	+++	a,b,d
Nu. Bas. of Meynert	+/-		+/-		Stra. Radiatum	++	d	++	d
Nu. H. L. Diag Band	+	a,b,c			Stra. Lacu. Mol.	+++	d	+++	d
Lateral Septal Nu.	+++	e	+++	e	CA1				
Medial Septal Nu.	+/-		+/-		Stra. Oriens	++	d	+	d
Dentate Gyrus					Stra. Pyramidal	+++	a,c	+/++	a,c
Mol. Layer	+++	d	++	d	Stra. Radiatum	++	d	+	d
Granule Cells					Stra. Lacu. Mol.	+++	d	++	a,d
Superior blade	++	c	+	c	Subiculum	+	d	++	a,d
Inferior blade	+/-	c	+	c	Presubiculum	+	d	+/-	d
Hilus	++	a,c	++	a,b,c,d	Parasubiculum	+	d	+/-	d

*Signal: staining intensities of +++ strong, ++ moderate, + light, +/- background level

Type: a = cell body, b = principal neurite, c = diffuse neurite, d = diffuse, e = fiber

Diencephalon					**Mesencephalon**				
Thalamus					Superior Colliculus	+	d	+	d
Paraventricular	+/-	d			Oculomotor (III) Nu.	+++	a,d	+/-	
Lateral Dorsal	+++	a,d	+/-	d	Red Nucleus	+++	a,b,c	+/-	
Lateral Posterior	++	d	+/-	d	Ventral Tegm. Area	+/-		+++	
Anterior Dorsal			++	a,d	Substantia Nigra				
Anterior Medial			+/-	d	Compacta	+/-		+++	a,b,c
Medial Dorsal	+++	a,d			Reticulata	+/-		+++	c
Ventral Medial	+	a,d	+	a,d	Inferior Colliculus	+	a,d		
Ventral Lateral	+++	a,d	+	a,d	**Metencephalon**				
Ventral Posterior	+++	a,d			Postdors Tegm Nu.	++	a,d		
Central Medial	++	a,d			Lat. Dors. Tegm Nu.	+	a,b,d		
Central Lateral			+	a,d	Locus Coeruleus	+/-	a,d	+/-	
Gelatinosus	++	a,d			Mesen. Nu. Trig. N.	+++	a	+/-	
Rhomboid	+/-	d			Motor, Sens Trig. Nu.	++	a,c,d		
Reuniens	+/-	d			Pontine Reticul. Nu.	+	a,b,c,d		
Reticular	++	c	+	c	Raphe Pontine Nu.	++			
Posterior	+++	a,d			Cerebellum				
Parafascicular			+	a,d	Mol. Layer	+	d	+	d
Hypothalamus					Granule Cel Layer				
Ant. & Lateroant.	+/-	a,d	+/-		Granule Cell	+	a	+/-	
Lateral	+	a,b,c,d			Glomeruli	+++		+++	
DM, VM, Tu. Cin	+/-				Purkinje Cells	+/-		+/-	
Entopeduncular Nu.	+/-	c	+/-		Deep Cerebel. Nu.	++		++	
Ant. Pretectal Nu.	+/-				Spinal Cord	+/-			
Deep Mesen. Nu.	+/-	c							

staining for GIRK1 and GIRK2 was found in the hypothalamus, locus coeruleus, and nucleus basalis of Meynert, regions where electrophysiological studies have revealed regulation of K_{ir} channels by specific neurotransmitters (Table 1).

GIRK1 and GIRK2 immunoreactivities were observed mostly in the somatodendritic subcellular compartment and sometimes in the axon-like fibers (e.g. lateral septal nucleus), and in some brain regions they may exist in both compartments (see Table 1 for details). These observations are consistent with a previous study of GIRK1 protein distribution (Ponce et al., 1996). Both GIRK1 and GIRK2 antibodies strongly stained cell bodies and dendrites of cells of the hippocampal formation (Fig. 3D-E). GIRK2 immunoreactivity was prominent in the cell body and apical dendrite of layer V pyramidal cells in both the forelimb and the hindlimb somatosensory cortex (Fig. 3B) and in both cell body and dendrites of the dopaminergic neurons in the substantia nigra (SN) (Fig. 3G-H). Unlike the predominantly somatodendritic localization of GIRK1 and GIRK2 immunoreactivities in the above brain structures, GIRK1 and GIRK2 immunoreactivities in the lateral septal region seemed to exist in axon-like fibers (data not shown).

In some brain regions, GIRK1 and GIRK2 antibodies seemed to stain synaptic regions, implying that these channel subunits may exist in pre- and/or post-synaptic membranes. In the cerebral cortex, GIRK1 immunoreactivity appeared to mark the whisker barrels in the primary somatosensory cortex (Fig. 3A). The barrels in layer IV of the somatosensory cortex are composed of postsynaptic stellate neurons as well as ascending presynaptic axon terminals from the ventral posterior thalamus (Waite and Tracey, 1995). Since both layer IV of cerebral cortex and ventral posterior thalamus express high levels of GIRK1 mRNA (DePaoli et al., 1994; Karschin et al., 1994, 1996), GIRK1 protein may be present in pre- and/or postsynaptic membranes. Both GIRK1 and GIRK2 antibodies strongly stained the terminal fields of the perforant pathway (part of the trisynaptic circuit and serial/parallel sensory information processing system) in the hippocampus (stratum lacunosum moleculare or slm) and dentate gyrus (outer molecular layer), as well as

commissural projection field (stratum radiatum or sr) that contain intra-hippocampal connections (Fig. 3D-E, Table 1). The cerebellar glomeruli, synaptic regions containing granule cell dendrites and mossy fiber terminals, also exhibited strong GIRK1 and GIRK2 immunoreactivity (Fig. 3K-L arrowheads). Both GIRK1 and GIRK2 staining outlined the border of the glomeruli, suggesting that the staining may be on the post-synaptic membrane.

This pattern for GIRK1 protein distribution correlated well with the GIRK1 mRNA distribution determined by in situ hybridization (DePaoli et al., 1994; Karschin et al., 1994, 1996). To test whether the GIRK2 expression revealed by immunohistochemistry was also consistent with the GIRK2 mRNA expression pattern, we carried out in situ hybridization experiments with specific oligonucleotide probes (for sequence and controls see Materials and Methods). Strong GIRK2 mRNA signal was present in substantia nigra pars compacta (SNc) (Fig. 2C and inset), ventral tegmental area (VTA) (Fig. 2C inset), hippocampus (HP) (Fig. 2C), and cerebellar granule cell layer (g) (Fig. 2C). Light staining can be seen in cerebral cortex (CTX) (Fig. 2C). Low to undetectable signal was observed in striatum [e.g. caudate-putamen (CP)], thalamus (Th), hypothalamus, substantia nigra pars reticulata (SNr) and cerebellar Purkinje cell layer. The pattern we observed is consistent with that of Kobayashi et al. (1995). Thus, the overall GIRK2 mRNA distribution confirmed the immunoreactive pattern.

<u>Antibodies against GIRK1 or GIRK2 co-immunoprecipitate GIRK1 and GIRK2 channel subunits from rat cerebral cortex, hippocampus, and cerebellum.</u>

Antibodies against the N-terminus of GIRK1 immunoprecipitated GIRK1 protein of around 62 kD from cerebral cortical, hippocampal, and cerebellar membranes, as revealed by western analysis using biotinylated antibodies against GIRK1 C-terminus (Fig. 4A) (See Materials and Methods for an explanation of background bands and rationale for biotinylating primary antibodies). Less immunoreactivity was present in hippocampus, and the band on the western blot was not evident in the exposure used in Fig. 4A. The GIRK2

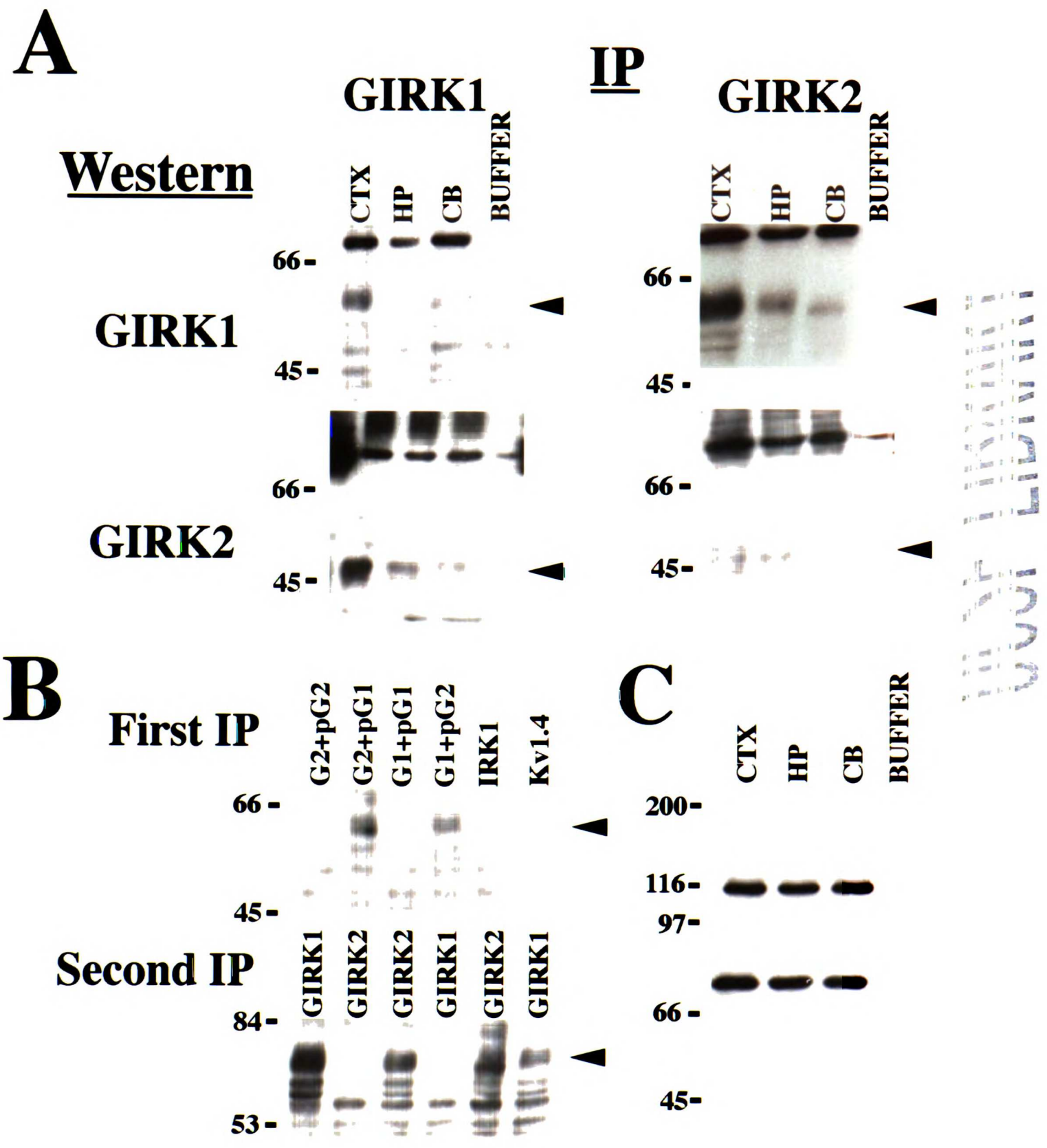

A
Western
GIRK1
IP
GIRK2
CTX
HP
CB
BUFFER
66-
45-
GIRK1
GIRK2
B
First IP
G2+pG2
G2+pG1
G1+pG1
G1+pG2
IRK1
Kv1.4
Second IP
GIRK1
GIRK2
84-
53-
C
200-
116-
97-

Figure 4: GIRK1 and GIRK2 are co-immunoprecipitated from rat cerebral cortex, hippocampus, and cerebellum

A. Immunoprecipitation and co-immunoprecipitation of GIRK1 and GIRK2 from membranes of cerebral cortex, hippocampus, cerebellum, or buffer control. Membranes were immunoprecipitated with different channel antibodies (labeled on top under IP) and probed with biotinylated antibody against GIRK1 C-terminus or GIRK2 N-terminus (labeled on the left under Western). **B.** Controls for immunoprecipitation experiments. The antibodies used for immunoprecipitation are listed above each lane (First IP). After the first immunoprecipitation, the "immunodepleted" supernatants were then subjected to a second immunoprecipitation (Second IP), and the lanes in the two blots are matched exactly. **G2+pG2**: GIRK1 is not co-immunoprecipitated by GIRK2 antibody in the presence of competitive GIRK2 peptide. **G2+pG1**: GIRK1 is co-immunoprecipitated by GIRK2 antibody in the presence of noncompetitive GIRK1 peptide. **G1+pG1**: GIRK1 is not immunoprecipitated by GIRK1 antibody in the presence of competitive GIRK1 peptide. **G1+pG2**: GIRK1 is immunoprecipitated by GIRK1 antibody in the presence of noncompetitive GIRK2 peptide. **IRK1**: GIRK1 is not co-immunoprecipitated by IRK1 antibody. **Kv1.4**: GIRK1 is not co-immunoprecipitated by $K_V1.4$ antibody. See Results and Methods for details. **C.** In the presence of competitive peptide, biotinylated antibody against GIRK2 did not stain GIRK2 bands, but the background 85 and 120 kD bands remained.

antibodies also immunoprecipitated GIRK2 proteins of 48-50 kD that stained with biotinylated antibodies against the N-terminus of GIRK2 (Fig. 4A). It seemed that more GIRK1 and GIRK2 were immunoprecipitated from the cerebral cortex than from the hippocampus or cerebellum.

We found that antibodies against GIRK1 or GIRK2 co-immunoprecipitated both GIRK1 and GIRK2 channel subunits. When membranes were immunoprecipitated with GIRK1 antibody but probed for the presence of GIRK2, we observed co-immunoprecipitation of the 48-50 kD GIRK2 doublet (Fig. 4A). Conversely, the GIRK1 protein band was co-immunoprecipitated by GIRK2 antibodies, as indicated by a broad band of 62 kD GIRK1 immunoreactivity (Fig. 4A). Again, the cortex band was the most intense, though GIRK1 and GIRK2 from all three brain regions were co-immunoprecipitated.

To make sure that the immunoprecipitation and co-immunoprecipitation results were specific, we carried out some control experiments. The addition of respective antigenic peptides to GIRK1 or GIRK2 antibodies resulted in the absence of immunoprecipitation of the GIRK1 band by GIRK1 antibody (Fig. 4B G1+pG1) and no co-immunoprecipitation of the GIRK1 band by the GIRK2 antibody (G2+pG2) from the cerebral cortex, whereas the addition of non-antigenic peptides had no effects (Fig. 4B G1+pG2 and G2+pG1). Such results were due to the ability of the antigenic peptides to prevent immunoprecipitation rather than a lack of channel subunits in the membrane, as shown by a second immunoprecipitation of proteins from the "immunodepleted" supernatant after the first immunoprecipitation (Fig. 4B, Second IP). For example, while GIRK2 antibody could not co-immunoprecipitate GIRK1 band in the presence of GIRK2 peptide (Fig. 4B G2+pG2), GIRK1 protein could be immunoprecipitated from the "immunodepleted" supernatant (Fig. 4B GIRK1 lane below the G2+pG2 lane). In the presence of non-antigenic peptide, GIRK2 antibody could co-immunoprecipitate GIRK1 protein (Fig. 4B G2+pG1), but could not further co-immunoprecipitate GIRK1 from the "immunodepleted" supernatant (Fig. 4B GIRK2 lane below the G2+pG1 lane). The same was true for GIRK1 antibody immunoprecipitation.

GIRK1 could not immunoprecipitate the GIRK1 band in the presence of GIRK1 peptide (Fig. 4B G1+pG1); from the (non) "immunodepleted" supernatant GIRK2 antibody could co-immunoprecipitate the left-over GIRK1 protein (Fig. 4B GIRK2 lane below the G1+pG1 lane). In the presence of non-antigenic peptide, GIRK1 antibody could immunoprecipitate GIRK1 protein (Fig. 4B G1+pG2), and no more GIRK1 protein was present in the "immunodepleted" supernatant (Fig. 4B GIRK1 lane below the G1+pG2 lane).

Using antibodies against the K_{ir} channel IRK1 or a voltage-gated K^+ channel $K_V1.4$ (Sheng et al., 1993), we were not able to co-immunoprecipitate GIRK1 (Fig. 4B IRK1 and $K_V1.4$) or GIRK2 (data not shown) from the cerebral cortex. The IRK1 and $K_V1.4$ antibodies could immunoprecipitate IRK1 and $K_V1.4$ channel subunits, respectively, from brain membranes (data not shown; Sheng et al., 1993). Furthermore, we showed that GIRK1 was present in the cortical membrane used for IRK1 and $K_V1.4$ immunoprecipitations. In the IRK1-immunodepleted supernatant, GIRK1 protein could be co-immunoprecipitated by GIRK2 antibody (Fig. 4B GIRK2 lane below the IRK1 lane); and in the $K_V1.4$-immunodepleted supernatant, GIRK1 could be immunoprecipitated by GIRK1 antibody (Fig. 4B GIRK1 lane below the $K_V1.4$ lane). Taken together, these results indicated that the interaction between GIRK1 and GIRK2 as revealed by co-immunoprecipitation was specific.

Distribution of GIRK1 and GIRK2 immunoreactivity in mice is similar to that in rats.

By using antibody against the N- or C-terminus of GIRK1 or GIRK2, we found GIRK1 and GIRK2 channel proteins in C57BL/6 mice in similar brain regions as well as relative intensities as in Sprague-Dawley rats, though some species differences of GIRK2 staining in the cortex and thalamus were apparent. Whereas GIRK1 but not GIRK2 was found in the whisker barrels in the rat, both GIRK1 and GIRK2 immunoreactivity was observed in the whisker barrels in the mouse (data not shown). In the rat thalamus, GIRK2 is found in a few nuclei (Table 1), while in the mouse GIRK2 staining was observed in many thalamic nuclei (data not shown). We could observe this more broad distribution of GIRK2 in the mouse when using antibodies against either the N- or C-terminus, although the antibody against the C-terminus consistently gave lighter staining (compare Fig. 7B, E with C, F).

Decrease in GIRK2 and GIRK1 protein levels in *weaver* mice.

Western blots of membranes prepared from *wv* and wildtype littermate showed that the GIRK2 band in *wv* mice was less intense than that from wildtype littermate (Fig. 5A). The decrease in the 48 kD GIRK2 band was consistent with the decrease in immunostaining (see below). The 58-60 kD GIRK1 band in *wv* brain membrane also seemed slightly less intense than that of the wildtype littermate (Fig. 5B). The additional 55 kD GIRK1 band in *wv* brain may reflect an elevated level of unglycosylated GIRK1 proteins. Antigenic peptide against either GIRK2 or GIRK1 antibody competed off all immunoreactive bands.

Abnormal GIRK2 expression in cerebellum and substantia nigra of the *weaver* mice.

We looked for changes in protein expression in the *wv* mouse as a result of the GIRK2 G156S mutation, particularly in the cerebellum and substantia nigra which are known to exhibit cell death. In the cerebellum, we used antibodies against GIRK1, GIRK2, IRK1 (found in Purkinje cell body and dendrites; Y.J.L., unpublished data), calbindin (calcium-

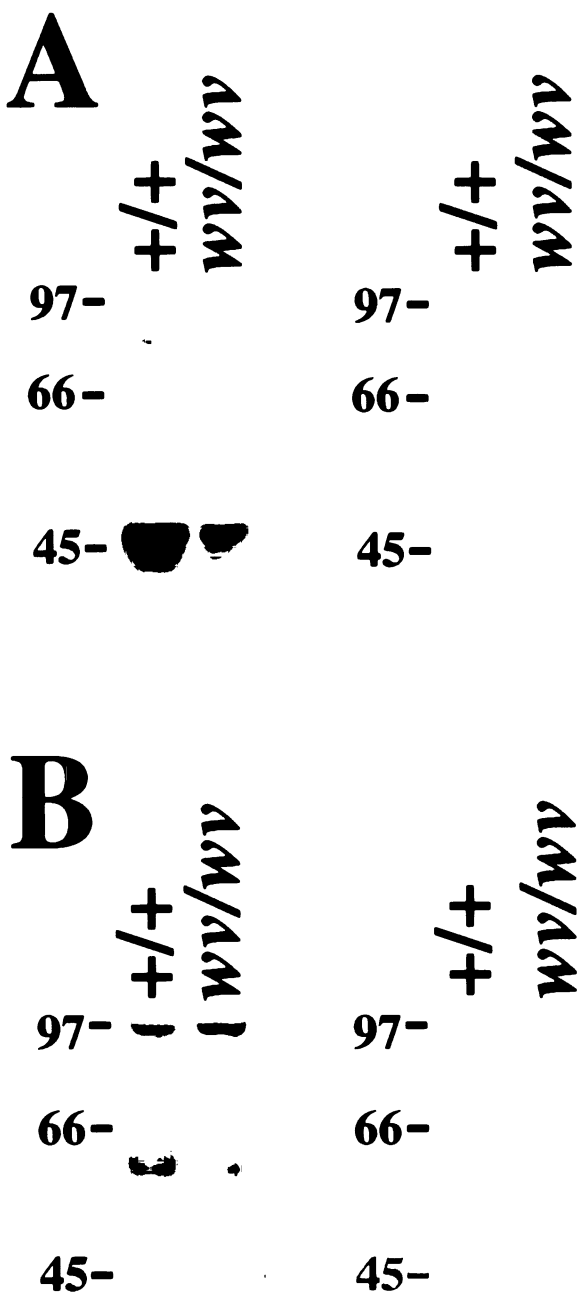

A
+/+
wv/wv
97-
66-
45-
+/+
wv/wv
97-
66-
45-
B
+/+
wv/wv
97-
66-
45-
+/+
wv/wv
97-
66-
45-

Figure 5: Decreased level of both GIRK2 and GIRK1 proteins in the *weaver* brain.

