A Novel Cortical Mechanism for Top-Down Control of Water Intake

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SUMMARY

Water intake is crucial for maintaining body fluid homeostasis and animals' survival [1-4]. In the brain, complex processes trigger thirst and drinking behavior [1-5]. The anterior wall of the third ventricle formed by the subfornical organ (SFO), the median preoptic nucleus, and the organum vasculosum of the lamina terminalis (OVLT) constitute the primary structures sensing thirst signals and modulating water intake [6-10]. These subcortical regions are connected with the neocortex [11]. In particular, insular and anterior cingulate cortices (IC and ACC, respectively) have been shown to receive indirect innervations from the SFO and OVLT in rats [11] and to be involved in the control of water intake [12-15]. Type-1 cannabinoid receptors (CB_1) modulate consummatory behaviors, such as feeding [16-26]. However, the role of CB_1 receptors in the control of water intake is still a matter of debate [27-31]. Here, we show that endogenous activation of type-1 cannabinoid receptors (CB_1) in cortical glutamatergic neurons, of the anterior cingulate cortex (ACC), promotes water intake. Notably, presynaptic CB_1 receptors of ACC glutamatergic neurons are abundantly located in the basolateral amygdala (BLA), a key area in the regulation of water intake. The selective expression of CB_1 receptors in the ACC to BLA projecting neurons is sufficient to stimulate drinking behavior. Moreover, chemogenetic stimulation of these projecting neurons suppresses drinking behavior, further supporting the role of this neuronal population in the control of water intake. Altogether, these data reveal a novel cortico-amygdalar mechanism involved in the regulation of drinking behavior.

KEYWORDS

51 water intake; CB_1 receptors; anterior cingulate cortex; basolateral amygdala;
52 neuronal circuit
53
54 **RESULTS**
55 **CB_1 Receptors are Necessary for the Control of Stimulated Water Intake.**
56 To examine the role of CB_1 receptors in the control of water intake, we first tested *CB_1*
57 knockout mice (*CB_1*-KO) [32] under different experimental conditions (**Figures S1A**
58 **and S1B**). No significant differences were observed between wild-type (*CB_1*-WT) and
59 *CB_1*-KO littermates in daily water intake (**Figure S1C**). However, *CB_1*-KO mice drank
60 less water than *CB_1*-WT after 24-hour water deprivation (**Figures 1A and S1D**),
61 without any change in food intake (**Figure S1E**). This indicates that CB_1 receptors are
62 necessary for the control of drinking behavior induced by 24-hour water deprivation.
63 Water deprivation triggers both intracellular and extracellular dehydration that can
64 promote water intake through different specific pathways [1-5]. To discriminate the
65 impact of CB_1 receptor signaling on either of these mechanisms, we first applied
66 systemic (intraperitoneal, i.p.) or local (intracerebroventricular, i.c.v.) administration of
67 sodium chloride (NaCl), which is known to induce water intake by mimicking
68 intracellular dehydration [5]. As compared to *CB_1*-WT, *CB_1*-KO mice displayed a lower
69 water intake induced by either i.p. or i.c.v. NaCl administration (**Figures 1B, 1C, and**
70 **S1F**). Extracellular dehydration promotes the production of angiotensin II (Ang II),
71 which can induce drinking behavior and salt appetite [1-5]. Notably, the water intake
72 induced by the i.c.v. injections of Ang II was blunted in *CB_1*-KO mice **(Figure 1D)**. In

addition, the acute systemic pharmacological blockade of CB_1 receptors decreased drinking under water deprivation or i.p. NaCl injection (**Figures 1E and 1F**). This further supports the role of CB_1 receptors in drinking behavior, and excludes potential developmental alterations caused by the long-lasting deletion of the *CB_1* gene in *CB_1*-KO mice [32]. Besides its abundant brain expression, CB_1 receptors are also present in peripheral organs [20, 23-26], suggesting that peripheral control of body water levels or blood osmolality might underlie the CB_1-dependent regulation of water intake. However, measurements of body water composition and blood osmolality did not reveal any differences between *CB_1*-WT and *CB_1*-KO mice (**Figures S1G and S1H**). Altogether, these results indicate that endogenous activation of CB_1 receptors contributes to the regulation of drinking behavior induced by either intracellular or extracellular dehydration, likely through central mechanisms.