A. left panel Western blotting with antibody against GIRK2 shows that there is less GIRK2 protein in the *weaver* brain than in wildtype brain. Fifty μg of membrane from wildtype littermate or *weaver* brain was loaded onto each lane. **B. left panel** Western blotting with antibody against the N-terminus of GIRK1 demonstrates that there is a slight decrease in the 58-60 kD GIRK1 band, but there is an additional 55 kD band, which may represent an unglycosylated form of GIRK1 in the *weaver* brain. **A. and B. right panels** Peptide competition controls for antibodies against the N-terminus of GIRK2 or GIRK1, respectively.

binding protein found in cerebellar molecular layer and Purkinje cells; Baimbridge and Miller, 1982; McRitchie et al., 1996), and P65 synaptotagmin (found in synapses; Matthew et al., 1981) to assess differences between *wv* mice and wildtype littermates. At postnatal day (PND) 19 (Fig. 6B, C) and PND27 (data not shown), antibodies against GIRK2 and GIRK1 gave nearly uniform staining in the cerebellum of *wv* mice compared to the discrete staining patterns in the wildtype littermates. This difference is consistent with the observation that GIRK1 and GIRK2 are normally expressed in granule cells (Kobayashi et al., 1995; Slesinger et al., 1996; Kofuji et al., 1996; Navarro et al., 1996), and that most granule cells in *wv* mice have disappeared by PND19 (Fig. 6 compare A with inset). IRK1, calbindin, and P65 synaptotagmin immunoreactivity was observed in both wildtype and *wv* mice (Fig. 6D; data not shown), suggesting that Purkinje cells in *wv* mice can still differentiate to some extent. The IRK1-positive Purkinje cell bodies and dendrites in *wv* cerebellum were disorganized, and they filled up the entire cerebellar cortex (Fig. 6D inset), consistent with previous observations of Purkinje cell abnormalities in *wv* mice (Rakic and Sidman, 1973a,b; Smeyene and Goldowitz, 1990).

We examined the substantia nigra (SN) of wildtype and *wv* mice for distribution of GIRK1 (absent in SN; this study), GIRK2, tyrosine hydroxylase (TH; present in substantia nigra pars compacta [SNc] and ventral tegmental area [VTA] dopaminergic neuronal cell body and dendrites; Roffler-Tarlov and Graybiel, 1984), $K_V1.4$ (found in axons in substantia nigra pars reticulata [SNr]; Sheng et al., 1992), calbindin (found in SNr; Gaspar et al., 1994; McRitchie et al., 1996), and P65 synaptotagmin (in synapses; Matthew et al., 1981). At PND19, we found reduced cell number and dendritic staining of GIRK2-positive cells in the SNc and VTA. With increasing age, we found even fewer GIRK2-positive cells in SNc and VTA (compare Fig. 7B, C with E, F). The number of TH-positive cells in the SNc also decreased with age, but there was no observable difference in the number of TH-positive cells in the VTA (Fig. 7 compare A and D). We found little difference in the staining of

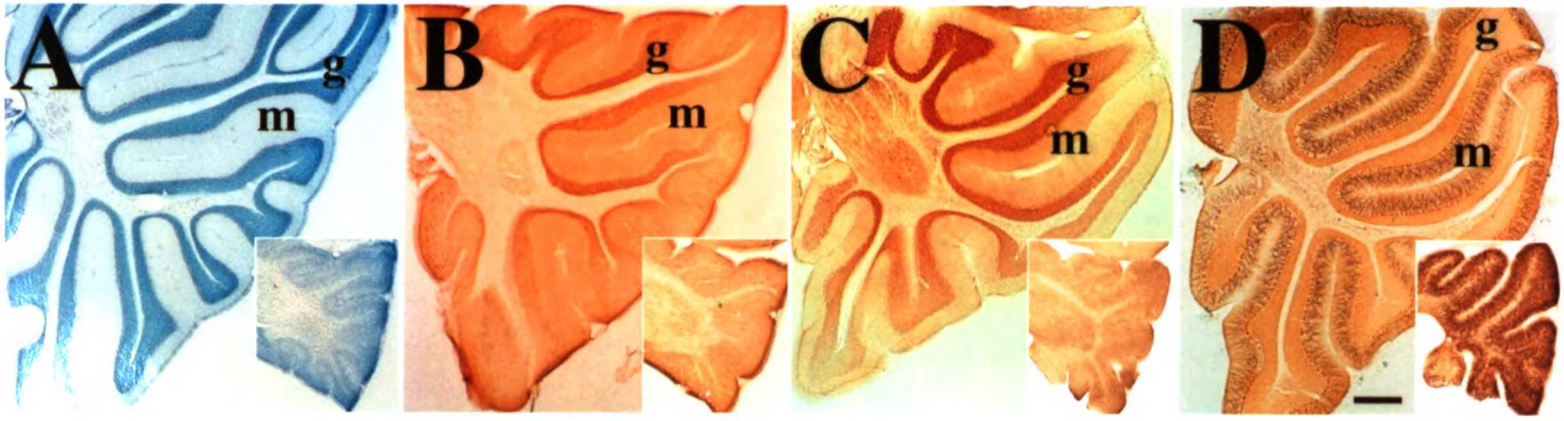
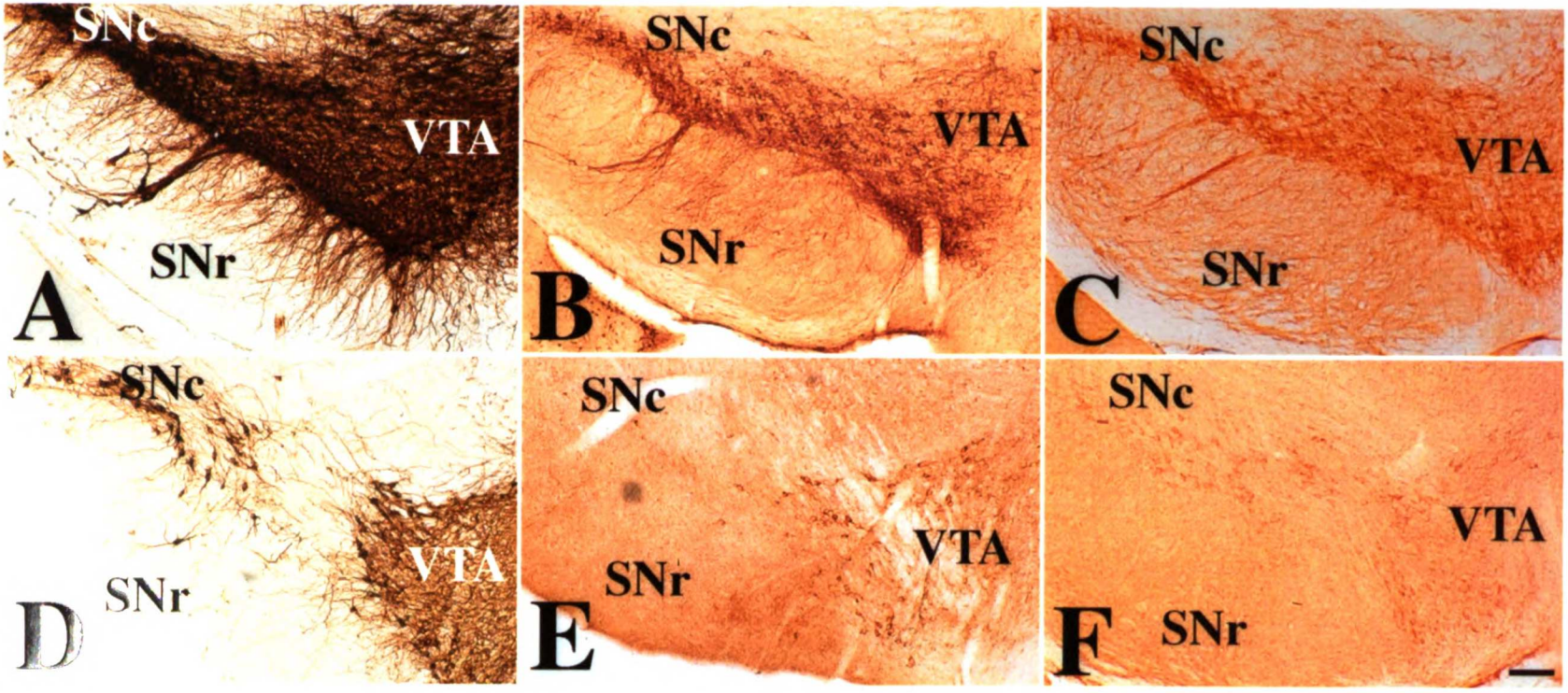


Figure 6: Comparison of GIRK1, GIRK2, and IRK1 staining between wildtype and *weaver* mouse cerebellum.

Parasagittal views of cerebella from wildtype mice and from PND19 *weaver* mice (Inset) counterstained with toludine blue (A) or stained with antibodies against GIRK2 (B), GIRK1 (C), and IRK1 (D). There is a dramatic loss of the granule cell layer (g) and corresponding reduction in the staining of GIRK2 and GIRK1. The IRK1 staining of Purkinje cell body (p) and dendrites still persists in the *weaver* cerebellum. The magnification is the same in each panel and insets. Scale bar: 0.2 mm.


SNc
VTA
SNr
A
SNc
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B
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C
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D
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VTA
SNr
E
SNc
VTA
SNr
F

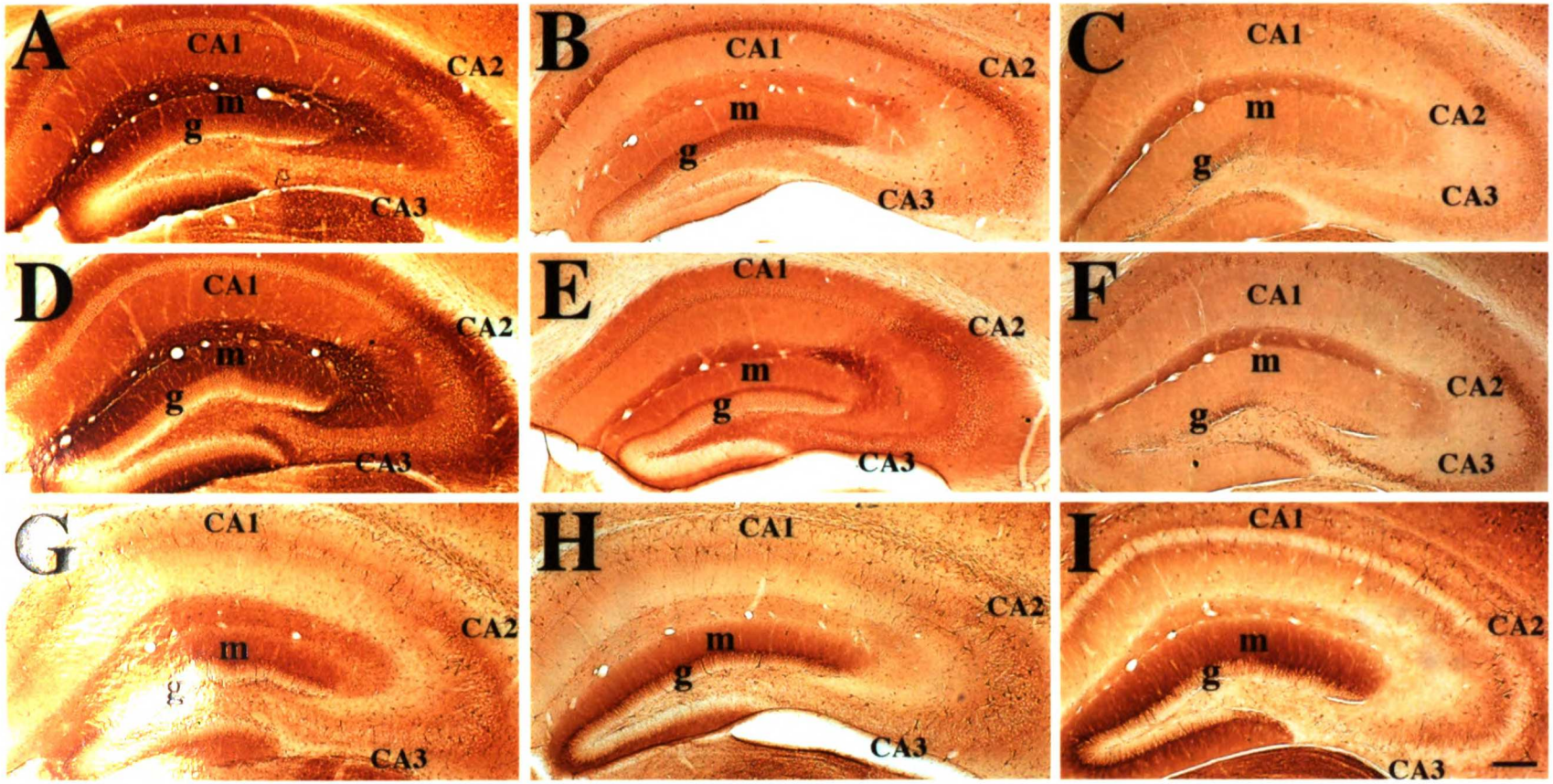
Figure 7: Reduction in dendritic and cell body staining of GIRK2 in the *weaver* substantia nigra pars compacta (SNc) and ventral tegmental area (VTA).

Comparison of coronal sections of wildtype and *weaver* midbrain stained with antibodies against tyrosine hydroxylase (**A, D**), N-terminus of GIRK2 (**B, E**), or C-terminus of GIRK2 (**C, F**). There is a dramatic decrease in both cell number as well as in dendritic staining of GIRK2-positive neurons in the SNc and VTA, while the number of TH-positive neurons decreases in the SNc but remains about the same in the VTA. **Scale bar**: 0.1 mm.

$K_V1.4$, calbindin, and P65 synaptotagmin between *wv* and wildtype substantia nigra (data not shown).

The hippocampal formation of *weaver* mice exhibits abnormal GIRK2 as well as GIRK1 staining.

The hippocampus of PND19, PND27, and PND95 *wv* and wildtype littermate mice were examined for immunoreactivity with antibodies against GIRK1, GIRK2, IRK1 (found in cell bodies as well as dendrites of dentate granule cells and CA3-1 pyramidal cells; Y.J.L., unpublished observation), $K_V1.4$ (found in axons and axon terminals; Sheng et al., 1992), calbindin (calcium-binding protein in dentate granule cell bodies and dendrites; Baimbridge and Miller, 1982), parvalbumin (calcium-binding protein in basket pyramidal interneurons), and P65 synaptotagmin (in synapses; Matthew et al., 1981). At PND19, there was no dramatic difference between *wv* and wildtype hippocampi (data not shown). By PND27 (Fig. 8B, E) and also at PND95 (Fig. 8C, F), significant change in expression of GIRK2 as well as GIRK1 became apparent. The *wv* hippocampus no longer showed intense dendritic staining of GIRK2 in the molecular layer of the dentate gyrus and strata oriens and radiatum of CA3-1 areas, though the cell bodies still exhibited light immunoreactivity comparable to that in the wildtype (compare Fig. 8D to E, F). Interestingly, there was a similar reduction in GIRK1 immunoreactivity in *wv* hippocampus (compare Fig. 8A to B, C). We observed less prominent differences between wildtype and *wv* hippocampus in their staining pattern of IRK1 (Fig. 8G-I) and $K_V1.4$ (data not shown), two channel subunits that were not found to associate with GIRK1 and GIRK2 (this paper). There was also relatively little difference in the staining patterns for calbindin, parvalbumin, and P65 synaptotagmin in these mice (data not shown). Consistent with the lack of report on hippocampal abnormalities in *wv* mice, no gross reduction in hippocampal cell number or size was observed. Thus, the *wv* mutation appeared to affect the expression of GIRK1 and GIRK2 specifically.


A
CA1
CA2
m
g
CA3
B
CA1
CA2
m
g
CA3
C
CA1
m
CA2
g
CA3
D
CA1
CA2
m
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CA3
E
CA1
CA2
m
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CA3
F
CA1
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CA3
G
CA1
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g
CA3
H
CA1
CA2
m
g
CA3
I
CA1
CA2
m
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CA3

Figure 8: The GIRK2 mutation in *weaver* mice results in defects in not only GIRK2 but also GIRK1 expression patterns in the hippocampus.

Serial sections of hippocampi from PND27 wildtype littermate (**A, D, G**), PND27 *weaver* (**B, E, H**), and PND95 *weaver* (**C, F, I**) mice for GIRK1 (**A-C**), GIRK2 (**D-F**), and IRK1 (**G-I**) staining. There is a dramatic decrease in dendritic staining of both GIRK1 and GIRK2 in the *weaver* hippocampi, while light level of immunoreactivity persists in the cell bodies. There is relatively little difference in IRK1 staining and in the number of cell bodies and structure of the hippocampus between *weaver* and wildtype littermates. See Results for further details. **Scale bar**: 0.2 mm. **Abbreviations: g:** granule cell layer in the dentate gyrus; **m:** molecular layer of the dentate gyrus; **CA1-3:** regions of the hippocampus proper.

Discussion

Using antibodies specific for GIRK1 or GIRK2, we have determined the expression patterns for these two G protein-gated K_{ir} channel subunits in mammalian brain and verified that these patterns of protein expression match the mRNA distributions (Karschin et al., 1994, 1996; Stoffel et al., 1994; Kobayashi et al., 1995; this study) and are consistent with the reported GIRK1 protein distribution (Ponce et al., 1996). We further show that GIRK1 and GIRK2 are specifically co-immunoprecipitated from cerebral cortex, hippocampus, and cerebellum. These findings, together with previous studies which suggest that GIRK1 and GIRK2 in heterologous systems can co-assemble to form heteromeric channels (Kofuji et al., 1995, 1996; Duprat et al., 1995; Lesage et al., 1995; Slesinger et al., 1996; Navarro et al., 1996), indicate that GIRK1/GIRK2 heteromultimeric channels exist in the mammalian brain. The dramatic alterations in GIRK1 as well as GIRK2 protein expressions in the *weaver* mice suggest that a significant proportion of GIRK1 channel subunits exists in complex with GIRK2 in the mouse brain.

GIRK channels of different composition exist in different parts of mammalian brain.

GIRK channels may exist in the brain as homomeric channels composed of one subunit type or as heteromeric complexes of two or more subunit types. GIRK2 can form functional homo- or heteromeric channels in heterologous expression systems. It is therefore conceivable that homomeric GIRK2 channels and heteromeric channels containing GIRK2 may be found in different proportions in different regions of the brain. Given that GIRK1 does not seem to form functional homomeric channels in heterologous systems (Kofuji et al., 1995; Duprat et al., 1995; Krapivinsky et al., 1995a,b; Lesage et al., 1995; Hedin et al., 1996), its association with GIRK2 *in vivo* may form the molecular basis for some of the GIRK1-containing channels in central neurons. Since GIRK1 and GIRK2 channel proteins overlap in many brain regions in the rat and even more so in the mouse, the GIRK1 subunit may be encountered in heteromeric complexes with GIRK2 in many brain regions. In

regions where GIRK1-4 all seem to be expressed such as the hippocampus, cerebral cortex, and thalamus (Karschin et al., 1994, 1996; Kobayashi et al., 1995; Ponce et al., 1996; Spauschus et al., 1996; this study), heteromeric GIRK channels may also consist of more than two different channel subunits, and channels with different subunit stoichiometry could conceivably exist in the same neuron.

Potential transmitter receptors for *in vivo* regulation of K_{ir} channels composed of GIRK1 and/or GIRK2 subunits.

Transmitter receptors that activate K_{ir} channels via G proteins include those for acetylcholine (M_2), adenosine (A_1), ATP (P_2), dopamine (D_2), γ-aminobutyric acid ($GABA_B$), opioid (μ, δ, κ), serotonin (5-HT_1; 5-HT_2), norepinephrine (α2), and somatostatin (North et al., 1987; North, 1989; Brown, 1990; Nicoll et al., 1990; Inoue et al., 1992; Hille, 1992, 1994). Other receptors such as those for substance P, neurotensin, thyrotrophin-releasing hormone, and angiotensin II inhibit K_{ir} channels and cause membrane depolarization (reviews listed above; Stanfield et al., 1985; Yamaguchi et al., 1990; Takano et al., 1995). In vitro co-expression experiments have shown that GIRK channel subunits can form homo- or heteromeric channels that are activated by neurotransmitter receptors such as M_2 acetylcholine receptor (Kubo et al., 1993; Dascal et al., 1993; Krapivinsky et al., 1995a,b; Slesinger et al., 1996), β_2-adrenergic receptor (Lim et al., 1995), δ opioid receptor (Dascal et al., 1993; Lesage et al., 1994), μ opioid receptor (Chen and Yu, 1994; Kovoor et al., 1995), κ opioid receptor (Ma et al., 1995; Henry et al., 1995), and 5-HT_{1A} receptor (Dascal et al., 1993; Kovoor et al., 1995).

Through electrophysiological and distribution studies, many neurotransmitter receptors that regulate K_{ir} via G proteins have been found in brain regions that also express GIRK1 and/or GIRK2. While *in vitro* evidence shows that many neurotransmitter receptors have the potential to regulate K_{ir} channels composed of GIRK1-4 subunits, it is not known just which combination of receptors and GIRK channels mediates the neurotransmitter

effects in different brain regions. We provide here a brief listing of potential receptors that may regulate K_{ir} channels containing GIRK1 and/or GIRK2 channel subunits (for review see North et al., 1987; North, 1989; Nicoll et al., 1990; Brown, 1990; Inoue et al., 1992; acetylcholine receptor: Levey et al., 1991; Karschin et al., 1994; Butcher et al., 1995; adenosine A1 receptor: Schwabe et al., 1991; Weber et al., 1990; dopamine D_2 receptor: Mansour et al., 1990; opioid receptors: Mansour et al., 1987; Yasuda et al., 1993; Meng et al., 1993; Thompson et al., 1993; Mestek, 1995, Arvidsson et al., 1995; somatostatin receptor: Breder et al., 1992; Gonzalez et al., 1992; Karschin et al., 1994; Reisine and Bell, 1995). In brain regions that express both GIRK1 and GIRK2, the cerebral cortex expresses the adenosine A_1 receptors, receptors for opioid peptides, and somatostatin receptors. The hippocampus and dentate gyrus express the A_1 receptors, $GABA_B$ receptors, D_2 dopamine receptors, 5-HT_{1A} receptors, μ receptors, and somatostatin receptors. The cerebellar granule cells express the A_1 receptors and somatostatin receptors (transiently during development). Of regions that express GIRK2 but only low levels of GIRK1, the A_1 adenosine receptors, κ_1 and μ opioid receptors, and D_2 dopamine receptors are found in the substantia nigra pars compacta and ventral tegmental area. The present study, combined with previous studies of transmitter receptor distributions, provides a basis for further characterization of the molecular composition of K_{ir} channels that are effectors of various transmitter receptors.

The somatodendritic localization of GIRK1 and GIRK2 channel subunits in some brain regions suggests that these channels may be involved in specific functions.

The relatively ubiquitous GIRK2 and GIRK1 immunoreactivity and their regulation by many different transmitter receptors when expressed in heterologous systems indicate that they are likely to contribute to a significant fraction of the G protein-gated inwardly rectifying potassium channels *in vivo*. The presence of mRNA and the somatodendritic localization of GIRK1 and GIRK2 proteins in certain central neurons (e.g. cerebral cortical pyramidal cells, substantia nigra dopaminergic neurons, and cerebellar granule cells) suggest

that they are on post-synaptic membranes. Many G protein-coupled receptors are found in dendrites and cell bodies, where they may mediate some of the longer lasting effects of transmitters released from nerve terminals of presynaptic neurons. Similar receptors are also present in nerve terminals, where they may regulate transmitter release. In the substantia nigra, the D_2 autoreceptors in dopaminergic neurons may regulate the dendritic release of dopamine, a neurotransmitter believed to be important for the self-regulation of dopaminergic neurons (Cheramy et al., 1981). Since GIRK2 is highly expressed in the substantia nigra pars compacta (Fig. 3G-H, Fig. 7B-C) but in low or background level in the striatum (Fig. 2B), it seems more likely that GIRK2 is involved in controlling dendritic dopamine release than in controlling release from nigral dopaminergic axon terminals. In many other regions of the brain, GIRK1 and GIRK2 are localized to sites of synapses such as the glomeruli of the cerebellar granule cell layer (Fig. 3K-L), perforant pathway terminal fields in the hippocampal formation (Fig. 3D-E; Fig. 8A, D), and layer IV of the barrel cortex (Fig. 3A). To elucidate the function of the GIRK channel subunits in these regions, it will be important to use electron microscopy to determine their pre- and/or postsynaptic localization and to identify transmitter receptors that may regulate them *in vivo*. Recently, Ponce et al. (1996) have shown by electron microscopy that GIRK1 is found in the post-synaptic membrane of the granule cell dendrites, and they also show that GIRK1 may be present presynaptically in the thalamic projections to the layer IV of the cerebral cortex and stratum lacunosum moleculare of the hippocampus.

Down-regulation of GIRK1 as well as GIRK2 protein expression and distribution in the *weaver* mice may be a result of their association *in vivo*.

It is surprising that the *wv* mutation, a single nucleotide change, could have resulted in the decrease in protein level as well as the dramatic alteration in protein distribution of both GIRK1 and GIRK2 channel subunits. Given that GIRK1 and GIRK2 interact *in vivo* to form heteromeric complexes, the simplest explanation for the down-regulation of both

proteins is the removal of GIRK1/GIRK2 channel complexes that contain abnormal GIRK2*wv* protein. Central neurons may target the GIRK1/GIRK2*wv* channel complex for degradation due to either abnormal structure or function. The selective removal of aberrant channel complexes may take place soon after protein synthesis in the endoplasmic reticulum or at the level of protein targeting. Some of the down-regulation of expression may also take place after the aberrant GIRK1/GIRK2*wv* complex has reached the plasma membrane, where its abnormal electrophysiological properties would be evident. Since abnormal current as a result of the *wv* mutation can be recorded from the cell body of dissociated cerebellar granule cells (Kofuji et al., 1996; Surmeier et al., 1996; P.A. Slesinger, unpublished observation), some GIRK channel complex containing the GIRK2*wv* subunit is clearly inserted into the plasma membrane of the cell body. Since more of the unglycosylated form of GIRK1 can be found in the *weaver* mice, it seems likely that some of the GIRK1/GIRK2*wv* complex is being retained in the endoplasmic reticulum.

Mutation in GIRK2 may directly or indirectly give rise to cerebellar and dopaminergic dysfunctions in *weaver* mice.

The G156S mutation in GIRK2 causes a range of abnormalities of channels expressed in *Xenopus* oocytes, including loss of potassium selectivity of GIRK2 channels and reduction of function of heteromultimers of GIRK1 and GIRK2 (Slesinger et al., 1996; Kofuji et al., 1996; Navarro et al., 1996). It thus appears that the *wv* mutation could qualitatively exert different effects in different neurons, depending on the level of expression of GIRK2 and other K_{ir} channel subunits such as GIRK1. In neurons that express high levels of GIRK2 but not GIRK1, such as the dopaminergic neurons in the substantia nigra, the *wv* mutation could lead to chronic or inhibitory transmitter-induced depolarization and potentially cell death. In other neurons which normally produce heteromeric channels containing GIRK1 and GIRK2 subunits such as those in the cerebral cortex, hippocampus, thalamus, and cerebellum, this mutation may weaken or abolish certain signaling processes. It seems likely that cerebellar

granule cells could belong to this latter category. Although no dramatic anatomical defects in hippocampus, cerebral cortex, and thalamus have been reported (but see Sekiguchi et al., 1995), we find that the subcellular distribution of both GIRK1 and GIRK2 channel subunits in these brain regions is severely altered in the *wv* mice. It is possible that neurons in these brain regions have compensatory mechanisms which prevent them from dying, unlike the cerebellar granule cells and substantia nigra dopaminergic neurons. These mechanisms may facilitate the removal of free calcium from the cytoplasm, removal of mutant channel complexes from the plasma membrane, or substitution of GIRK2 functions with other GIRK channel subunits. To understand the roles of G protein-gated K_{ir} channels *in vivo* and in the different *wv* mutant phenotypes, it will be important to determine the channel compositions and transmitter receptors that regulate these channels in different cell types during development and in the adult.

Chapter 3:

Mice lacking G protein-coupled inwardly rectifying K+ channel GIRK2 are susceptible to seizures but have normal midbrain and cerebellum

Abstract

G protein-gated inwardly rectifying K^+ channels (GIRK) are effectors of G protein-coupled receptors for neurotransmitters and hormones (Sakman et al., 1983; Breitwieser et al., 1985; Pfaffinger et al., 1985) and may play an important role in the regulation of neuronal excitability (Hille, 1992; Jan and Jan, 1994; Kubo, 1994; Wickman and Clapham, 1995). GIRK channels may be important in neurodevelopment as suggested by the recent finding that a point mutation in the pore region of GIRK2 (G156S) is responsible for the *weaver* (*wv*) phenotype (Patil et al., 1996), which includes extensive cerebellar granule cell death (Rakic and Sidman, 1973a,b; Hatten et al., 1984), age-dependent dopaminergic neuronal loss (Schmidt et al., 1982; Roffler-Tarlov and Graybiel, 1984), seizures (Eisenberg and Messer, 1989) and male infertility (Harrison and Roffler-Tarlov, 1994). The GIRK2G156S gene gives rise to channels that exhibit a loss of K^+ selectivity and may also exert dominant-negative effects on $G_{\beta\gamma}$-activated K^+ currents (Slesinger et al., 1996; Kofuji et al., 1996; Navarro et al., 1996). Electrophysiological recordings from *weaver* (*wv/wv*) and wildtype cerebellar granule cells have yielded conflicting reports, supporting either a loss of K^+ selectivity (Kofuji et al., 1996) or a loss of channel function (Surmeier et al., 1996). To investigate the physiological role of GIRK2, we generated mutant mice lacking GIRK2 (GIRK2-/-). Unlike *wv/wv* mutant mice, GIRK2-/- mice are morphologically indistinguishable from wild-type mice, suggesting that the *wv* phenotype is likely due to abnormal GIRK2 function. Like *wv/wv* mice, GIRK2-/- mice have much reduced GIRK1 expression in the brain. They also develop spontaneous seizures and are more susceptible to pharmacologically induced seizures using a GABA antagonist. Moreover, *wv/-* mice exhibited much milder cerebellar abnormalities than *wv/wv* mice, indicating a dosage effect of the GIRK2 G156S mutation. Our results indicate that the *weaver* phenotypes arise from a gain-of-function mutation of GIRK2 and that GIRK1 and GIRK2 are important mediators of neuronal excitability *in vivo*.

Results and Discussion

The GIRK2 gene was disrupted in embryonic stem cells by homologous recombination using a targeting vector in which exon 2 was disrupted and partially deleted by a pgk-neomycin resistance casette (Fig.1a). An embryonic stem (ES) cell clone that carried the targeted allele was used to generate chimeric male animals which passed the mutant allele to their offspring. GIRK2+/- mice were indistinguishable from wildtype mice and were inbred to produce GIRK2-/- mice (Fig.1b). No normal GIRK2 mRNA could be detected in brains of adult GIRK2-/- mice by RT-PCR analysis, but a truncated GIRK2 mRNA was present (Fig.1c). No GIRK2 immunoreactivity was detectable by western blot analysis and immunohistochemistry using antibodies against either the N- or C-terminus of GIRK2 (Fig. 2a, 3a, b; data not shown). We conclude, therefore, that we have generated GIRK2 null mice.

GIRK2-/- mice are born at the expected frequency and are viable. Given that GIRK2 and GIRK1 have partly overlapping temporal and spatial expression patterns and are known to form functional heteromultimers *in vitro* (Slesinger et al., 1996; Kofuji et al., 1996; Navarro et al., 1996), (Kobayashi et al., 1995; Duprat et al., 1995; Lesage et al., 1995) and *in vivo* (Liao et al., 1996), we examined the expression of GIRK1 and other related inward rectifier channels by using affinity purified polyclonal antibodies against GIRK1, IRK1, and GIRK4 in western and immunohistochemical studies of GIRK2+/+, +/- and -/- mice. Immunoblot analysis showed that GIRK1 levels were reduced in brain membranes of GIRK2+/- mice and nearly undetectable in -/- mice, while IRK1 protein levels remained constant in mice of all three genotypes (Fig.2a). RT-PCR analysis showed that GIRK1, GIRK4 and IRK1 mRNA were similar in all animals, suggesting that the downregulation of GIRK1 in GIRK2-/- mice occurred post-transcriptionally (Fig.2b). Immunohistochemical analysis showed dramatic reduction of GIRK1 immunoreactivity in many brain regions in GIRK2-/- mice, while IRK1 and GIRK4 immunoreactivities were normal in these GIRK2

mutants (Fig.3a,b, data not shown). The extent of reduction in GIRK1 varied with the brain regions; expression of GIRK1 in the cerebral cortex and hippocampus was virtually undetectable whereas in the cerebellum significant amounts of GIRK1 remained in the granule cell layer (Fig.3a,b). The reduction of GIRK1 protein levels throughout the brain suggests that the majority of GIRK1 proteins in the brain interacts with GIRK2 and, in the absence of GIRK2, there is a concurrent loss of GIRK1 subunits which normally would form heteromultimers with GIRK2.

The GIRK2-/- and *wv/wv* mice showed striking differences. Visual inspection and histological examination of the brain and other organs of GIRK2-/- animals revealed no anomalies. GIRK2-/- mice exhibited normal cerebellar morphology except for the reduced GIRK1 and GIRK2 protein expression (Fig.3b, d). Midbrain dopaminergic neurons and their dendrites also appeared normal despite the absence of GIRK2 protein (Fig.3c). While male *wv/wv* mice are infertile, male GIRK2-/- mice are fertile; superovulated CD-1 mice mated with either GIRK2-/- males or their wildtype littermates produced comparable number of fertilized eggs. The apparent normal phenotype in GIRK2-/- mice provides strong evidence that loss of homomeric GIRK2 channel and/or heteromeric GIRK1/GIRK2 channel function is not the primary cause of the *weaver* phenotype.

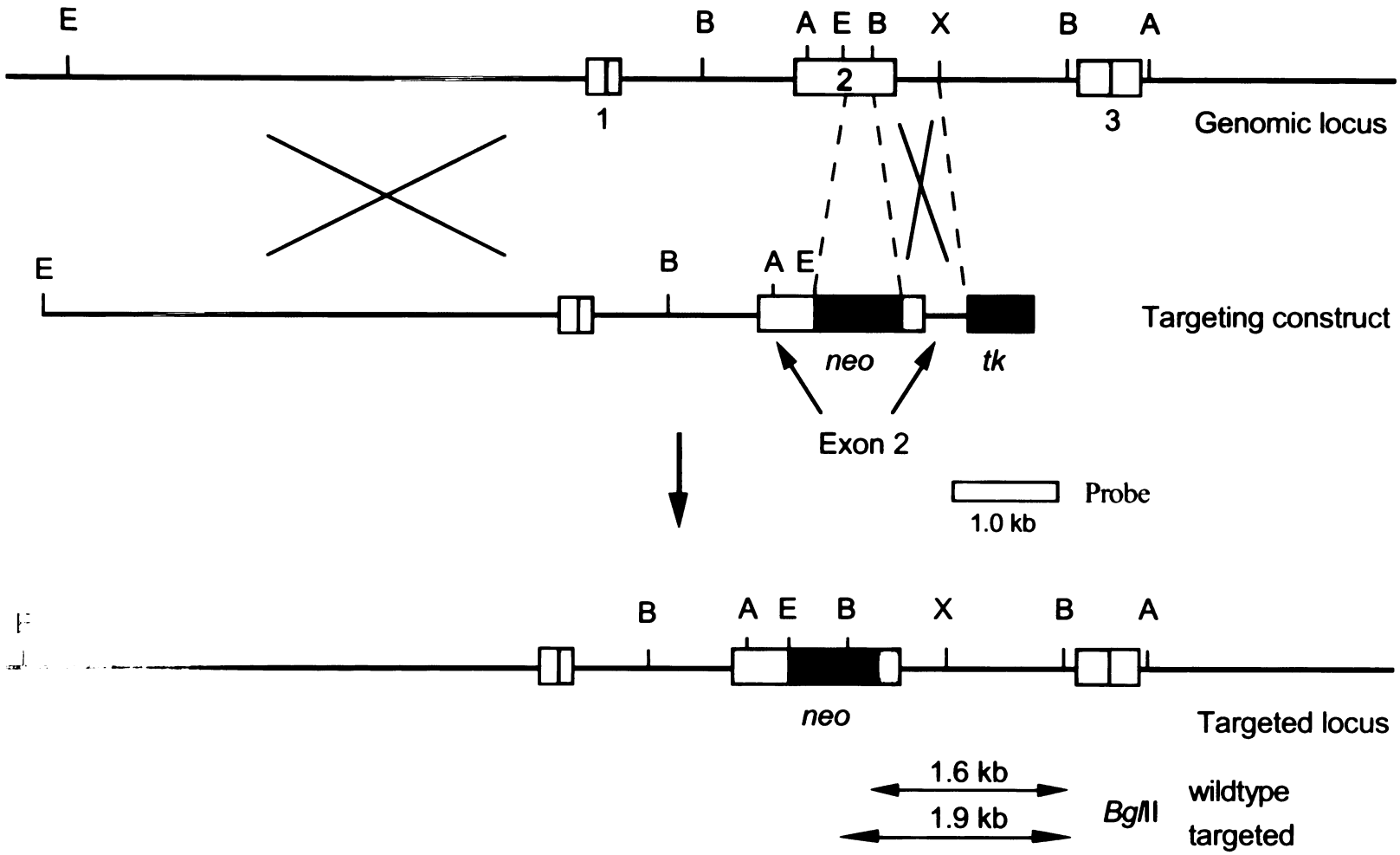
When GIRK2-/- mice were compared to mice carrying one or two copies of the *wv* allele (GIRK2*wv*/-, *wv/wv*), we found that both -/- and *wv*/- mice exhibit normal locomotive behavior, unlike the *wv/wv* mice. In +/+, -/- and *wv*/- animals, there was no obvious loss of TH-positive neurons or dendrites in the substantia nigra pars compacta (SNc) or in the ventral tegmental area (VTA), while substantial cell loss was evident in the SNc of the *wv/wv* midbrain (Fig.3c). In the SNc and VTA of *wv*/- mice, GIRK2 immunoreactivity was present but of lower intensity. In contrast to the heterozygous GIRK2+/- mice, most of the GIRK2 immunoreactivity in the *wv*/- mice was found in the cell bodies of the dopaminergic neurons; the GIRK2 immunoreactivity in the dendrites was much reduced (Fig.3c). The size and gross morphology of the cerebellum of *wv*/- animals are not significantly different from that of the

wildtype animals. Histologically, the *wv*/- cerebellum appeared more similar to that of the *wv*/+ (Rakic and Sidman, 1973a,b) than the cerebellum of +/+ or *wv*/*wv* mice. The *wv*/- granule cell layer often appeared thinner. The Purkinje cell layer was disorganized in various locations, and some of the Purkinje cells were found deep in the granule cell layer (Fig. 3d). The similarity between *wv*/- and *wv*/+ cerebella and the difference between *wv*/- and *wv*/*wv* cerebella suggest that cerebellar development is sensitive to the dosage of the GIRK2G156S mutant gene.

The GIRK2-/- mice exhibited sporadic seizures characterized by jerking of head and body, vocalization and sometimes progression to a tonic-clonic seizure. Typically, the episodes lasted for 30 seconds and were followed by complete physical inactivity. All witnessed seizures occurred when some kind of stress was exerted on the animal (changing cages, setting up matings), and the behaviour of mice returned to normal after the seizure. Seizures were never observed before weaning and seemed to occur at equal frequencies in young and old mutant mice. Pharmacological challenge with the convulsant agent pentylenetetrazol (PTZ) (Orloff et al., 1949), a GABA antagonist, revealed that GIRK2-/- mice were hyperexcitable when challenged with a single injection of PTZ (50 mg/kg). At this dose, 70% of GIRK2-/- mice but only 25% of heterozygous or wildtype littermates developed severe stage 3 tonic-clonic seizures frequently associated with death ($P<0.004$ Mann-Wilcoxon rank sum test). The severity of seizure, in the range from 0-3, was shifted towards increased severity in GIRK2-/- mice as compared to +/- and +/+ controls. No statistically significant difference was seen between heterozygous and wildtype mice (Fig.4A). The time taken to develop seizure activities was significantly shorter in GIRK2-/- mice compared to +/- and +/+ animals ($P<0.002$, unpaired *t*-test) (Fig.4B). Seizure activity has previously been noted in *weaver* mice and might be due to altered or reduced G protein-activated K^+ channel function (Eisenberg and Messer, 1989). Our observation that GIRK1/GIRK2-deficient mice are susceptible to spontaneous and pharmacologically-induced seizures was consistent with numerous studies demonstrating that agonists of G protein-

coupled receptors, such as receptors for opioid peptides, somatostatin and dopamine, can have significant effects on seizure thresholds in several different experimental seizure model systems (Schwartzkroin, 1993).

In conclusion, we show that GIRK2 deficient mice have greatly reduced GIRK1 protein levels in the brain, suggesting that a significant proportion of GIRK1 proteins in the brain associate with GIRK2. Phenotypic characteristics of GIRK2-/-, *wv*/-, *wv*/+ and *wv*/*wv* mice suggest that gain-of-function and gene dosage mechanisms are responsible for the developmental defects in *weaver* mutants. Moreover, loss of GIRK2 function results in sporadic seizures and increased susceptibility to a convulsant agent, implicating GIRK1 and GIRK2 in the control of neural excitability *in vivo*.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B A
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Genomic locus
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Probe
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Targeted locus
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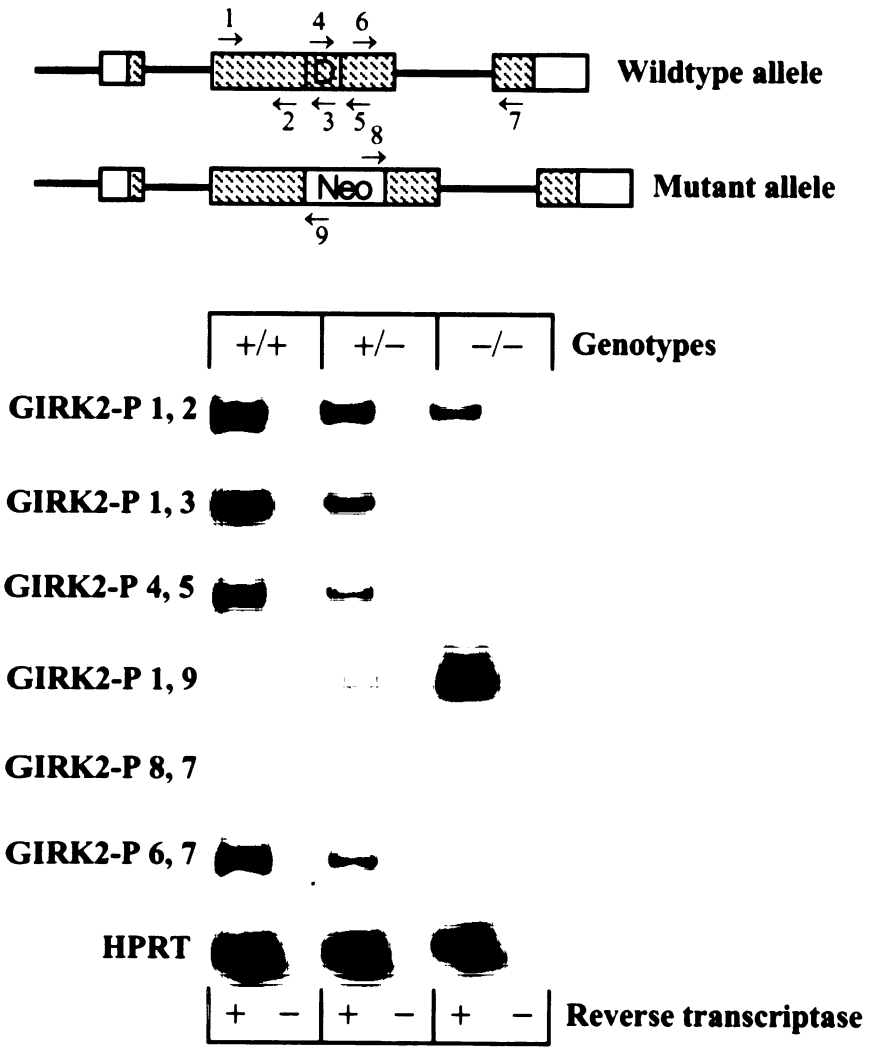
1.9 kb
BglII
wildtype
targeted

Fig.1b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Wildtype allele
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3
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Mutant allele
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Genotypes
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GIRK2-P 1, 3
GIRK2-P 4, 5
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HPRT
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Reverse transcriptase