CB_1 Receptors Expressed in Cortical Glutamatergic Neurons are Sufficient for the Control of Stimulated Water Intake.

CB_1 receptors are present in different brain regions and in distinct cell types [19, 20, 22]. To identify the specific cell types involved in CB_1 receptor-dependent control of water intake, we used conditional mutant mice carrying a deletion of the *CB_1* gene in cortical glutamatergic neurons (*Nex*-Cre:*CB_1*-Flox, hereafter called Glu-*CB_1*-KO) [33, 34], forebrain GABAergic neurons (*Dlx5/6*-Cre:*CB_1*-Flox, hereafter called GABA-*CB_1*-KO) [33, 34], glial fibrillary acidic protein-positive cells (mainly astrocytes, *GFAP*-Cre-ERT2:*CB_1*-Flox, hereafter called GFAP-*CB_1*-KO) [33, 35] or dopamine receptor D_1-positive cells (*D_1*-Cre:*CB_1*-Flox, hereafter called D_1-*CB_1*-KO) [33, 36],

respectively. All these cell types have been implicated in the control of water intake [6-10, 17, 19, 37-42]. Surprisingly, however, none of these mutant lines displayed significant phenotypes in drinking behavior induced by water deprivation or NaCl treatment (**Figures S1I-S1P**).

This puzzling observation on how global, but not cell type-specific, CB_1 deletion impact water intake might be due to the redundancy of CB_1 receptor-dependent pathways controlling a vital function as water intake [20]. In this context, despite the general necessary role of CB_1 receptors in promoting drinking behavior during stimulated conditions, this redundancy would decrease the specific *necessity* of selected subpopulations of these receptors. This, however, does not exclude that CB_1 receptor-dependent control of specific cell populations might play *sufficient* roles in controlling stimulated water intake [20].

To address this possibility, we adopted a rescue approach and used mice carrying specific and exclusive re-expression of the CB_1 protein in specific cell types [43, 44]. A “floxed-stop” cassette prevents the expression of CB_1 receptors in the Stop-*CB1* mutant mouse line, similarly as in global *CB1*-KO mice [43, 44]. Viral or transgenic expression of the Cre recombinase, however, induces re-expression of the CB_1 receptors in particular brain regions and/or cell types over a “knockout-like” background [43, 44]. First, we verified that Stop-*CB1* mice displayed the same impaired water intake as *CB1*-KO mice and that global re-expression of the CB_1 protein was able to fully rescue water intake under water deprivation or NaCl treatment (*CMV*-Cre:Stop-*CB1* for CB_1 "rescued", hereafter called *CB1*-RS; **Figures**

1G and 1H) [43, 44]. Re-expression of CB_1 receptors in GABAergic neurons (*Dlx5/6*-Cre:Stop-*CB1*, hereafter called GABA-*CB1*-RS) [43, 44], which include the large majority of brain CB_1 receptors [19, 20, 22], did not rescue drinking behavior either after water deprivation or i.p. NaCl injections (**Figures S1Q and S1R**). Interestingly, re-expression of CB_1 receptors in cortical glutamatergic neurons (Nex-Cre:Stop-*CB1*, hereafter called Glu-*CB1*-RS) [43], which represents a minority of the receptor in the brain [19, 20, 22], rescued large part of water intake induced by either water deprivation, by injection of NaCl, or by Ang II administration compared to *CB1*-WT or *CB1*-RS mice (**Figures 1I-1L**). These data indicate that the presence of CB_1 receptors in cortical glutamatergic neurons is sufficient to promote stimulated water intake.

CB_1 Receptors in ACC Glutamatergic Neurons are Sufficient for the Control of Stimulated Water Intake.