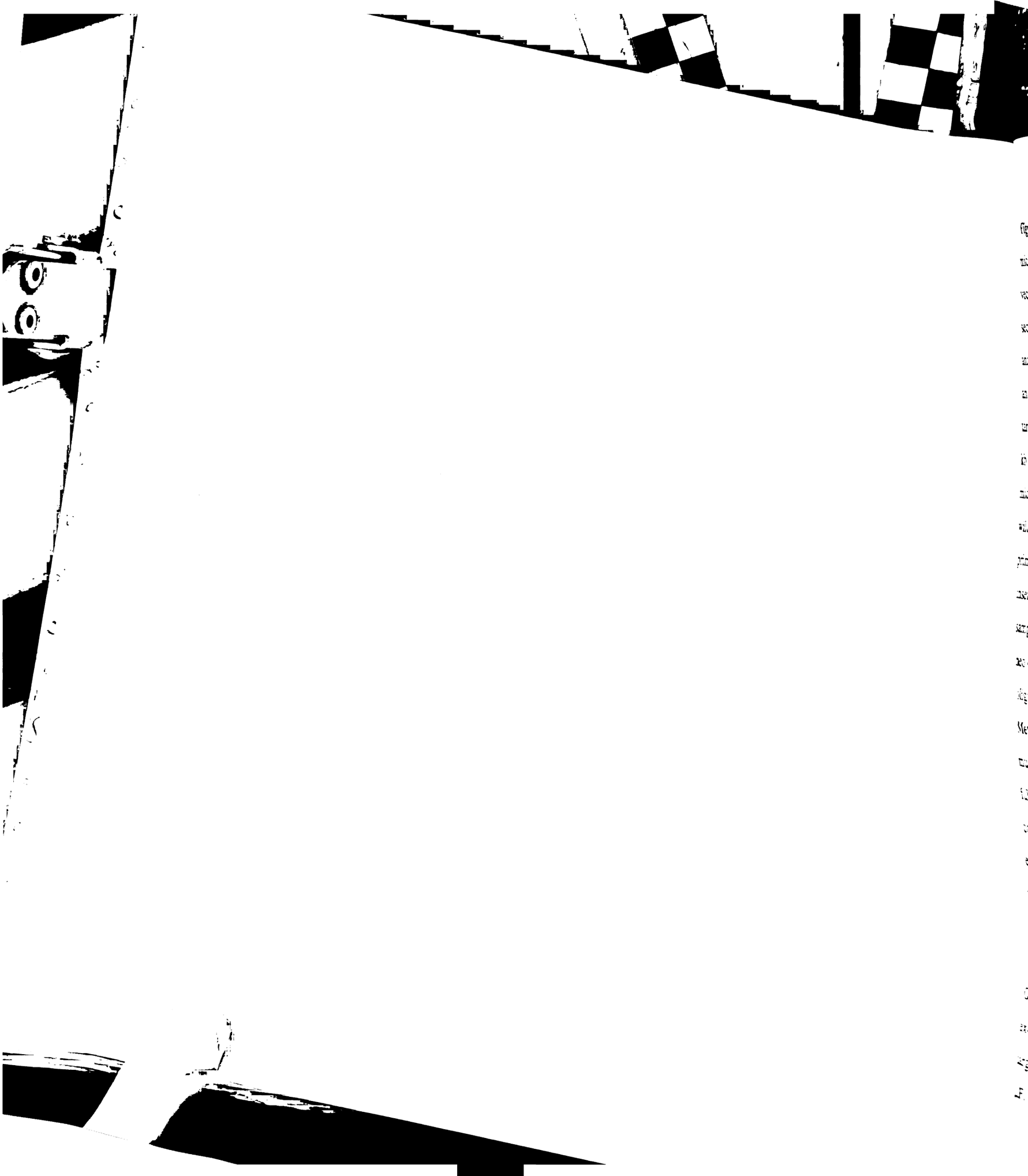
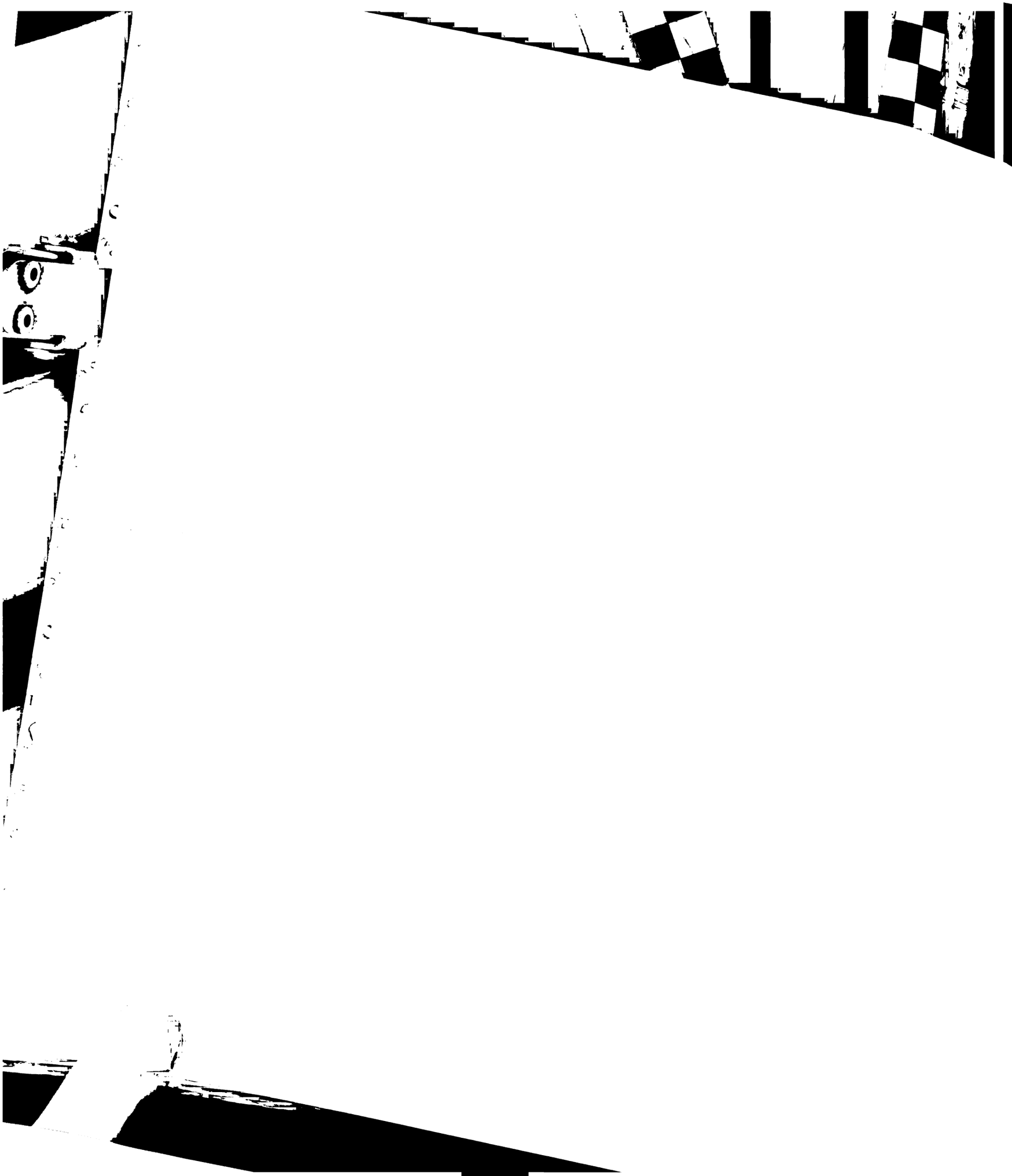


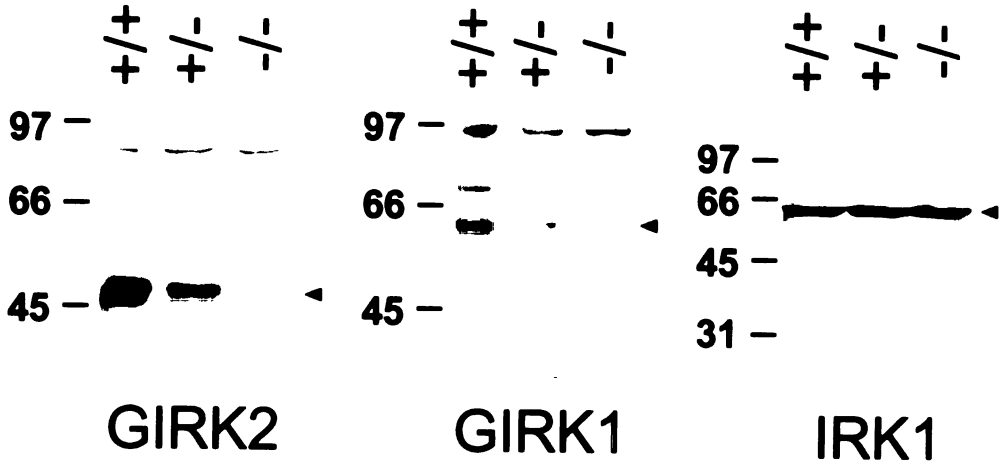
Figure 1 A. Targeted disruption of the mouse GIRK2 gene in embryonic stem cells and mice. Genomic structure and restriction map of the mouse GIRK2 gene locus and targeting vectors pPNT-76 used to disrupt the GIRK2 gene are shown. Grey boxes represent coding sequences of the exons (boxes). The probe 3' of the deletion is used for Southern blot analysis and shown as a white bar. B, *Bgl*II; A, *Acc*I; E, *Eco*RI; X, *Xba*I. **B.** Southern analysis of transfected ES cells; 1: parental clone containing the normal 1.6 kb fragment, 2: targeted clone containing the targeted allele, as assessed by the presence of an additional 1.9 kb *Bgl*II fragment. Lanes 3-9 show genotypes from tail biopsies of GIRK2 +/+, +/- and -/- mice. **C.** RT-PCR analysis from brain mRNA of GIRK2+/+, +/- and -/- mice. Top: GIRK2 wild-type and mutant allele is shown with the position of oligonucleotides used as PCR primers. D stands for deleted region into which pgk-neomycin resistance cassette was inserted. No primer-pair amplified products in the absence of reverse transcriptase. Each sample started with an equal amount of cDNAs. A 5'- truncated mRNA terminating in pgk-neo can be detected by PCR at reduced levels in +/- and -/- animals. The sequences of the oligonucleotide used are available upon request.

Methods: Genomic clones containing the murine GIRK2 gene were isolated from a λFIX II murine 129/Sv genomic library (Stratagene, LaJolla, CA) by screening the library using the full length hamster GIRK2 cDNA as a probe (Tsaur et al., 1995). Two identical phage clones containing the entire murine GIRK2 gene were identified and three exons containing the entire open reading frame were mapped. To generate the GIRK2 targeting vector pPNT-76, an ≈8kb *Eco*RI fragment containing exon 1 and part of exon 2 was inserted into the targeting vector pPNT (Tybulewicz et al., 1991) such that its 3' end was adjacent to the PGK promotor upstream of the neo gene. The 3' end of the targeting construct was generated from the same GIRK2 genomic clone and contained a 0.47 kb *Bgl*II-*Xba*I fragment that was inserted into the exon 2 *Eco*RI/*Bgl*II deletion and included sequences from exon and intron 2 (Fig.1A). The targeting vector was linearized by *Not*I and electroporated into R1 ES cells at 200 V and 800 μF. Stable colonies were grown under double selection in 350 mg/ml of G418 and 0.2 μM



FIAU in ES cell medium (Robertson, 1987). 150 colonies were analyzed for homologous recombination by Southern blotting. One clone (G2) was identified by the presence of a 1.9 kb *Bgl*II band and was microinjected into blastocysts to generate GIRK2-deficient mice (Fig.1b).

A.


+/+
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66 –
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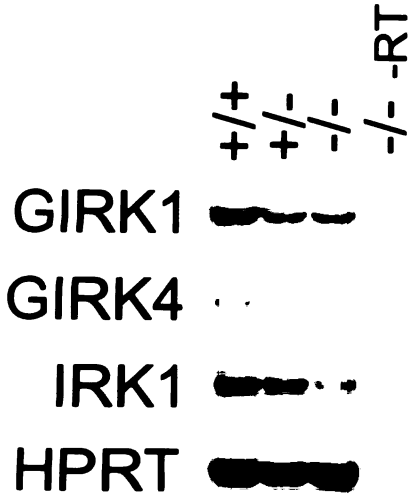
31 –
IRK1

B.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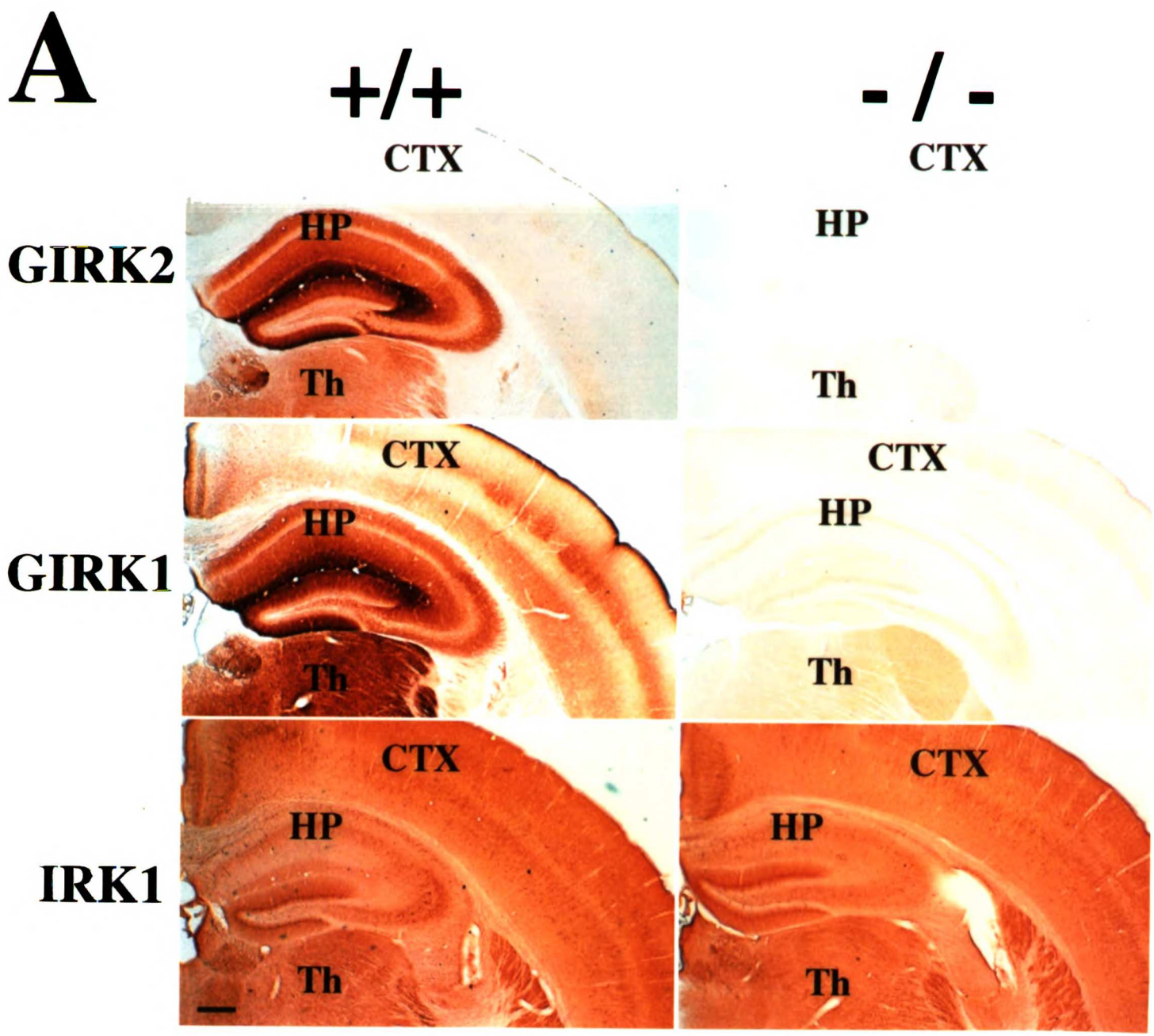
-/-
-/- -RT
GIRK1
GIRK4
IRK1
HPRT

Figure 2 A. Western blots of membrane prepared from GIRK2+/+, +/-, and -/- brains show that both GIRK2 and GIRK1 protein levels are reduced in the GIRK2 knockout mice. **B.** RT-PCR showing equal mRNA expression of GIRK1 in +/+, +/-, and -/- mouse brains. No product is amplified by GIRK1 primers in the absence of reverse transcriptase (GIRK1 -RT) (lane 4), confirming that all products were amplified from cDNA rather than contaminating genomic DNA. HPRT primers amplify a comparable level of product in all samples, indicating that same amount of template is present. Amplified products by GIRK1, GIRK4, IRK1 and HPRT primers are of expected sizes.

Methods: Fifty μg of mouse brain membrane, prepared as described (Liao et al., 1996), was solubilized in 2% SDS-sample buffer (125 mM Tris, pH 6.8, 20% glycerol, 5% ß-mercaptoethanol) and loaded onto each lane. Western blots were probed with 1 μg/ml of affinity-purified rabbit polyclonal antibodies against the N-terminus of GIRK2 or GIRK1 and against the C-terminus of IRK1 (Liao et al., 1996). Donkey anti-rabbit-HRP was used as secondary antibody at 1:5000 dilution. The blots were developed with ECL reagents (Amersham) and exposed to Hyperfilm-ECL (Amersham).


A
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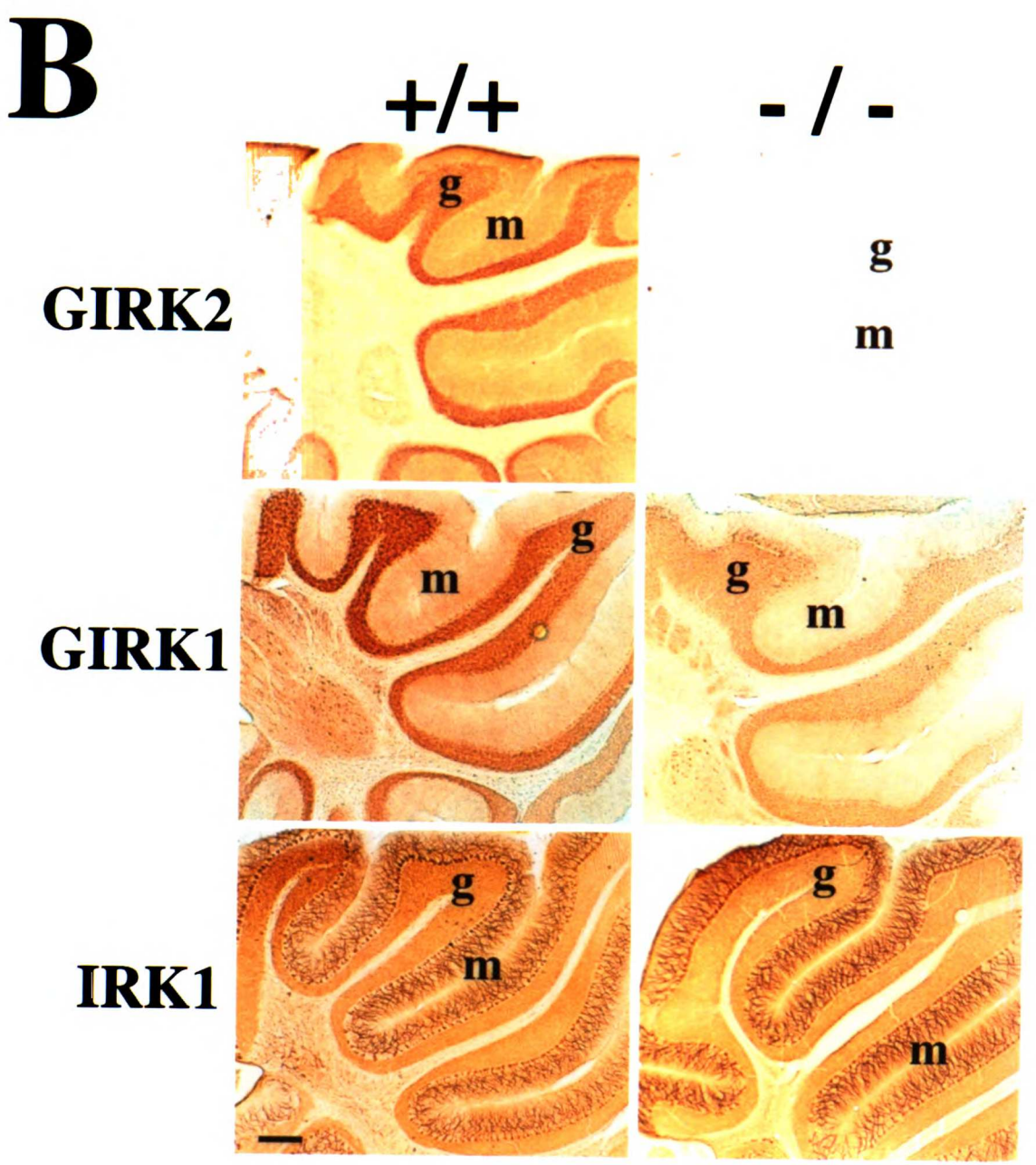
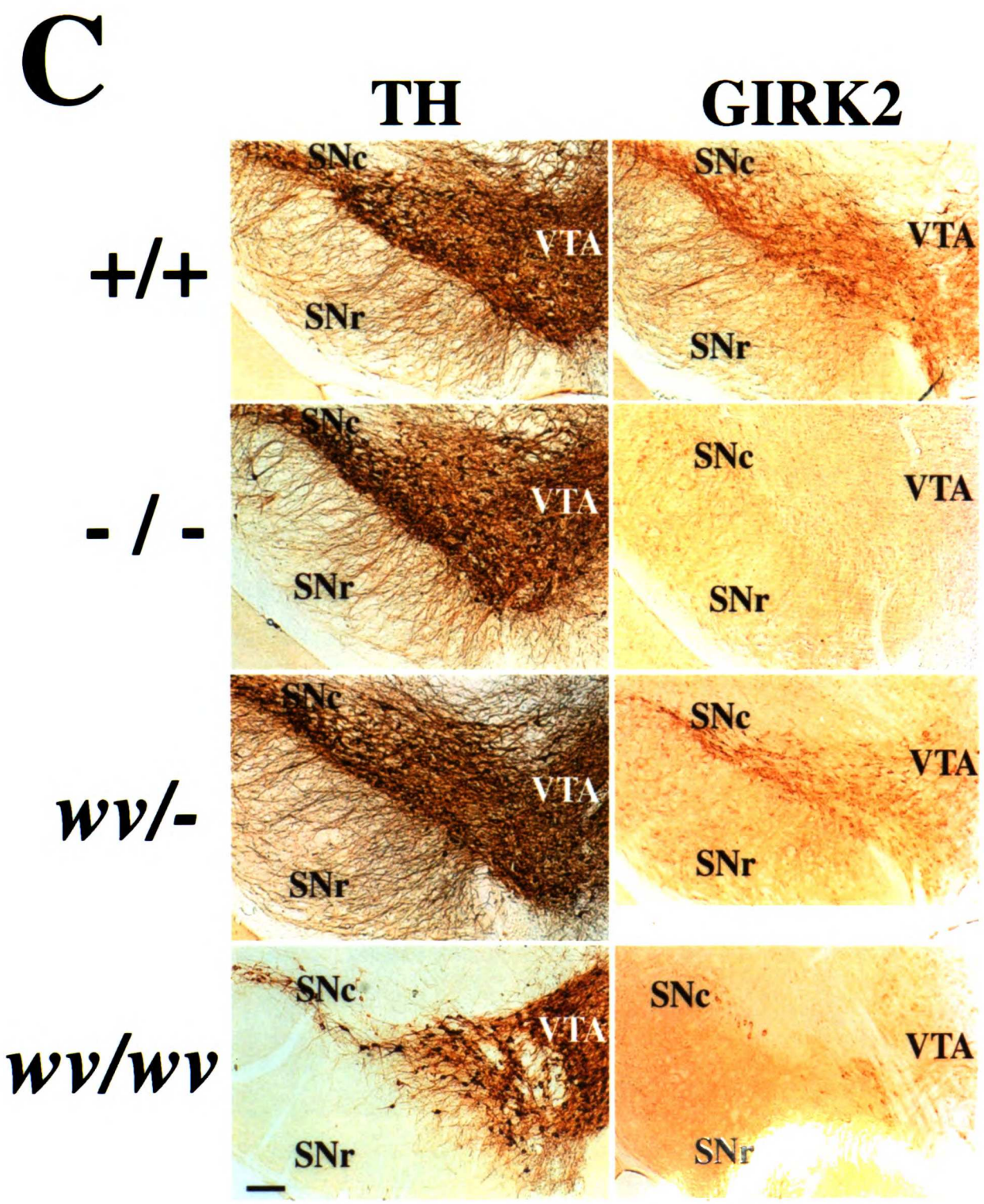
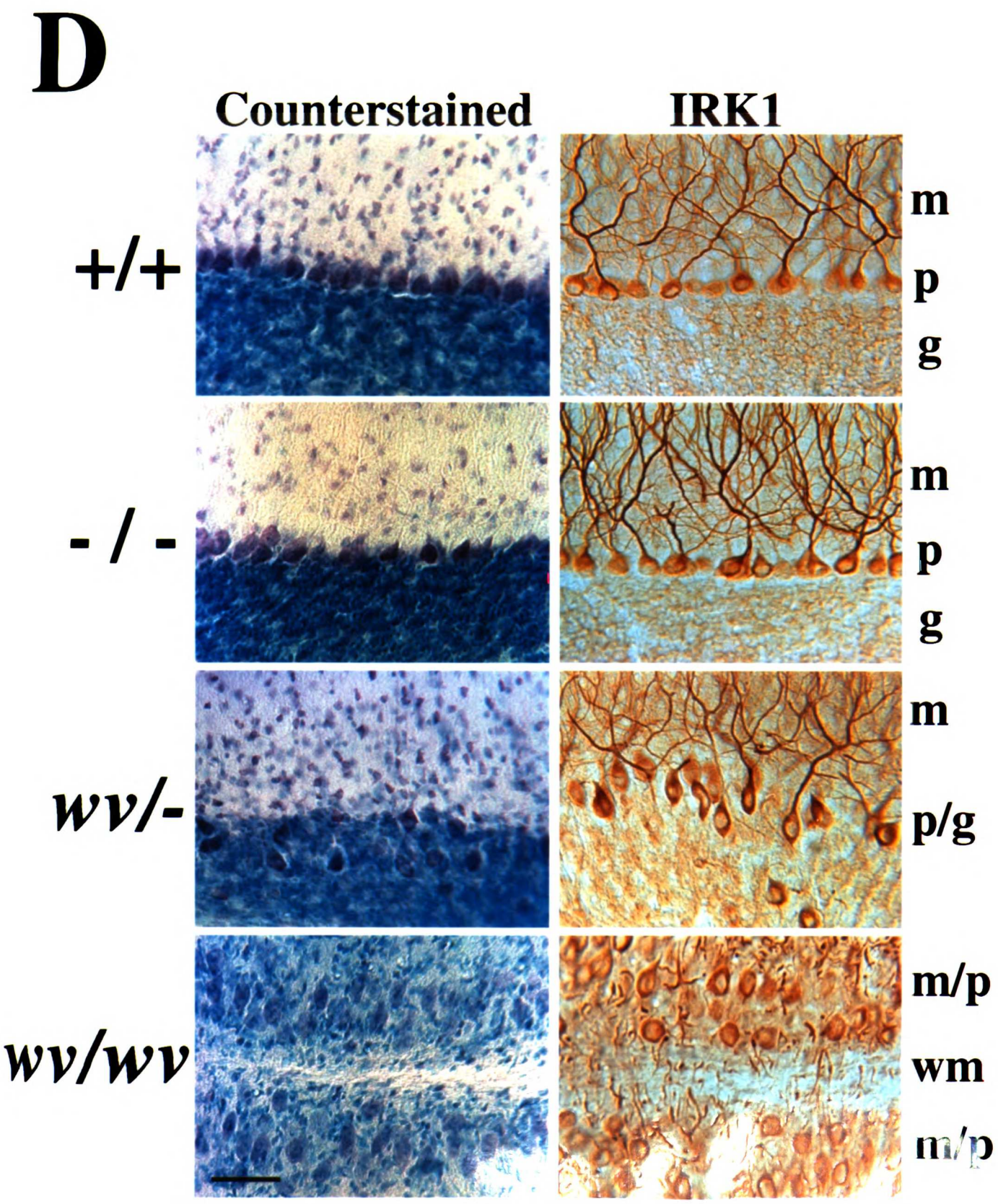

B
+/+
- / -
GIRK2
GIRK1
IRK1
g
m


C
TH
GIRK2
+/+
- / -
wv/-
wv/wv
SNc
VTA
SNr


D
Counterstained
IRK1
+/+
m
p
g
- / -
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p
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wm
m/p

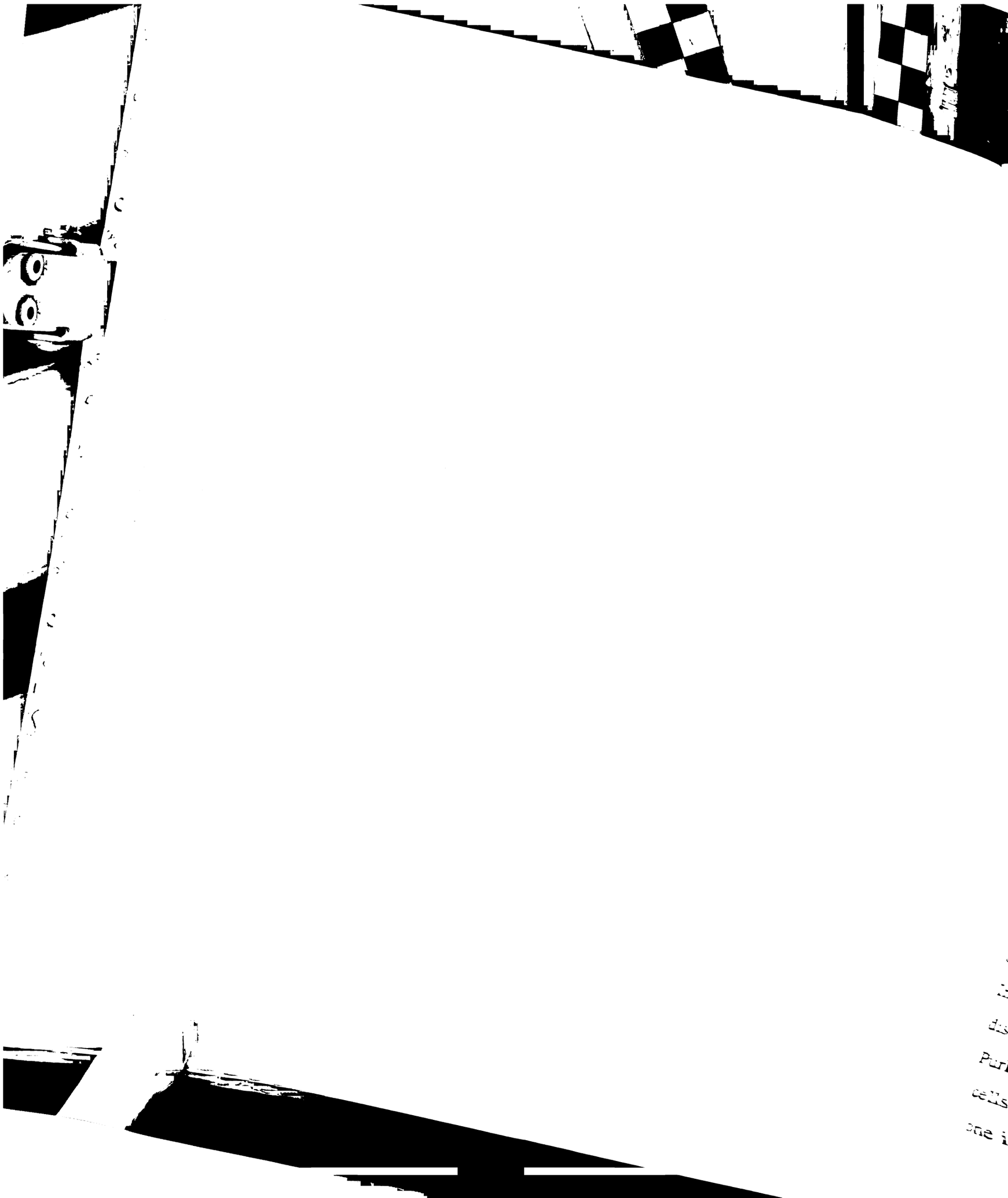

Figure 3 **A.** Coronal sections of brain from GIRK2+/+ and -/- mouse brain stained with antibodies against the N-terminus of GIRK2, N-terminus of GIRK1, and C-terminus of IRK1, respectively, show that there is no detectable GIRK2 and dramatically reduced GIRK1 immunoreactivity in the GIRK2-/- mice. The IRK1 staining patterns are the same for these mice. CTX: cerebral cortex, HP: hippocampus, Th: thalamus. Scale bar: 1 mm. **B.** Sagittal views of cerebella from GIRK2+/+ and -/- mice stained with the same antibodies described in A. While there is no detectable GIRK2 protein in the -/- mice, there is still significant GIRK1 staining, while the level of IRK1 expression appears to be the same. g: granule cell layer, m: molecular layer. Scale bar: 0.5 mm. **C.** Coronal sections of ventral midbrain from +/+, -/-, *wv*/-, and *wv*/*wv* mice are stained with antibodies against tyrosine hydroxylase (TH) and N-terminus of GIRK2, respectively. The TH staining of +/+, -/-, and *wv*/- midbrain appears similar, while there are fewer TH-positive neurons in the substantia nigra pars compacta (SNc) of the *wv*/*wv* midbrain. GIRK2 immunoreactivity is absent in the -/- midbrain but can still be found in the *wv*/- and *wv*/*wv* mice, though the dendritic staining is dramatically reduced. SNc: substantia nigra pars compacta, SNr: substantia nigra pars reticulata, VTA: ventral tegmental area. Scale bar: 0.1 mm. **D.** High magnification views of parasagittal cerebellar sections from +/+, -/-, *wv*/-, and *wv*/*wv* mice. The sections are counterstained with toluidine blue or stained with antibody against the C-terminus of IRK1, which stains the cell body and dendrites of the Purkinje cells as well as the dendrites in the granule cell layer. The *wv*/- cerebellum is mostly wildtype in appearance except for regions with a Purkinje cell layer more disorganized and broader than that in GIRK2+/+ and -/- mice. Some Purkinje cells and their dendrites can be found in the granule cell layer of *wv*/- mice. In the *wv*/*wv* cerebellum, there is no granule cell layer, and the Purkinje cells with disorganized dendrites are scattered throughout the cerebellum. m: molecular layer, p: Purkinje cell layer, g: granule cell layer, p/g: cell layer where both Purkinje cells and granule cells are found, m/p: cell layer where molecular layer and Purkinje cell layers collapse into one in the absence of granule cell layer, wm: white matter. Scale bar: 0.1mm.

Methods: Mice were perfused intracardially with 4% formaldehyde and 0.1% glutaraldehyde in phosphate-buffered saline, pH 7.4. Brains were dissected and postfixed overnight at 4°C. Fifty μm vibratome sections were collected in 0.1 M Tris pH 7.6; blocked with 2% H_2O_2; washed in 50 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Triton X-100 (TBST); and then blocked in 4% normal goat serum (NGS) and 3% BSA in TBST. Rabbit antibodies were affinity-purified, and sections were incubated in 1-2 μg/ml primary antibody for overnight (Liao et al., 1996; Sheng et al., 1992). Monoclonal antibodies against tyrosine hydroxylase (Pel-Freeze) were used at 1:1000. Biotinylated donkey anti-rabbit or anti-mouse IgG Fab (Jackson labs) were used at 1:200, and sections were developed with the ABC kit (Vectastain) and diaminobenzidine.

Fig.4a

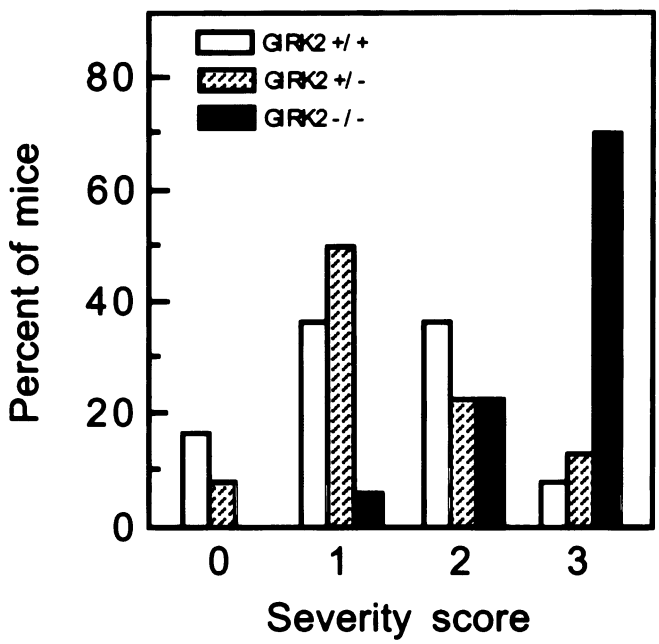
GRK2 +/ +
GRK2 +/ -
GRK2 - / -
Percent of mice
80
60
40
20
0
0
1
2
3
Severity score

Fig.4b

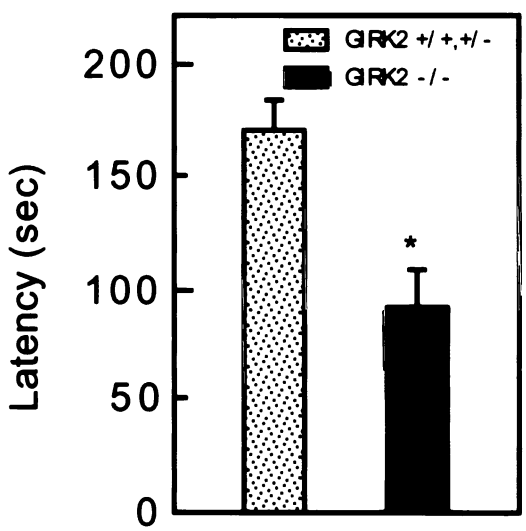
GRK2 +/ +,+/ -
GRK2 - / -
Latency (sec)
200
150
100
50
0
*

Figure 4 Susceptibility of GIRK2 deficient mice to pentylenetetrazol (PTZ) induced seizures. **A.** Response of mice receiving one injection of 50 mg/kg PTZ i.p. 0: no response, 1: isolated twitches, 2: tonic-clonic convulsions, 3: tonic extension and/or death. GIRK2-/- mice (n=16) tend to progress to more severe stages than +/+ or +/- mice (n=13 and 12, respectively). ($P<0.004$, Mann-Whitney U-Wilcoxon rank sum test). No statistically significant difference was observed between +/+ and +/- animals. **B.** Seizure latency. The PTZ seizure latency was defined as the time elapsed from PTZ injection to the first obvious sign of tonic-clonic convulsion or tonic extension. The latency to seizures was shorter for the -/- mice ($^*P<0.002$, unpaired test).

Methods: PTZ (Sigma) was dissolved in phosphate buffered saline and injected i.p. at a dose of 50 mg/kg in ≈0.1 ml. Animals were housed in a room with controlled light-dark cycle (12 hr light/12 hr dark) and temperature (23°C). All experiments were performed between 11 a.m. and 1 p.m. Animals were injected and observed without prior knowledge of their genotype. Each mouse was placed in a transparent cage and observed for 30 min. after injection. All mice were littermates between 10 and 14 weeks of age and weighed ≥ 20 grams.

Chapter 4: Discussion

The predominantly somatodendritic distribution of G protein-gated Kir channels suggests that they may be involved in different functions.

Kir channels are believed to be important in the maintenance of membrane potential, modulation of neurotransmitter release and effects, and buffering of extracellular K^+ ions. The relatively ubiquitous GIRK2 and GIRK1 immunoreactivity in neurons and their regulation by many transmitter receptors when expressed in heterologous systems indicate that they are likely to contribute to a significant fraction of the G protein-gated inwardly rectifying potassium channels *in vivo*. The presence of mRNA and the somatodendritic localization of GIRK1 and/or GIRK2 in certain central neurons (e.g. cerebral cortical pyramidal cells, substantia nigra dopaminergic neurons, and cerebellar granule cells) suggest that they are on post-synaptic membranes. GIRK1 and GIRK2 may be important in the modulation of neurotransmitter effects from the post-synaptic membrane. Many G protein-coupled receptors are found in dendrites and cell bodies, where they may extend or shorten the effects of transmitters released from nerve terminals of presynaptic neurons. In the substantia nigra, GIRK2 is likely to be a downstream effector of the D_2 autoreceptor, which is important in the regulation of dopamine released from the cell body and dendrites (Cheramy et al., 1981), since GIRK2 protein is found in the cell bodies and dendrites of dopaminergic neurons in the midbrain. GIRK1 and GIRK2 are also found in axon-like fibers and may control presynaptic neurotransmitter release. Both GIRK1 and GIRK2 are also found in axon-like fibers in the lateral septal nucleus, the barrel cortex where presynaptic fibers from the thalamus synapse with layer IV neurons, and the terminal fields of the lateral perforant pathway in the hippocampus (Ponce et al., 1996; this study).

Disruption of the functions of G protein-gated Kir channel leads to seizures

A seizure phenotype is found in GIRK2-/-, *wv/wv*, and *wv*/+ mice (Eisenberg and Messer, 1989; this study). While K^+ channels generally have a hyperpolarizing effect on cells, the phenotype of a K^+ channel knockout mouse depends on where the channel is

normally expressed. If it is expressed on an excitatory neuron, knocking the channel out may slightly depolarize the resting membrane potential, making the cell more excitable. If such a cell is found in a circuit of synapses that is seizure-genic, such as the trisynaptic pathway present in the hippocampus, the removal of a K^+ channel may give rise to a seizure-prone mouse. If the K^+ channel is normally expressed in an inhibitory neuron, such as an interneuron, the removal of such channels could increase the firing potential of the inhibitory cell, leading to reduced output from the circuit. If, however, this inhibitory neuron (neuron 1) is important in the inhibition of another inhibitory cell (neuron 2), increase activity in neuron 1 may release the inhibition on a third neuron, giving rise to an overall elevated output from this circuit. Since GIRK channels are expressed in excitatory as well as inhibitory neurons, it is hard to know what is the cause for seizures in the GIRK2-/-, *wv/wv*, and *wv/+* mice. Superficially, the seizure in the GIRK2-/- mice could be due to loss of GIRK channel function, and the seizure in the *wv/wv* and *wv/+* mice could be due to gain of depolarizing Na^+ current. The neurons responsible for the seizure phenotype likely are different in the loss-of-function and the gain-of-function mutant mice. There is however, a severe depletion of GIRK1 and GIRK2 immunoreactivities in the neuronal processes of *weaver* neurons. It is possible, then, that seizures in both GIRK2 knockout and *weaver* mice are due to loss of GIRK channel functions in similar neurons. While seizure is not an uncommon phenotype in different transgenic and knockout mice and may represent abnormalities in many brain functions, it would be interesting to identify the brain regions or specific neurons that are responsible for this phenotype in the different GIRK2 mutant mice.

Alteration in expression and function of GIRK2 can lead to concomitant down-regulation of GIRK1 in gain-of-function as well as loss-of-function mouse models

In regions where GIRK1-4 are all expressed, such as the hippocampus, cerebral cortex, and thalamus (Karschin et al., 1994, 1996; Kobayashi et al., 1995; Ponce et al., 1996;

Spauschus et al., 1996; this study), heteromeric GIRK channels may also consist of more than two different channel subunits, and channels with different subunit stoichiometry could exist in the same neuron.

It is striking that the expression of both GIRK1 and GIRK2, but not GIRK4 and IRK1, channel subunits are down-regulated in the *weaver* and GIRK2 knockout mice. GIRK1 and GIRK2 are known to form heteromeric channels *in vitro* (Kofuji et al., 1995, 1996; Duprat et al., 1995; Slesinger et al., 1996; Navarro et al., 1996) as well as *in vivo* (this study), and their expression overlaps in many brain regions (Kobayashi et al., 1995; Karschin et al., 1996; Slesinger et al., 1996; this study). The simplest explanation for their concomitant down-regulation in the *weaver* mice is the selective removal of GIRK1/GIRK2 heteromeric channel complexes as a result of the G156S mutation in GIRK2. The GIRK2*wv* protein is presumably made since the defect is only a missense mutation, but abnormal structure and/or function of homo- and heteromultimeric GIRK channels containing the GIRK2*wv* subunit lead(s) to their degradation. In the GIRK2 knockout mice, there are two possible explanations for the reduction of GIRK1 expression. First, GIRK1 in the brain predominantly associates with GIRK2; in the absence of the GIRK2 subunit, GIRK1 cannot form functional channels and is degraded. Consistent with this theory, GIRK1 is not capable of forming functional homomeric channels in heterologous expression systems (Kofuji et al., 1995; Duprat et al., 1995; Hedin et al., 1996), and GIRK4, known to associate with GIRK1 in the heart, has low level of expression in the brain (Krapivinsky et al., 1995b; this study). Although a truncated GIRK2-neo mRNA is transcribed in the GIRK2 knockout mice, it is possible that GIRK2 protein is not made because the truncated GIRK2-neo mRNA is somehow not translated. Consistent with this hypothesis, GIRK2-neo cRNA injected into *Xenopus* oocytes does not give rise to a truncated protein (P. A. Slesinger and Y. J. Liao, unpublished observation). It is also possible that a GIRK2 truncated protein is made but unable to associate with GIRK1 because a region important for multimerization is missing or the truncated GIRK2-neo protein may assume an unfavorable configuration. Second, an

abnormal and short-lived GIRK2-neo protein is made, and its association with GIRK1 leads to the selective removal of the whole complex. If the second theory were correct, the reduction of GIRK1 and GIRK2 protein expression in *weaver* and GIRK2 knockout mice is both due to the presence of abnormal GIRK2 subunit. The drastic reduction in GIRK1 protein expression in both *weaver* and GIRK2 knockout mice suggests that most of GIRK1 in the brain exists in heteromeric complex with GIRK2.