Amongst neocortical areas, the insular cortex (IC) has been directly shown to regulate water intake [12, 13]. Therefore, we tested whether specific re-expression of CB_1 receptors in this brain region rescue the impairment of water intake observed in Stop-*CB1* mice. Multiple local injections of an adeno-associated virus expressing Cre recombinase (AAV-CAG-Cre) [45] into the IC of Stop-*CB1* mice resulted in a consistent CB_1 re-expression in the neurons of both the anterior and the posterior IC (IC-*CB1*-RS; **Figures S2A-S2C**). However, this manipulation did not rescue the water intake associated with the lack of CB_1 receptors (**Figures S2D and S2E**). Recent evidence showed that activation of the anterior IC (aIC) increases water intake [13],

whereas posterior IC (pIC) activation exerts opposite effects [12, 13]. Considering that activation of the pIC inhibits drinking behavior and CB_1 receptors generally reduce neuronal activity [19, 22], we hypothesized that exclusive CB_1 receptor-dependent control of the pIC might lead to decreased neuronal activity and thereby promote drinking behavior. To test this possibility, we re-expressed CB_1 receptors exclusively in the pIC of Stop-*CB1* mice (pIC-*CB1*-RS, **Figures S2F and S2G**). This specific re-expression did not rescue the phenotype of Stop-*CB1* mice (**Figures S2H and S2I**), suggesting that CB_1 receptors in the IC do not play a sufficient role in the control of water intake.

The anterior cingulate cortex (ACC) participates in the regulation of water intake [1, 3, 4, 11, 14, 15]. Therefore, we generated ACC-CaMKIIα-*CB1*-RS mice, in which CB_1 receptors were re-expressed only in ACC principal neurons (**Figures 2A-2C**) [46]. Notably, ACC-CaMKIIα-*CB1*-RS mice displayed significantly higher water intake than control Stop-*CB1* littermates (ACC-CaMKIIα-*CB1*-SS) upon either water deprivation or i.p. NaCl injection (**Figures 2D and 2E**). This indicates that activation of CB_1 receptors in ACC principal neurons is sufficient to promote stimulated drinking behavior.

CB_1 Receptors in the ACC to BLA Projecting Neurons are Sufficient for the Control of Stimulated Water Intake.

As the ACC is a heterogeneous structure targeting multiple downstream regions (**Figure S3**, neural projections in ACC-CaMKIIα-GFP mice), we next aimed at identifying which CB_1-positive projections from the ACC might be responsible for the

stimulation of drinking behavior. In order to analyze the expression of presynaptic CB_1 receptors in these ACC projections, we evaluated the distribution of the CB_1 protein in ACC-CaMKIIα-*CB1*-RS mice. Interestingly, CB_1 receptor-positive fibers were mainly present in the basolateral amygdala (BLA), the claustrum, the entorhinal and perirhinal cortices among the innervated brain structures (**Figures 2F-2H, S3, and Video S1**). Interestingly, the BLA is involved in the control of drinking behavior [13, 47]. According to the abundant distribution of CB_1 receptor-positive fibers in the BLA (**Figure 2G**), we hypothesized that re-expression of CB_1 receptors in the ACC to BLA projecting neurons (**Figure 3A**) would be sufficient to promote drinking. To rescue the CB_1 protein in the ACC neurons projecting to the BLA, we used a retrograde viral approach in the Stop-*CB1* mice. The injection of a retrograde AAV (rAAV2-retro) expressing flippase (FLIPo) coupled to the enhanced blue fluorescent protein (rAAV2-retro-hSyn1-chI-FLIPo-EBFP) into the BLA of Stop-*CB1* mice was associated with the simultaneous infusion of another AAV carrying a FLIPo-dependent expression of Cre recombinase (AAV-hEF1α-FRT-iCre-GFP) into the ACC (**Figure 3B**). These viral manipulations resulted in a major re-expression of CB_1 receptors in the BLA (ACC-BLA-*CB1*-RS mi□e; **Figures 3C-3E**), although slight expression in other ACC targeted regions is also observed (**Figures S4A-S4C**), suggesting there are collateral projections of ACC-BLA projecting neurons. Strikingly, upon water deprivation or i.p. NaCl injections, ACC-BLA-*CB1*-RS mice consumed significantly more water than Stop-*CB1* control mice (**Figures 3F and 3G**). Overall, these results reveal that CB_1 receptors in ACC neurons projecting to BLA modulate water intake.

Activation of the ACC Neurons Projecting to BLA Suppresses Stimulated Water Intake.

The ACC projections to BLA are mostly monosynaptic and glutamatergic [48]. By doing fluorescent *in situ* hybridization, we observed that around 97% of excitatory neurons (VGLUT1 positive cells) co-express CB_1 receptor mRNA in ACC neurons (**Figures S4D-S4F**), suggesting that a large proportion of the ACC to BLA projecting neurons express CB_1 receptors. Given the general inhibitory role of endocannabinoid signaling at synaptic transmission, the data presented so far suggest that, upon thirst induction, CB_1 receptors in ACC neurons projecting to BLA likely decreases glutamatergic neurotransmission, thereby promoting water intake. Thus, we hypothesized that the specific activation of ACC neurons projecting to BLA would suppress drinking behavior in wild-type mice. To test this hypothesis, a double viral approach was applied to express DREADDs hm3D(Gq) in the ACC neurons projecting to BLA [49, 50]. A retrograde virus expressing Cre recombinase (rAAV2-retro-hSyn1-chI-iCre-EBFP) was injected into the BLA in combination with the injection of an AAV, expressing hm3D(Gq) (AAV-hSyn-DIO-hM3D(Gq)-mCherry) or mCherry (AAV-hSyn-DIO-mCherry) in a Cre-dependent manner, into the ACC (**Figure 4A**). Interestingly, we observed that mCherry expression was abundantly located in the BLA (**Figure S3C**) and with a lower extent in the claustrum, the caudate putamen, the Ect, and the PRh, the paraventricular hypothalamic nucleus, the zona incerta, and other brain regions in ACC-BLA-mCherry mice compared to the ACC

projections in the ACC-CaMKIIα-GFP mice (**Figure S3**), revealing the specificity of ACC-BLA manipulation and collateral innervations of the ACC-BLA projecting neurons. Intraperitoneal injection of Clozapine-N-oxide (CNO) did not induce c-Fos expression in the ACC of ACC-BLA-mCherry control mice, but increased c-Fos positive cells in the ACC of ACC-BLA-hm3D(Gq) mice (**Figures 4B-4D**). Notably, CNO administration induced a significant decrease in water intake upon either water deprivation or i.p. injection of NaCl in ACC-BLA-hm3D(Gq) mice compared to the control mice (**Figures 4E-4H**). Altogether, our data indicate that the ACC neurons projecting to BLA play a crucial role in the control of stimulated water intake.

DISCUSSION

By addressing the role of CB_1 receptors in stimulated water intake, this study identifies a novel cortical mechanism involved in the top-down control of drinking behavior. In particular, our data indicate that the CB_1 receptor-dependent control of ACC neurons projecting to BLA participates in the regulation of stimulated water intake.

CB_1 Receptors are Necessary for the Control of Water Intake.

Our different experimental conditions together with the genetic approaches used here, reveal that CB_1 receptors are necessary for the control of stimulated drinking, but not in physiological conditions (i.e. daily water intake). This might be due to the fact that water intake is controlled by redundant, and tightly regulated, biological mechanisms [51, 52]. Accordingly, the observed changes in water intake after different dipsogenic

conditions can induce drastic effects on body fluids homeostasis thereby impacting physiological functions.

Potential Redundancy in the Control of Water Intake by Brain CB_1 Receptors.