The selective removal of aberrant GIRK1/GIRK2 complexes may take place either soon after protein synthesis in the endoplasmic reticulum, at the level of protein targeting to the plasma membrane of the cell body or neurites, or after the channel complexes have inserted into the plasma membrane. Assembly of multimeric protein complexes are known to take place as early as in the endoplasmic reticulum (ER). Since more of the unglycosylated form of GIRK1 can be found in the *weaver* mice, it seems possible that some of the GIRK1/GIRK2*wv* complex is being retained in the ER. As GIRK2 channels are processed and targeted to the subcellular location where they normally function, they may also be intercepted due to certain abnormalities. Selective removal of channel complexes in the ER or at the level of protein targeting implies that there may be detectable structural abnormalities in the GIRK2*wv* protein. It is also possible that the complexes containing the GIRK2*wv* subunit may form channels that are less selective, disrupting some electrochemical gradients in vesicles involved in post-translational processing or protein targeting. The abnormal channel properties may be detected in this manner, and vesicles containing abnormal channel complexes could be targeted for degradation. The down-regulation of GIRK protein expression may also take place after the aberrant GIRK1/GIRK2*wv* complex has reached the plasma membrane, where its abnormal electrophysiological properties would be evident. Since abnormal current as a result of the *wv* mutation can be recorded from the cell body of dissociated cerebellar granule cells (Kofuji et al., 1996; Surmeier et al., 1996; P.A. Slesinger, unpublished observation), some GIRK channel complex containing the

GIRK2*wv* subuit is clearly inserted into the plasma membrane. It is possible that the down-regulation of GIRK1 and GIRK2 channel proteins may occur at all of the above levels.

The *weaver* mouse phenotype is a result of gain-of-function mutation of GIRK2

The ataxia, dopamine deficiency, and infertility observed in the *weaver* mice are likely the result of the gain-of-function mutation of GIRK2 for three reasons. First, a functional knockout of the GIRK2 gene in the same genetic background as the *weaver* mice (C57BL/6) has relatively little phenotype. This discrepancy suggests that these *weaver* phenotypes are not simply due to the down-regulation of GIRK2 and GIRK1 protein expression, which occurs in both GIRK2 knockout and *weaver* mice. (The presence of seizures in both GIRK2 knockout and *weaver* mice suggests that this phenotype may be due to loss of GIRK2 function. See above for details). Second, expression of one copy of the *wv* gene in the presence or absence of normal GIRK2 (*wv*/+ or *wv*/-, respectively) gives rise to mice with some histological abnormalities in the cerebellum similar to those of mice expressing two copies of the *wv* gene. The *wv* mutation appears to be a dominant mutation, and the dosage of mutant gene seems to correlate with severity of the pathology. Third, cerebellar granule cells from *weaver* mice display reduced G protein-activated Kir currents and an abnormal, constitutive Na^+ current (Kofuji et al., 1996; P.A. Slesinger, unpublished data), similar to that found in *Xenopus* oocytes expressing GIRK2*wv* channels (Slesinger et al., 1996; Kofuji et al., 1996; Navarro et al., 1996; Tong et al., 1996 but see Surmeier et al., 1996 and Mjaatvedt et al., 1995). The *weaver* granule cells can be partly rescued and express marker of differentiation TAG1 by the application of cation channel blockers such as MK-801 and QX-314 (Kofuji et al., 1996). This rescue occurs presumably by blocking the GIRK2*wv* channels since the pharmacological blockade of G protein-activated Kir currents can also be seen in *Xenopus* oocytes co-expressing GIRK2*wv* and GIRK1 (Kofuji et al.,

1996). The inhibition of abnormal Na^+ current and subsequent phenotypic rescue suggests that the gain-of-function mutation is responsible for the abnormalities in *wv* granule cells.

Future directions

The biochemical analysis of G protein-activated Kir channels in normal and mutant mice has pointed to many areas for further research. I will discuss some areas that are particularly important: 1) More biochemical characterization of Kir channels *in vivo*, 2) Electron microscopy studies of GIRK channel distribution and potential compartmentalization with seven-transmembrane receptors, 3) Analysis of GIRK channel composition and formation of multimeric channels, and 4) Electrophysiological recordings of G protein-gated inwardly rectifying K^+ currents in cultured dissociated neurons or in brain slices.

Although many Kir channel subunits have been cloned (Table 1 in Introduction), the protein distribution and functional roles of channels containing these subunits are not well studied. To understand what other G protein-gated inwardly rectifying K^+ channel subunits could have substituted for the functions of GIRK2 and GIRK1 in the GIRK2 knockout mice or in the *weaver* mice, it would be important to determine which GIRK or Kir channel proteins are found in similar subcellular location. GIRK3, for example, may enhance or reduce GIRK currents when co-expressed with different members of the GIRK subfamily (Duprat et al., 1995; Kofuji et al., 1995). The mRNA of GIRK3 overlaps in many areas as GIRK1 and GIRK2 such as in the cerebellar granule cells and thalamus (Kobayashi et al., 1995; Karschin et al., 1996), but it is not known if GIRK3 protein distribution overlaps with those of GIRK1 and GIRK2 or if GIRK3 associates with GIRK1 and/or GIRK2 *in vivo*. While there is not much evidence so far, other members of the Kir family may also interact with members of the GIRK subfamily by either direct physical association or by functional substitution. A detailed study of the protein distribution of different Kir family members would sort out the combinations of Kir channels that may exist in the same neurons. Co-

expression of the potential combinations of normal and mutant Kir channel subunits in heterologous expression systems will facilitate the characterization of different Kir currents that exist in the same cell.

To elucidate the function of the GIRK channel subunits, it will be important to use electron microscopy (EM) to determine their pre- and/or postsynaptic localization and to identify transmitter receptors that may regulate them *in vivo*. Immunohistochemical experiments using light microscopy has shown that the distribution of GIRK1 and GIRK2 proteins overlaps with seven-transmembrane receptors such as the m2 muscarinic acetylcholine receptor (Levey et al., 1991), the D_2 dopamine receptor in the midbrain, and somatostatin and $GABA_B$ receptors in the cerebellum. These transmitter receptors, G proteins, and downstream effectors may be organized so the signal transduction occurs rapidly after receptor activation (Neubig, 1996). They may even be targeted to specific subcellular location as a complex. These proteins may be found relatively close to each other in a "compartment" and they may associate with each other directly or by attaching to common proteins. The "compartmentalization" of receptors and effector Kir channels could be evaluated by double-labeling immuno-EM studies to determine if they are found in clusters in similar subcellular location. They could also be co-immunoprecipitated from brain membranes or heterologous expression systems. If transmitter receptors and effector Kir channels are found in close proximity to each other, it would be interesting to look at the subcellular distribution of these receptors in mutant mice by EM for subtle changes in their organization.

Since heteromeric GIRK1/GIRK2 channels exist *in vivo*, it would be interesting to study the assembly of such heteromultimeric complexes *in vitro*. The GIRK2*wv* mutant channel subunit can be expressed with normal GIRK1 and/or GIRK2 to study the cellular mechanisms that detect and dispose of abnormal protein complexes. Cell lines that endogenously express GIRK1 and GIRK2, such as the neuron-like pheochromocytoma (PC12) cells (N. Patil, unpublished observation), are good systems to study the formation of

homo- and heteromeric GIRK1/GIRK2 complexes. Other heterologous expression systems such as the COS cells, CHO cells, or HEK293 cells may be transfected with normal and/or mutant GIRK channel subunits to address the same question. These *in vitro* systems can be more easily manipulated experimentally and can express greater amount of proteins. They also have some disadvantages. The higher level of GIRK proteins expressed, the ratio of homo- vs. heteromeric GIRK proteins, and the ratio of different GIRK subunits in heteromeric complexes may not simulate what occurs *in vivo*.

The association of GIRK1 and GIRK2 proteins may occur as early as during protein synthesis in the endoplasmic reticulum (ER) or it may occur in later steps as described above. Since more of the unglycosylated form of GIRK1 seems to be present in the *weaver* mice (Fig. 5, Chapter 2), it is possible that the GIRK1/GIRK2 complex form as early as in the ER and that the abnormal GIRK1/GIRK2*wv* complex is being retained there. To get some idea of the extent of heteromeric complex formation in the different membrane compartments of the cell such as ER, Golgi, and plasma membrane, one can fractionate the different membranes with the aqueous two-phase system (Walter and Johansson, 1994) and look for the overlap in protein distribution with electron microscopy and GIRK1-GIRK2 association by co-immunoprecipitation. To see if GIRK1 and GIRK2*wv* proteins are retained in the ER, one can use western analysis to examine the level of GIRK1 and GIRK2 proteins in the different membrane fractions prepared from normal and *weaver* mice. Pulse-chase experiments using ^{35}S-methionine can be used to examine the rate of protein synthesis, GIRK1-GIRK2 association, retention of channels containing the GIRK2*wv* subunit, and degradation of GIRK channel proteins expressed in cell lines. There may be interesting differences between cells expressing normal vs. GIRK2*wv* channel subunits.

Dissociated primary cell culture or brain slices from normal and mutant mice can be used to study the role of G protein-gated Kir currents in the developing and adult nervous system. Since gain of Na^+ current can be found in the *weaver* cerebellar granule cells, it would be interesting to see if the presence of such abnormal current correlates with the extent

of damage elsewhere in the *weaver* mice. The substantia nigra dopaminergic neurons and testicular germline and support cells are known to be defective in the *weaver* mice; while no major defect is found in the hippocampus, cerebral cortex, and thalamus of the *weaver* mice, despite the dramatic reduction in GIRK1 and GIRK2 protein expression. It seems that some compensatory mechanism may have prevented the cell death in neurons in the hippocampus, cerebral cortex, and thalamus. It would be interesting to see if such compensatory mechanisms occur at the level of ion currents or if they take place inside the cell, for example by Ca^{2+} buffering or synthesis of certain proteins.

Brain slices from wildtype and mutant mice can be used to assess the synaptic circuitry in different brain regions such as the midbrain and hippocampus. Midbrain slices can be used to study the activity of G protein-gated K^+ channels in dopaminergic neurons in response to different pharmacologic agents. The release of neurotransmitters such as dopamine in the GIRK2 knockout mice can be evaluated for the role of GIRK2 channels in these functions. Hippocampal slices can be used to evaluate the difference in G protein-gated Kir currents in normal, GIRK2 knockout, and *weaver* mice. One can assess the function of certain seven transmembrane receptors known to couple to Kir channels such as $GABA_B$, serotonin, and opioid receptors and study possible changes in phenomena such as long-term potentiation and long-term depression due to alterations in receptor-activated Kir currents. These experiments can help clarify the role these G protein-gated Kir channels play in the developing and adult nervous system.

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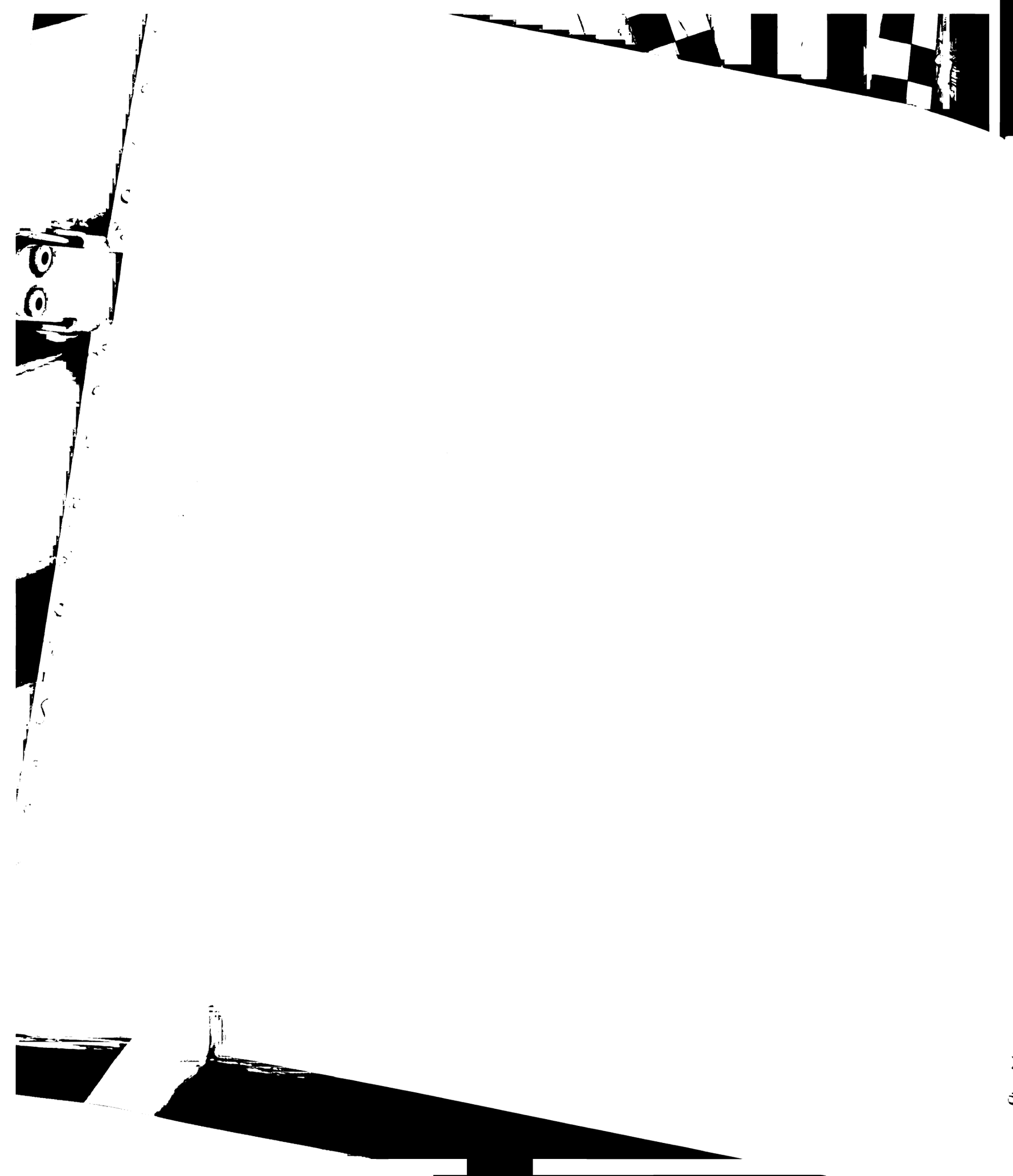

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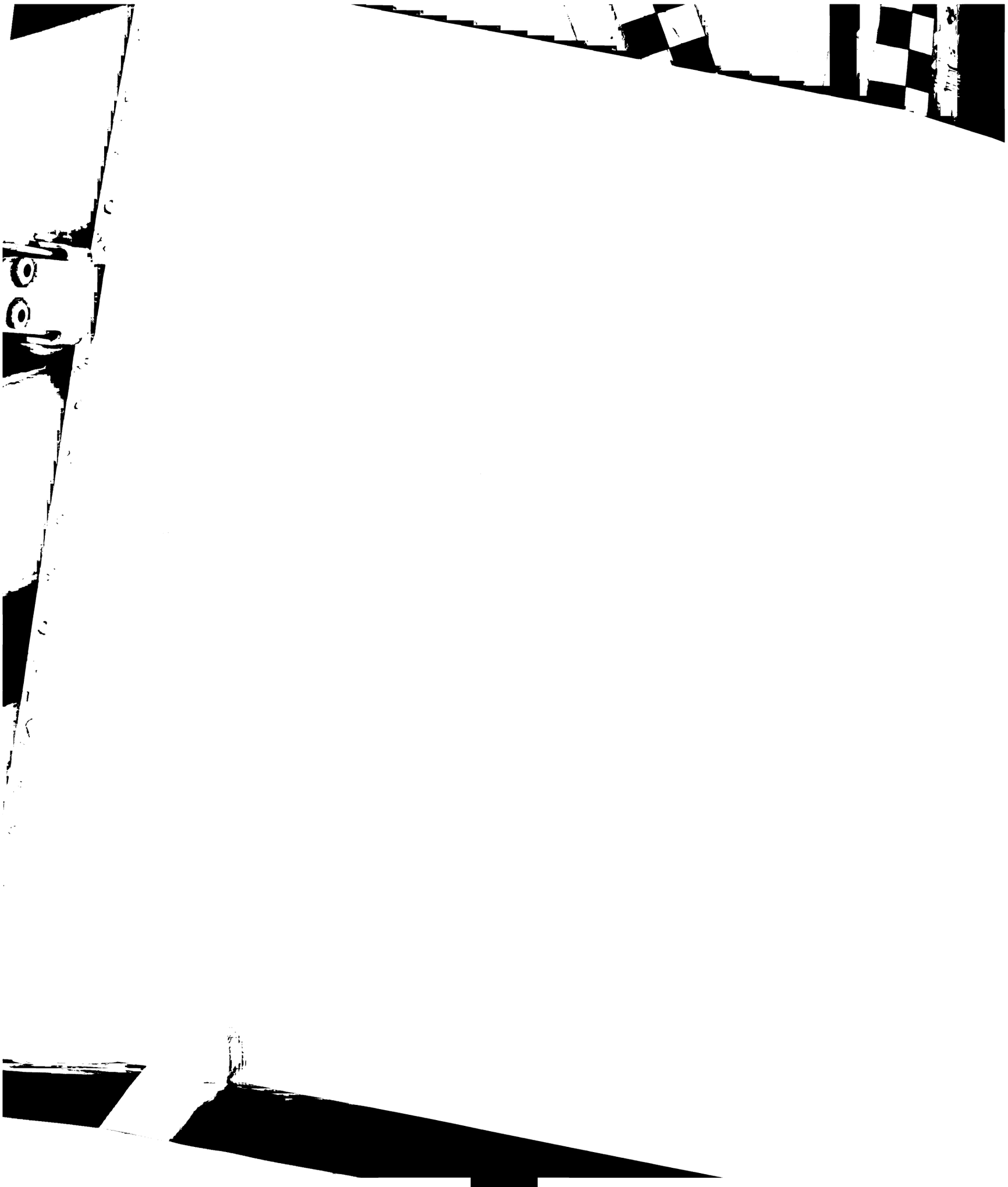

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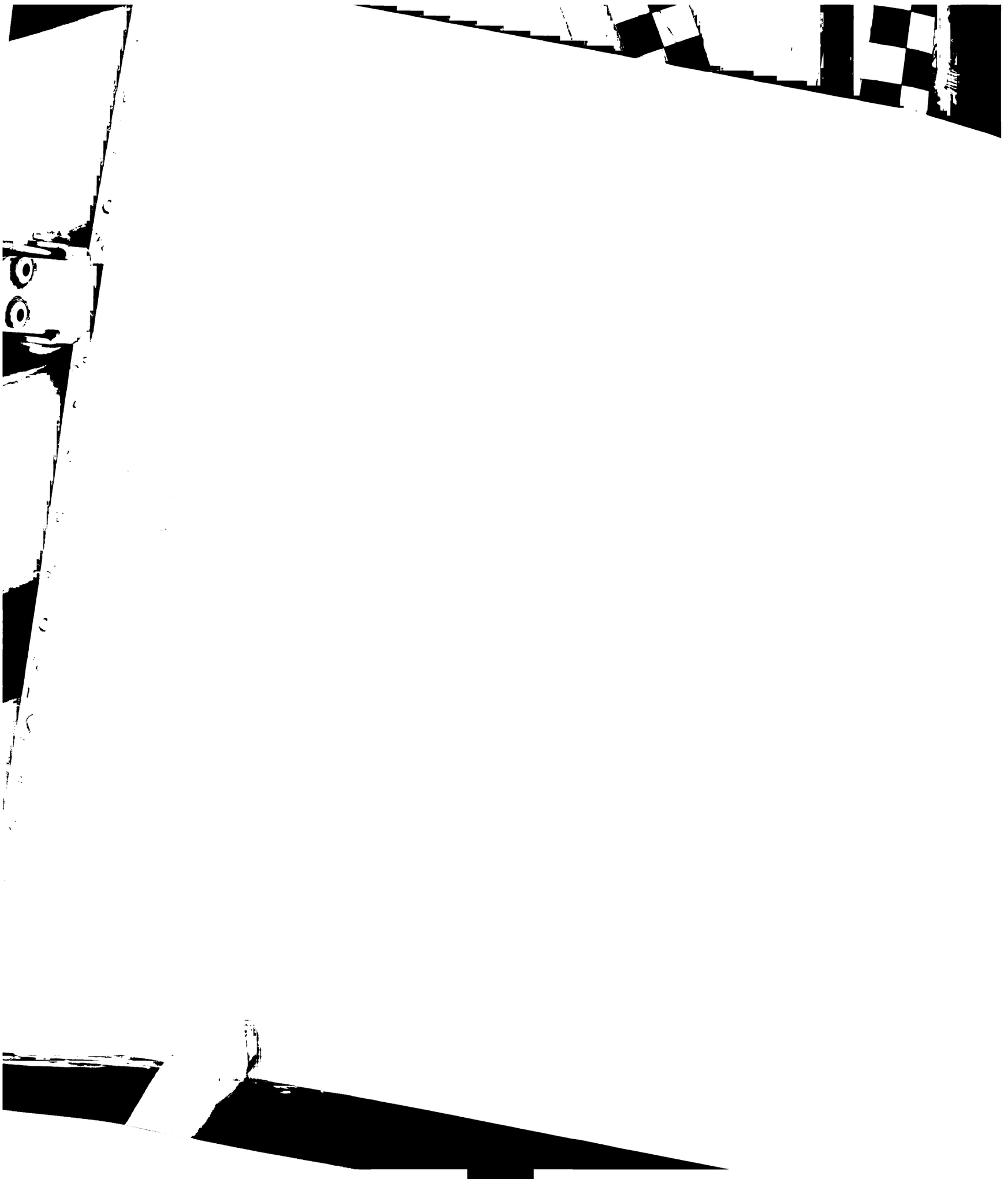