Several neuronal mechanisms have been suggested to control water intake and body fluid homeostasis. This includes the regulation of excitatory and inhibitory balance in different brain regions [6-10, 12, 13], the potential involvement of astrocytes [53, 54] or dopamine signaling [37-39, 42]. However, the specific deletion of CB_1 receptors in different neuronal types (Glu-*CB1*-KO, GABA-*CB1*-KO, and D_1-*CB1*-KO) or astrocytes (GFAP-*CB1*-KO) did not induce any observable water intake phenotype in mice. Thus, whereas global CB_1 receptors exert a positive control of water intake, this general necessary role is not due to any of the different subpopulations independently analyzed. This surprising result might be explained by different potential scenarios. (i) Since CB_1 receptors are expressed in many cell types and brain regions [19, 20, 22], it is possible that their activation in other cell types (not explored in the present study) might play a necessary role in the control of water intake. For example, our experiments did not address the role of CB_1 receptors in subcortical areas directly involved in the control of drinking behavior [6-10]. Future studies will address this question by using Cre lines able to induce the deletion of CB_1 receptors in subcortical glutamatergic neurons (e.g. VGLUT2-Cre mouse line) [55]. (ii) CB_1 receptors are also present in peripheral organs, including the ones involved in the control of body fluids [23-26]. It is, therefore, possible that the general necessary role of CB_1 receptors on water intake is mainly exerted at the peripheral level. Even if our data exclude a

CB_1-dependent control of blood osmolality and total body water content, they cannot definitely rule out that peripheral CB_1 receptors might be involved in some signaling events required for drinking behavior. (iii) Finally, another possibility is that a vital function like water intake is likely exerted through redundant pathways and mechanisms, which can compensate each other [20, 22].

In redundant biological systems, whereas the whole system is necessary for a certain function, each single element is not. However, single elements are likely to play a sufficient role for the same function [56]. This might be the case of the endocannabinoid system in vital body functions. In previous feeding behavioral experiments, specific deletion of CB_1 receptors in cortical glutamatergic neurons decreased food intake [17], whereas exclusive re-expression of CB_1 receptors in the same neuronal population promoted food intake after food deprivation [18]. In contrast, our data show that both re-expression of CB_1 receptors globally or exclusively in cortical glutamatergic neurons is sufficient to promote drinking behavior. Thus, whereas this CB_1 subpopulation is necessary and sufficient for feeding, it is only sufficient for drinking. Future studies will aim to better understand these redundant functions and will elucidate the specific temporal and spatial role of endocannabinoids in the control of water intake in cortical brain circuits.

ACC Neurons Projecting to BLA: A Novel Locus for Control of Drinking Behavior.

The sufficient role played by CB_1 receptors in ACC neurons projecting to the BLA suggests that this circuit might be an important relay in the brain systems dedicated to

271 the control of water intake. Indeed, our chemogenetic experiments clearly show that
272 the activation of these neurons is able to reduce drinking behavior in different
273 conditions of stimulation. Thus, these data indicate that, independently of CB_1
274 receptors, the activity of the ACC neurons projecting to BLA is a key element of the
275 control of water intake. However, there are still some important questions to be
276 addressed to further dissect the cortical descending mechanisms controlling water
277 intake. For instance, future studies using calcium imaging and specific inhibition will
278 address the relationship between hydration and drinking with the dynamic activity of
279 ACC neurons projecting to BLA. Moreover, our data show that these neurons have
280 collateral innervations to other brain regions. Therefore, it will be of great interest to
281 study the involvement of these additional targets in the control of drinking behavior.

282 Interestingly, both CB_1 re-expression and DREADD activation of ACC neurons
283 projecting to BLA produce stronger effects on drinking induced by water deprivation
284 than the one caused by NaCl injections. The reasons for this difference are presently
285 unknown. Water deprivation represents a globally stressful experience for individuals
286 and BLA is an important region for stress responses [57-59]. Therefore, it is tempting
287 to speculate that the cortical control of BLA activity might mitigate the
288 stress-component induced by water deprivation. Accordingly, CB_1 receptors regulate
289 stress responses [60] and it will be very interesting to investigate their functions in the
290 specific stress induced by water deprivation.