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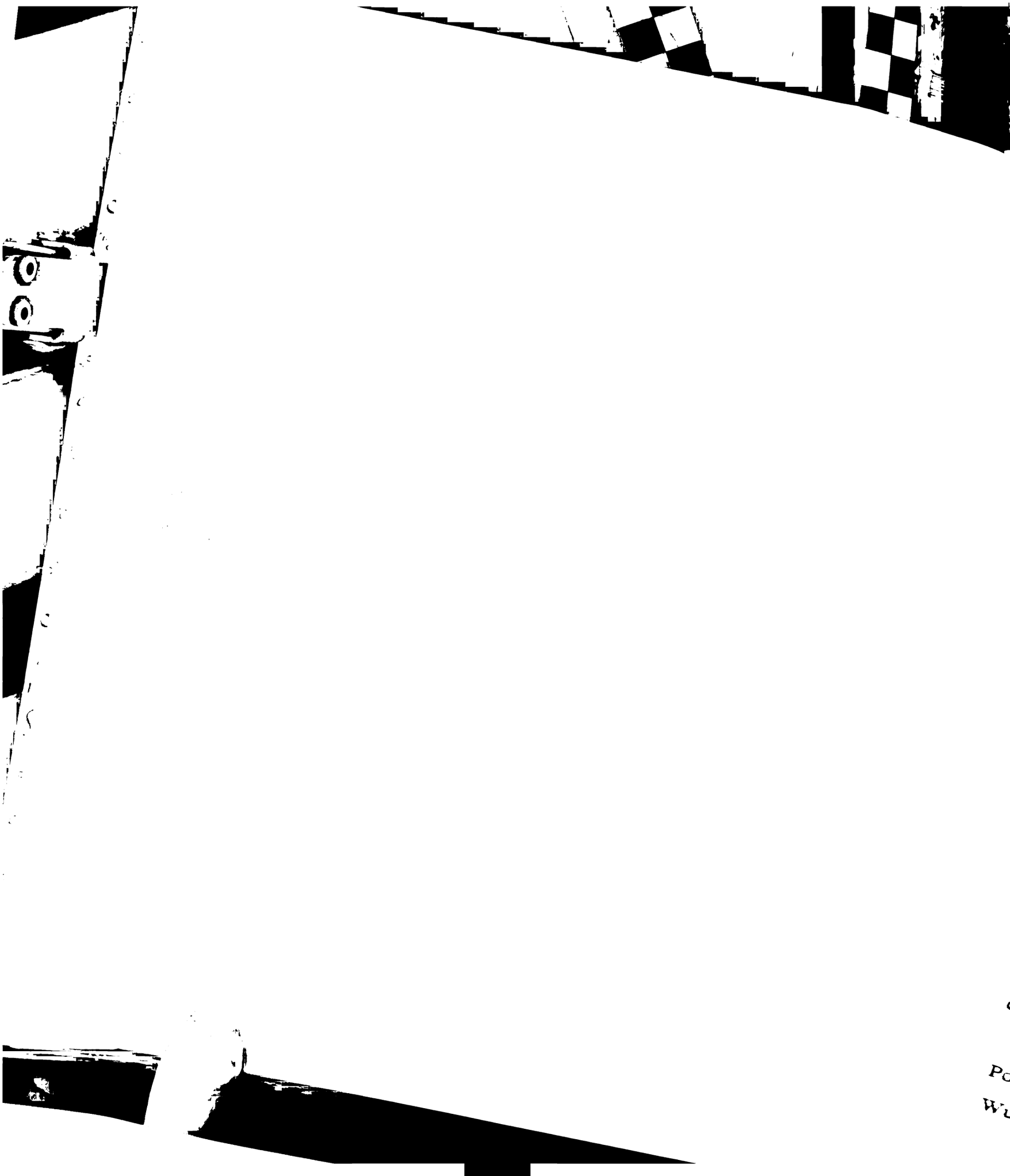

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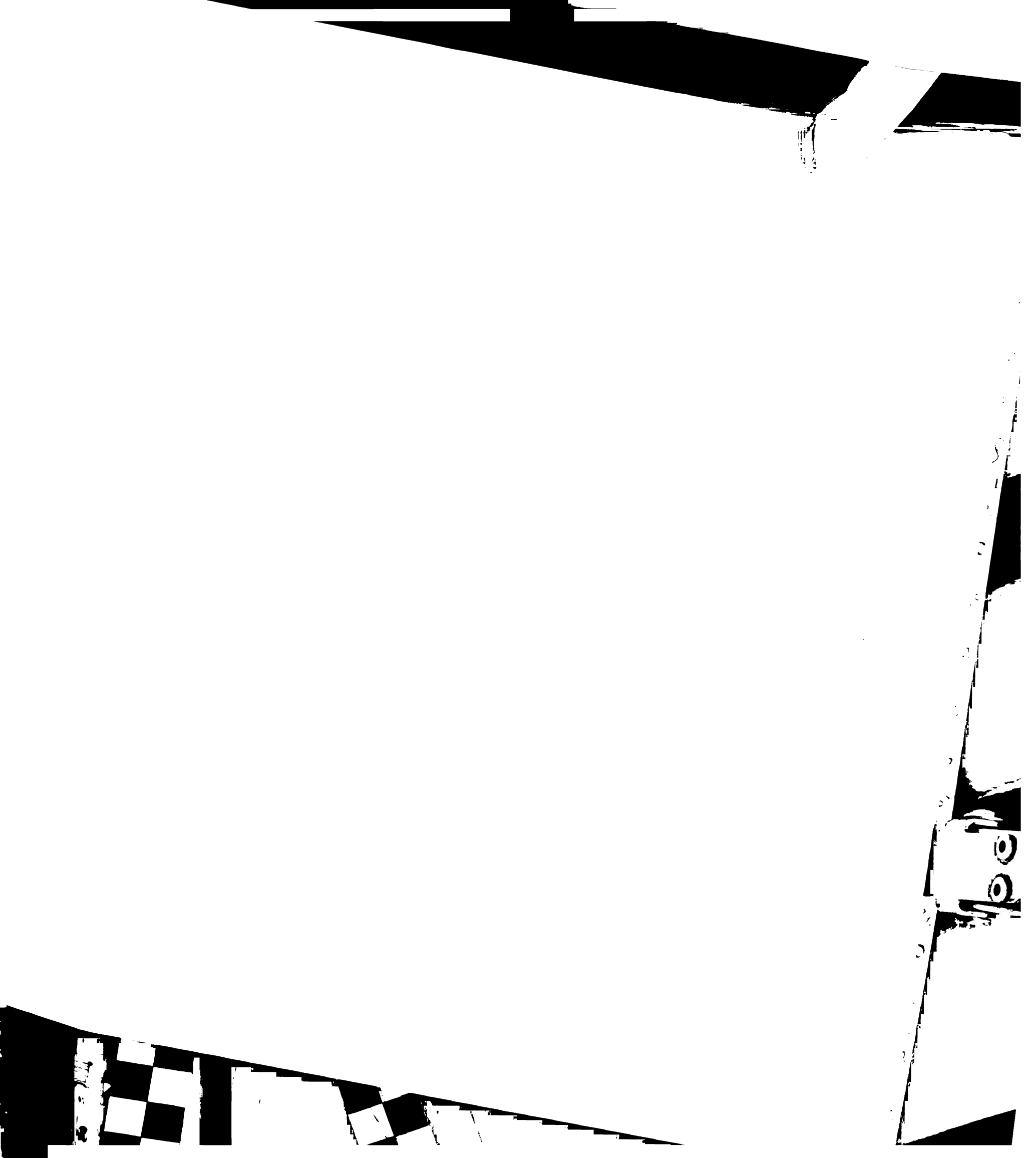

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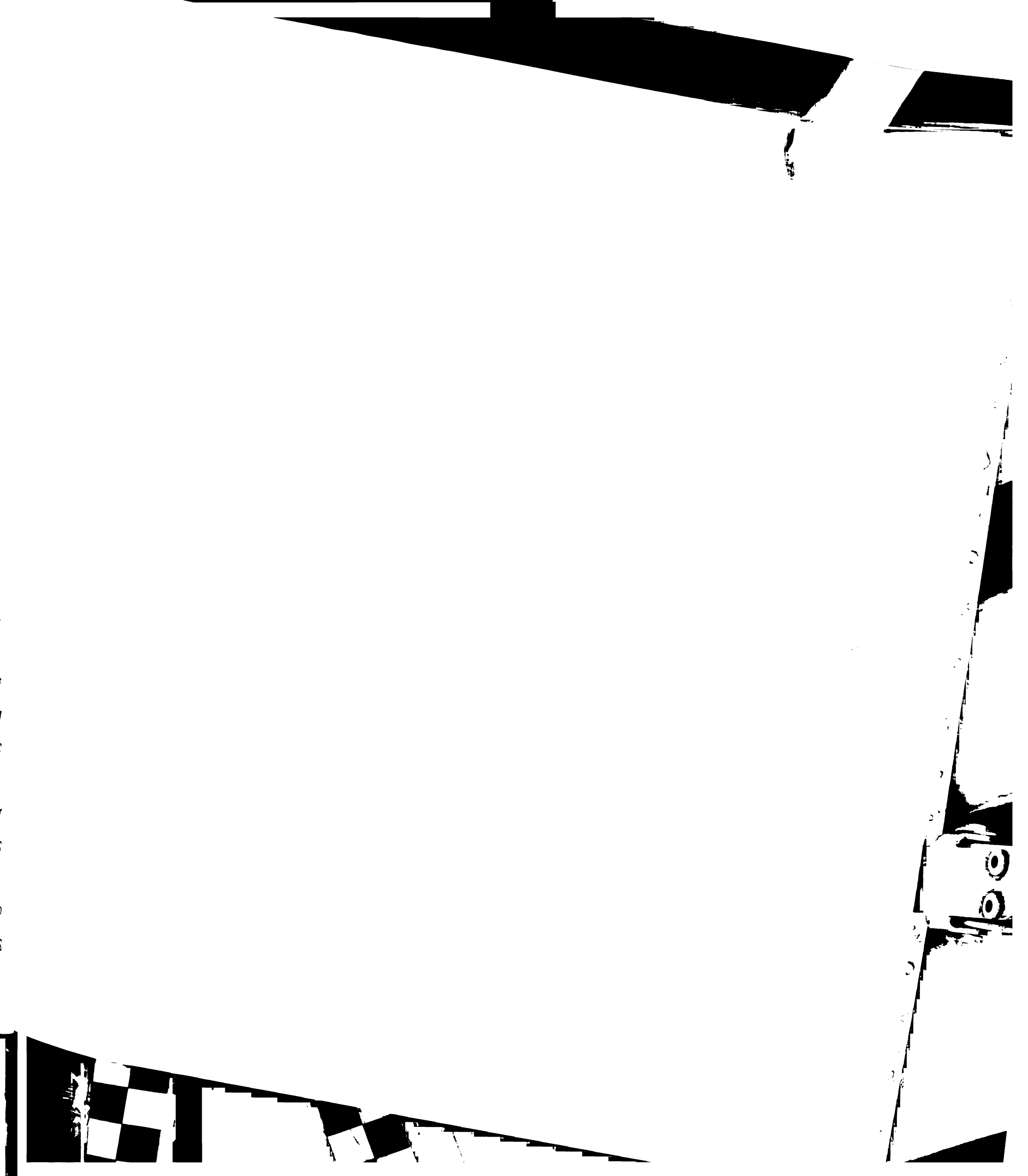

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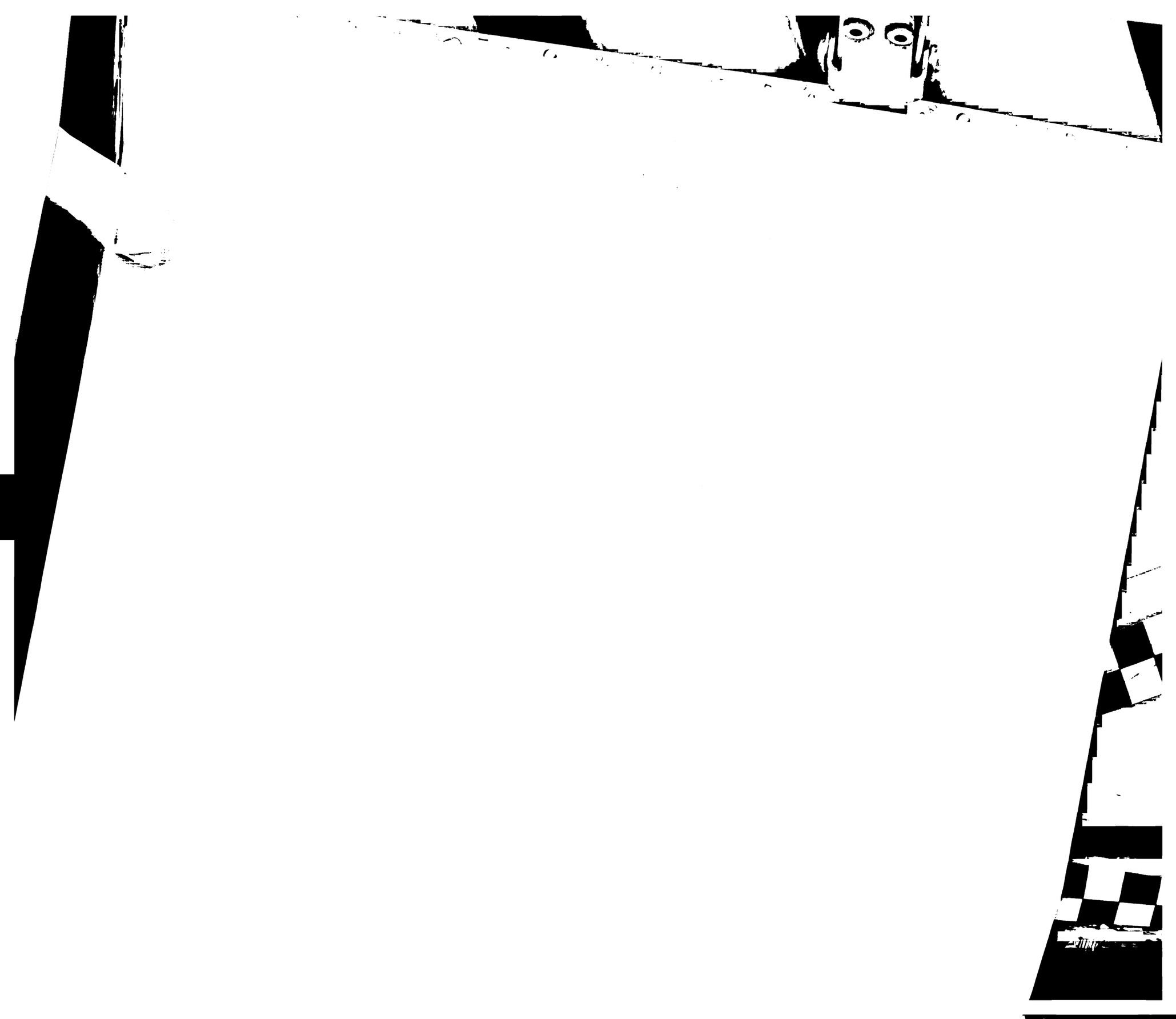

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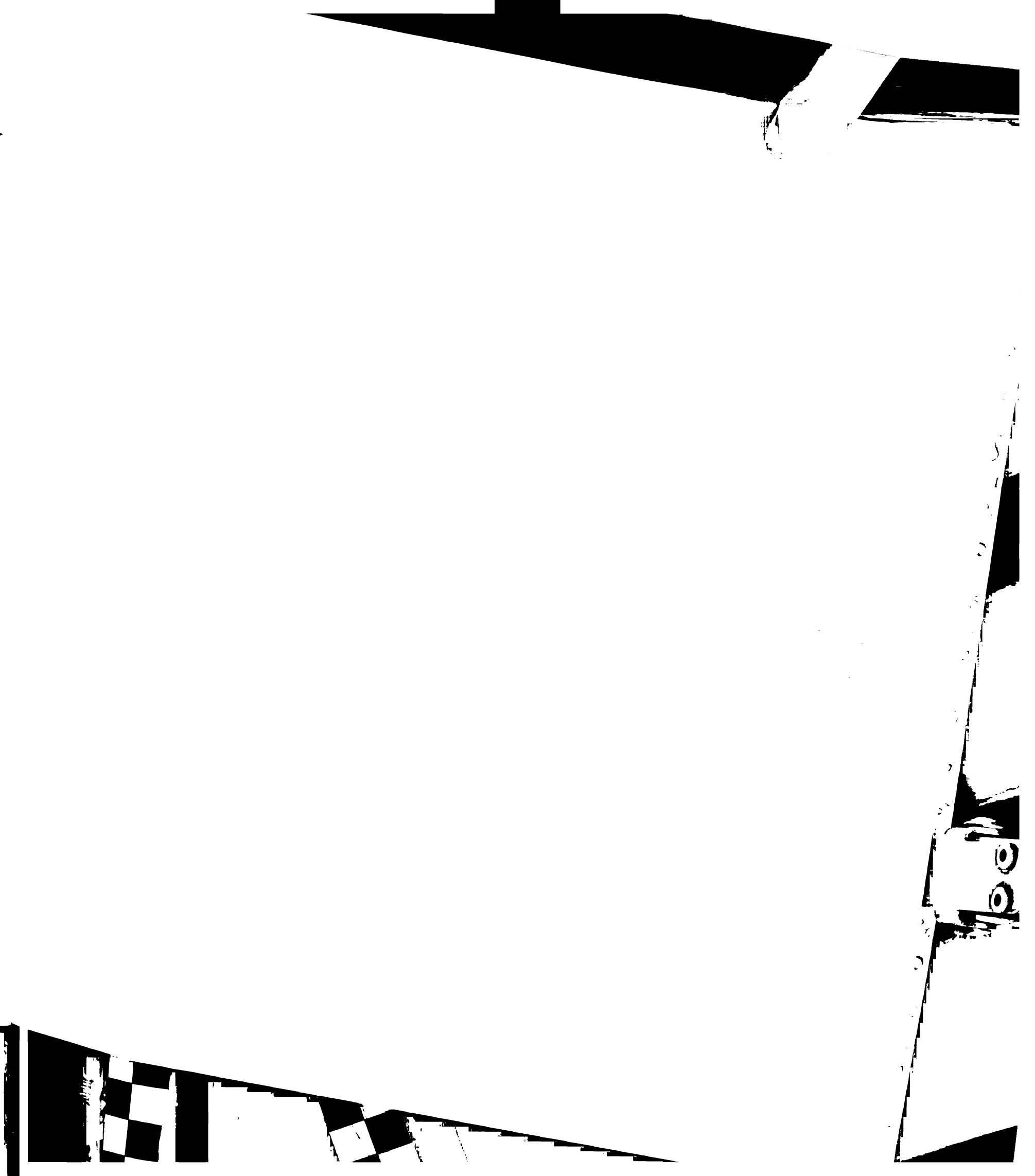

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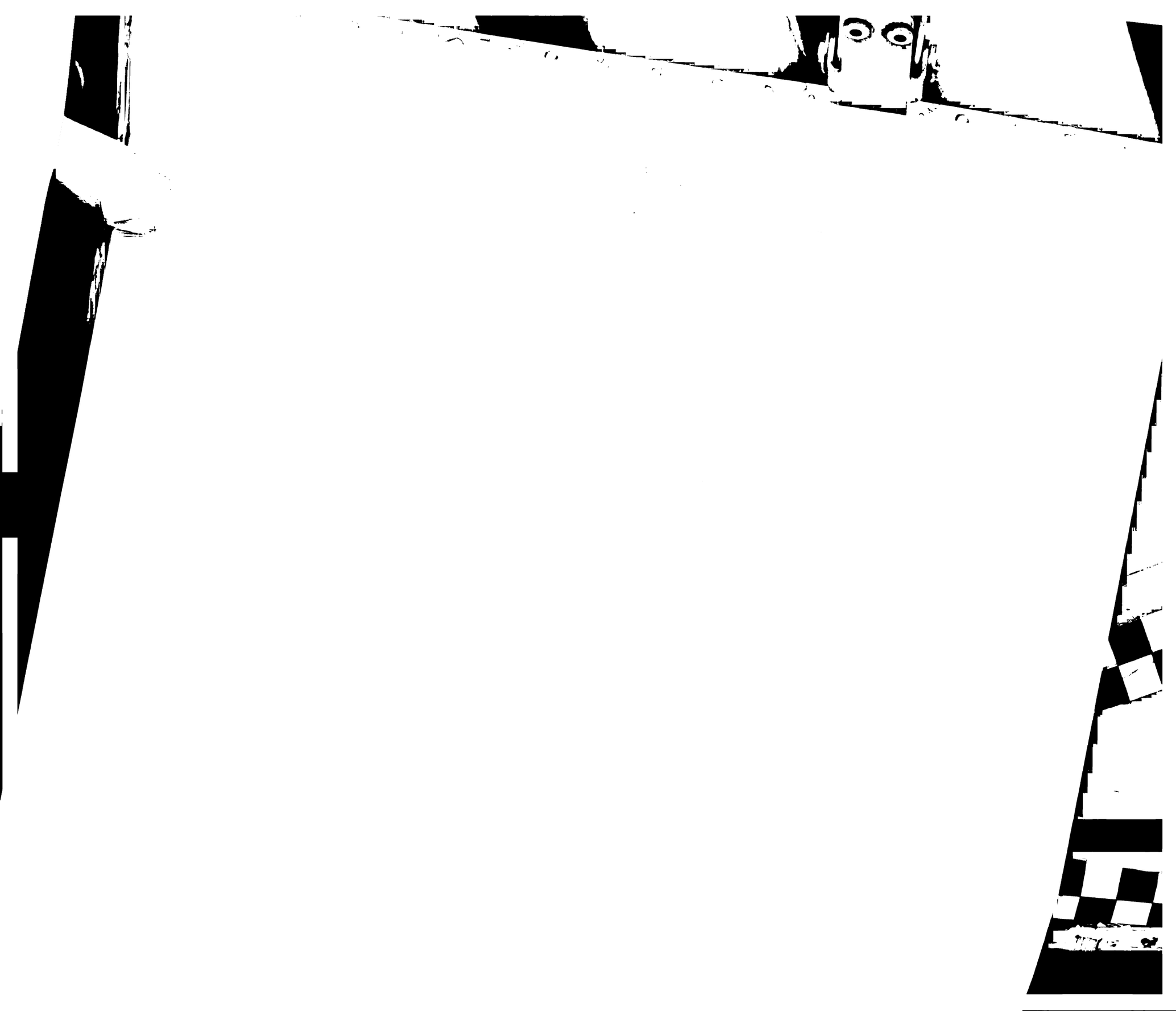

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Appendix

Presynaptic A-current based on heteromultimeric K+ channels detected *in vivo*

Abstract

A wide variety of voltage-gated K^+ channels are involved in the regulation of neuronal excitability and synaptic transmission. Their heterogeneity arises in part from the large number of genes encoding different K^+ channel subunits (Jan and Jan, 1990). In addition, heterologous expression studies indicate that assembly of distinct subunits into heteromultimeric channels may contribute further to K^+ channel diversity (Christie et al., 1990; Isacoff et al., 1990). A question has been whether heteromeric K^+ channels actually form *in vivo*, and if so, whether specific combinations of subunits could account for major K^+ currents identified in neurons. We present here biochemical evidence that Kv1.4 and Kv1.2, two K^+ channel subunits of the Shaker subfamily, co-assemble in rat brain. The Kv1.4/Kv1.2 heteromultimer combines features of both parent subunits, resulting in an A-type K^+ channel. Immunocytochemical evidence suggests that the heteromultimers are localized in axons and nerve terminals. We propose that Kv1.4/Kv1.2 heteromultimers may form the molecular basis of a presynaptic A-type K^+ channel involved in the regulation of neurotransmitter release.

Results and Discussion

Using in situ hybridization, Kv1.4 and Kv1.2 messenger RNAs exhibit distinct but overlapping expression patterns in rat brain, with co-expresssion in many neurons (Sheng et al., 1992; Tsaur et al., 1992). The Kv1.4 protein appears to be localized specifically to axons and terminals of neurons that express this gene (Sheng et al., 1992). Immunostaining of adjacent brain sections with gene-specific antibodies reveals that Kv1.2 immunoreactivity overlaps with Kv1.4 in several parts of the rat brain, particularly in the neuropil of the cerebral cortex, axon tracts of the corpus callosum (Fig. 1C, D), and in well defined terminal fields in the hippocampus. A striking example is the co-localization of Kv1.4 and Kv1.2 in the middle third of the dentate gyrus molecular layer and in the stratum lacunosum moleculare of CA1 (Fig. 1A, B), the layers of the hippocampal formation that receive the terminations of the medial perforant path projection (Steward and Scoville, 1976). The neurons of layer II of the medial entorhinal cortex whose axons form the medial perforant path projection express particularly high levels of both Kv1.4 and Kv1.2 mRNAs (Sheng et al., 1992; Tsaur et al., 1992). Both Kv1.4 and Kv1.2 immunoreactivities are also found as large puncta in the mossy fibre tract of the hippocampus, in a pattern and distribution characteristic of mossy fibre terminals (Fig. 1E, F). These large terminals represent en passant synapses made by the axons of dentate granule cells, a cell type in which Kv1.4 and Kv1.2 mRNAs are co-expressed (Sheng et al., 1992; Tsaur et al., 1992). These results indicate that Kv1.2 and Kv1.4 proteins may coexist in axons and terminals of neurons expressing both genes.

Direct association of Kv1.2 and Kv1.4 gene products in a mixed-subunit K^+ channel could underlie their immunohistochemical co-localization. This idea is supported by biochemical co-fractionation of Kv1.4 and Kv1.2 proteins (Fig. 2). Both Kv1.4 and Kv1.2 are readily solubilized in 2% Triton X-100 and are bound by an anion-exchange (DEAE-Sepharose) column, and after elution from DEAE-Sepharose, by a wheat-germ agglutinin

column. In these sequential chromatographic steps, Kv1.4 and Kv1.2 show virtually identical elution profiles with high salt and N-acetylglucosamine. The co-fractionation of Kv1.4 and Kv1.2 show some specificity, because another K^+ channel subunit, Kv4.2 (Baldwin et al., 1991), behaves quite distinctly on wheat-germ agglutinin chromatography, eluting over a much wider range of N-acetylglucosamine concentrations (Fig. 2). Interestingly, Kv4.2 (a member of the Shal subfamily) is predicted not to form heteromultimers with subunits of the Shaker subfamily (Covarrubias et al., 1991), and its gene product is targeted to the dendritic domain of neurons (Sheng et al., 1992).

Co-purification of Kv1.4 and Kv1.2 is consistent with protein-protein association in membranes, but could merely reflect highly similar physicochemical properties. We have used anti-peptide antibodies (raised against the non-conserved N- and C-terminal regions of the K^+ channel subunits) to determine whether Kv1.4 and Kv1.2 polypeptides could be co-immunoprecipitated by antibodies specific for either gene. In non-denaturing conditions, Kv1.4 antibodies consistently immunoprecipitated not only Kv1.4 protein, as detected by immunoblots of the immunoprecipitate, but also Kv1.2 in a lesser amount (Fig. 3). Conversely, anti-Kv1.2 immunoprecipitates contained not only the Kv1.2 protein, but also Kv1.4. In contrast, neither Kv1.4 nor Kv1.2 antibodies immunoprecipitated Kv4.2, and antibodies against Kv4.2 failed to bring down either Kv1.4 or Kv1.2, even though they were effective in immunoprecipitating their cognate antigen Kv4.2 (Fig. 3). These findings indicate that Kv1.4 and Kv1.2 subunits associate in the neuronal membrane with some degree of specificity, perhaps reflecting properties shared by members of the same K^+ channel gene subfamily. Identical co-immunoprecipitation results (not shown) were also obtained using samples of Kv1.4 and Kv1.2 that had been partially purified through two chromatographic steps, for example the 50 mM N-acetylglucosamine eluate from the wheat-germ agglutinin column (Fig. 2). The maintenance of the Kv1.4 and Kv.12 association through several columns makes it highly unlikely that actual co-immunoprecipitation occurred as a result of aggregates of membrane proteins formed during solubilization.

Immunoprecipitation of Kv1.4 by anti-Kv1.2, and of Kv1.2 by anti-Kv1.4, was abolished by boiling the membrane proteins in sodium dodecyl sulphate (SDS) before addition of antibodies. Immunoprecipitation of the respective cognate antigens, however, was relatively unaffected under these denaturing conditions (Fig. 3). These results indicate that co-immunoprecipitation of distinct subunits occurs through non-covalent interactions between Kv1.4 and Kv1.2, rather than through cross-reactivity of anti-Kv1.4 and anti-Kv1.2 antibodies. Further evidence against the cross-reactivity of the antibodies comes from the observation that, even in non-denaturing conditions, Kv1.2 antibodies fail to immunoprecipitate Kv1.4 protein from membrane extracts of GH3 cells, a cell line that expresses Kv1.4 but not the Kv1.2 gene (Lan Bo Shi and M.S., unpublished observations).

The association of Kv1.4 and Kv1.2 gene products in a multimeric complex in rat brain membranes is thus based on the following evidence: (1) co-expression of mRNAs in the same cells (Sheng et al., 1992; Tsaur et al., 1992); (2) co-localization of immunoreactivities in defined structures of the brain; (3) co-purification of the proteins through two chromatographic steps; and (4) most compellingly, co-immunoprecipitation with gene-specific antibodies.

By itself, Kv1.4 encodes a rapidly inactivating K^+ channel which recovers slowly from inactivation (Ruppersberg et al., 1990; Stuhmer et al., 1989). In contrast, Kv1.2 homomultimers are very slowly inactivating K^+ channels that are highly sensitive to dendrotoxin and 4-aminopyridine (4-AP) (Stuhmer et al., 1989). The Kv1.4/Kv1.2 heteromultimers combine features of both of the parent subunits, forming a novel channel that exhibits fast inactivation, rapid recovery from inactivation, and high 4-AP sensitivity (Po et al., 1993). These characteristics are typical of A-type K^+ channels (Rogawski, 1985; Rudy, 1988), a channel type widely implicated in the modulation of the presynaptic action potential and thus neurotransmitter output (Jan et al., 1977; Shimahara, 1992; Kaang et al., 1992; Tibbs et al., 1989). As the pattern of co-localized Kv1.4/Kv1.2 immunoreactivity correlates with an axonal and nerve terminal location, we speculate that Kv1.4/Kv1.2

heteromultimers may underlie a class of presynaptic A-type K^+ channels involved in relating neurotransmitter release and synaptic efficacy.

Although we have provided evidence that Kv1.4 and Kv1.2 form heteromultimeric K^+ channels in rat brain membranes, it should be emphasized that the overlap in distribution of Kv1.4 and Kv1.2 is partial at both the mRNA and protein levels. Hence only a subset of Kv1.4 and Kv1.2 subunits interacts with each other in the rat brain, leaving the rest to form homomultimers or to associate with other K^+ channel subunits. Such results are to be expected if combinatorial mechanisms between different subunits contribute to the diversity of K^+ channels in the brain. Adding another level of complexity, subunits encoded by other K^+ channel genes may also participate in, and refine the properties of Kv1.4/Kv1.2 heteromers, thereby contributing further to the heterogeneity of A-type K^+ channels observed *in vivo*.

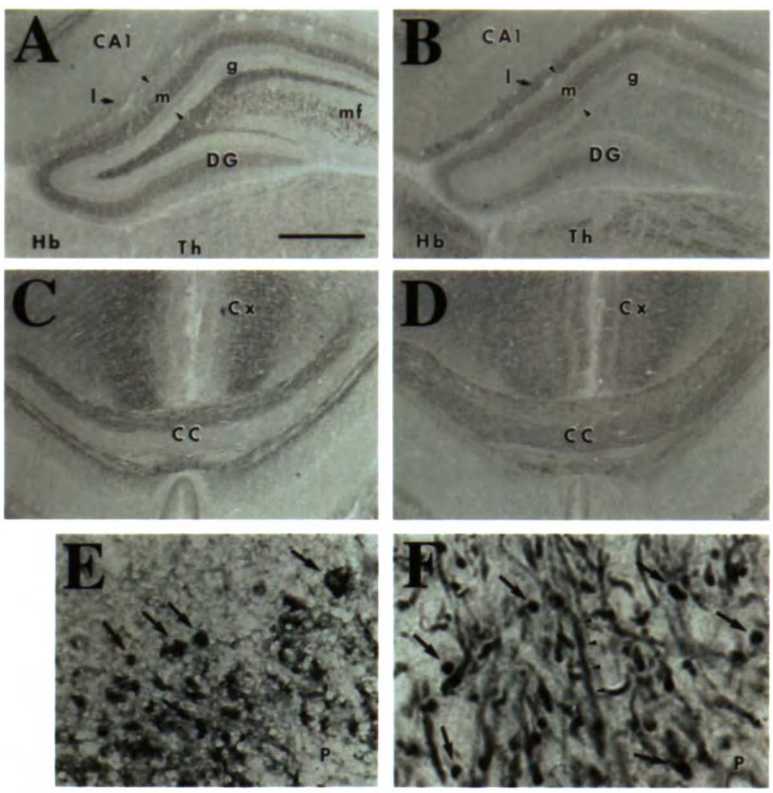
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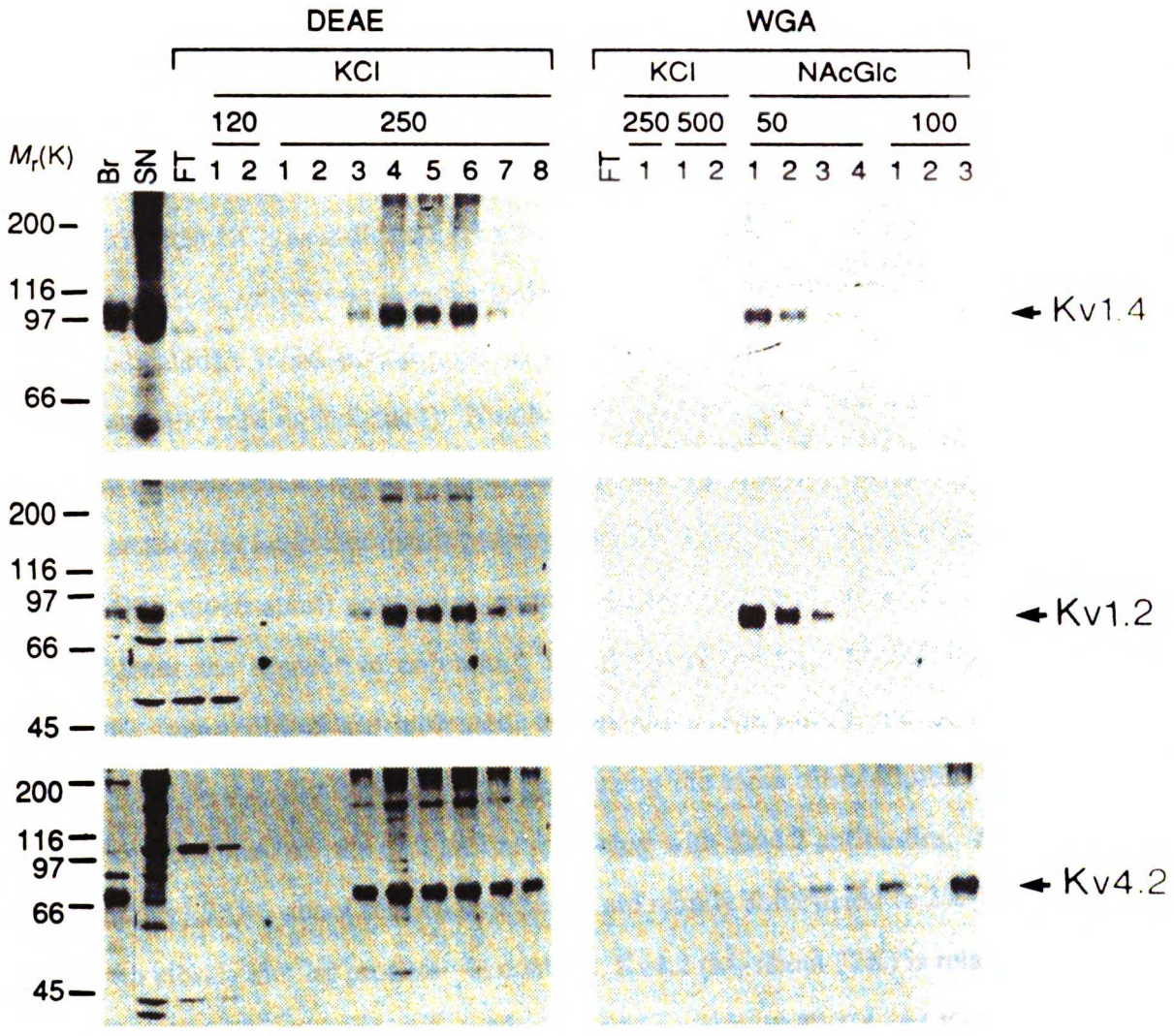
Figure 1. Co-localization of Kv1.4 and Kv1.2 proteins revealed by immunohistochemistry of adjacent coronal sections of rat brain.

A, C, and E. Staining pattern of Kv1.4 antibodies; B, D, and F, staining pattern of Kv1.2 antibodies. A and B, Hippocampal region. Kv1.4 and Kv1.2 immunoreactivities overlap prominently in the middle third of the molecular layer of the dentate gyrus ("m"; the full extent of the molecular layer is delineated by arrowheads), and in the stratum lacunosum moleculare ("l") of CA1. C and D, Kv1.4 and Kv1.2 immunostainings also overlap in axon tracts in the corpus callosum (CC), and in all regions of the cerebral cortex (Cx), including the retrosplenial cortex shown here. In cortex, both Kv1.4 and Kv1.2 are found in the neuropil but not in cell bodies. E and F, Numerous large terminals in the mossy fibre tract in the CA3 region of hippocampus are immunoreactive for both Kv1.4 and Kv1.2 (examples are marked by arrows). These terminals represent en passant synapses formed by the dentate granule cell axons with the proximal dendrites of CA3 pyramidal cells (the position of the CA3 pyramidal cell body layer is indicated by "P"). The overlap of Kv1.4 and Kv1.2 staining patterns is only partial. For instance, Kv1.2, but not Kv1.4, is present in CA3 pyramidal cell dendrites (an example is indicated by arrowheads). Additionally, whereas Kv1.2 is relatively evenly expressed throughout the thickness of the corpus callosum, Kv1.4 appears to be concentrated in the dorsal and ventral portions. Other abbreviations: DG, dentate gyrus; g, granule cell layer of dentate gyrus; Hb, habenular nucleus; Th, thalamus. Scale bars, 0.8 mm (A, B, C, D); 40 μm (E, F).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Kv1.4
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Kv4.2

Figure 2. Chromatographic co-fractionation of Kv1.4 and Kv1.2 subunits.

Triton-solubilized rat brain membrane proteins were subjected to sequential chromatography on DEAE-Sepharose and wheat-germ agglutinin (WGA)-agarose columns. The fractionation of different K^+ channel subunits was followed by immunoblotting with Kv1.4-, Kv1.2-, and Kv4.2-specific antibodies, as indicated. After binding of solubilized membrane proteins, the DEAE-Sepharose column was washed with 120 mM KCl; retained proteins were eluted by the application of 250 mM KCl, as indicated. Fractions positive for Kv1.4 were pooled and applied to the WGA column, which was then washed with KCl (at concentrations of 250 mM and 500 mM, as indicated). WGA-bound proteins were eluted with N-acetylglucosamine (NAcGlc; 50 mM and 100 mM as indicated). Numbers immediately above individual lanes refer to the fraction number collected after application of each wash or eluant. Other lanes are as follows: Br, starting rat brain membrane preparation; SN, 2% Triton extract of rat brain membranes (100,000g supernatant) containing solubilized membrane proteins; FT, flow-through fractions from the respective columns. Positions of the specific K^+ channel polypeptides on the immunoblots are indicated, as are molecular weight standards (in thousands). Kv1.2 and Kv4.2 immunoblots were done using the same nitrocellulose filter: Kv1.2 antibodies were stripped off the blot before reprobing with Kv4.2 antibodies. Kv1.4 (M_r about 95K) and Kv1.2 (M_r about 80K) polypeptides are readily solubilized in 2% Triton and fractionated with closely similar profiles. In contrast, Kv4.2 (M_r about 72K) is relatively poorly solubilized in 2% Triton and although it fractionates in part with Kv1.4 and Kv1.2 on a DEAE anion-exchange column, its interaction with WGA is different, with a substantial proportion being unable to bind WGA, and subpopulations of the rest being eluted at different concentrations of N-acetylglucosamine.

Methods. Crude membranes (Sheng et al., 1992) prepared from 4 rat brains were solubilized for 1 h at 4°C in 2% Triton X-100 in buffer A (20 mM HEPES, pH 7.4, 1 mM EDTA, 10% glycerol) containing 120 mM KCl. Insoluble material was pelleted at 100,000xg for 1.5 h. The supernatant (about 80 ml) was mixed with DEAE-Sepharose (Pharmacia; 50-ml bed

volume) in batch and poured into a column. After collection of flow through, the DEAE-Sepharose column was washed with buffer A with 0.1% Triton, and with 0.02% phosphotidylcholine (buffer B) containing 120 mM KCl. Proteins were eluted after application of buffer B containing 250 mM KCl and collected in small fractions (0.25 bed volumes). Fractions containing high levels of Kv1.4 were pooled and applied in batch to a 10-ml WGA-agarose column (Vector), which was then washed sequentially with buffer B containing 250 mM KCl, 500 mM KCl, then 150 mM KCl. Proteins retained on the column were eluted with N-Acetylglucosamine at 50 mM, then 100 mM, in buffer B containing 150 mM KCl. Immunoblots were made (Sheng et al., 1992) with affinity-purified antibodies at 1-2 μg/ml concentration. Filter-bound antigen was detected by donkey anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase and visualized using enhanced chemiluminescence and autoradiography (ECL, Amersham).

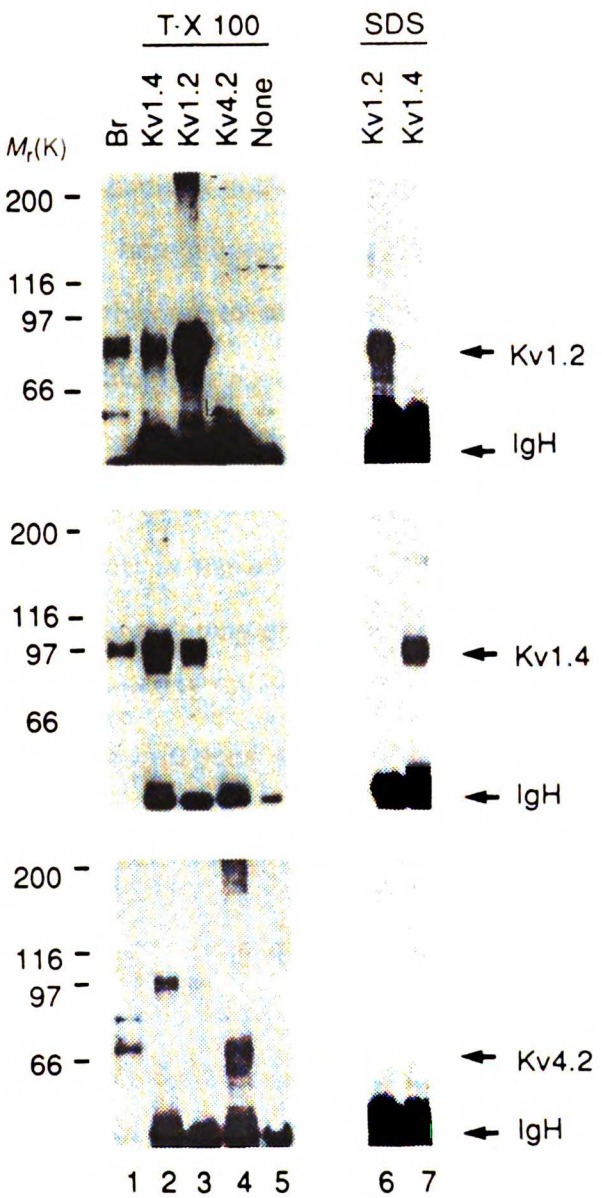
T·X 100
SDS
Br
Kv1.4
Kv1.2
Kv4.2
None
Kv1.2
Kv1.4
M_r(K)
200
116
97
66
Kv1.2
IgH
200
116
97
66
Kv1.4
IgH
200
116
97
66
Kv4.2
IgH
1 2 3 4 5
6 7

Figure 3. Co-immunoprecipitation of Kv1.4 and Kv1.2 subunits. Solubilized brain membrane proteins were immunoprecipitated with affinity-purified antibodies specific for Kv1.4, Kv1.2, or Kv4.2, or mock-immunoprecipitated with no addition of antibodies ("none"), as indicated, in either non-denaturing conditions ("TX100"), or after denaturation in boiling SDS ("SDS"). Immunoprecipitates were then separated by SDS-polyacrylamide gel electrophoresis and subjected to sequential immunoblot analysis using antibodies against Kv1.2 (top), Kv1.4 (middle), or Kv4.2 (bottom). The lane marked "Br" was loaded with 5 μg total membrane proteins from rat brain. Positions of the specific K^+ channel polypeptides, and the IgG heavy chain derived from the immunoprecipitating rabbit antibodies ("IgH") are indicated. Immunoblots in the bottom two panels (Kv1.4 and Kv4.2) were obtained by reprobing the filter after stripping off the previous antibody; note that there is some residual Kv1.4 signal (at M_r about 95K) in the Kv4.2 immunoblot.

Methods. Immunoprecipitations were performed from the 2% Triton X-100 extracts of whole brain membranes described in Fig. 2 (typically about 200 μg membrane protein), precleared for 1 h with protein A-Sepharose. Affinity-purified antibodies were added (3-6 μg/ml) for 2 h at 4°C, and immune complexes collected with protein A-Sepharose. Final non-denaturing conditions (1 ml reactions) were 1% Triton X-100, 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA. For denaturing conditions, the same amount of 2% Triton extract was boiled for 3-5 min in 0.5% SDS, 50 mM Tris, pH 8, and then diluted to 1 ml (final conditions: 1% Nonidet P-40, 0.5% deoxycholate, 0.4% Triton, 0.1% SDS, 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA) before addition of antibodies.

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