291 In conclusion, this study reveals a novel cortical mechanism for descending
292 control of a fundamental life function such as water intake. Altogether, these data

293 highlight the complexity of brain control of drinking behavior and underline the
294 importance of top-down regulatory circuits in these processes.
295

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AUTHOR CONTRIBUTIONS

Z.Z. and G.M. conceived the project. Z.Z., E.S-G, A.Covelo, L.B., A.B-G. and G.M. designed the experiments and analyzed data. Z.Z. performed the behavioral and imaging experiments. Z.Z., M.V. and F.J-K. performed immunohistochemistry experiments. M.V. performed fluorescent *in situ* hybridization experiments. A.Cannich, A.Castiglione, L.V., A.D., and P.Z. prepared reagents and assisted in performing behavioral experiments. A.B. and D.C. discussed the study. Z.Z., E.S-G., L.B., A. B-G., and G.M. wrote the manuscript. All authors read and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

MAIN FIGURE TITLES AND LEGENDS

Figure 1. Control of water intake through CB_1 receptors.

(A-D) Cumulative water intake of *CB_1*-WT (Black circles) and *CB_1*-KO (open circles) mice after 24-hour water deprivation **(A)** (*CB_1*-WT n=10, *CB_1*-KO n=8), the i.p. administration of 1M NaCl **(B)** (*CB_1*-WT n=10, *CB_1*-KO n=8), the i.c.v. infusion of NaCl **(C)** (*CB_1*-WT n=13, *CB_1*-KO n=10) and i.c.v. infusion of Ang II **(D)** (*CB_1*-WT n=11, *CB_1*-KO n=13). **(E-F)** Cumulative water intake in vehicle- (black circles) or rimonabant-treated (grey circles, rimonabant, 3mg/kg) mice induced by 24-hour water deprivation **(E)** (Vehicle n=9, Rimonabant n=10) or the i.p. administration of 1.5M NaCl **(F)** (Vehicle n=6, Rimonabant n=7). **(G-H)** Cumulative water intake induced by 24-hour water deprivation **(G)** (Stop-*CB_1* n=9, *CB_1*-RS n=12) and the i.p. administration of 1M NaCl **(H)** (Stop-*CB_1* n=9, *CB_1*-RS n=11) in Stop-*CB_1* (open squares) and *CB_1*-RS (black squares) mice. **(I-L)** Cumulative water intake induced by 24-hour water deprivation **(I)** (Stop-*CB_1* n=11, Glu- *CB_1*-RS n=11), the i.p. administration of 1M NaCl (**J**) (Stop-*CB_1* n=11, Glu-*CB_1*-RS n=11), the i.c.v. infusion of NaCl **(K)** (Stop-*CB_1* n=13, Glu-*CB_1*-RS n=11) and the i.c.v. infusion of Ang II **(L)** (Stop-*CB_1* n=15, Glu-*CB_1*-RS n=13) in Stop-*CB_1* (open triangles) and Glu-*CB_1*-RS (black triangles) mice. All data are shown as the mean ± SEM, and were analyzed by two-way ANOVA of repeated measures and shown in **Table S1**. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. For more relevant information, see **Figure S1**.

Figure 2. Re-expression of CB_1 receptors in the ACC is sufficient to promote water intake.

(A) Schematic representation of the CB_1 rescue approach in the ACC of Stop-*CB_1* mice. **(B and C)** CB_1 (red) immunostaining in the ACC of ACC-CaMKIIα-*CB_1*-SS (control) and ACC-CaMKIIα-*CB_1*-RS (rescue), respectively. Scale bar, 200 μm. **(D and E)** Cumulative water intake of ACC-CaMKIIα-*CB_1*-SS (open squares) and ACC-CaMKIIα-*CB_1*-RS (black squares) mice after 24-hour water deprivation **(D)** (ACC-CaMKIIα-*CB_1*-SS n=17, ACC-CaMKIIα-*CB_1*-RS n=20) or i.p. 1M NaCl **(E)** (ACC-CaMKIIα-*CB_1*-SS n=18, ACC-CaMKIIα-*CB_1*-RS n=20). **(F-H)** Presynaptic CB_1 receptors located in the CI **(F)**, BLA **(G)** and Ect/PRh **(H)** in an ACC-CaMKIIα-*CB_1*-RS mouse. Scale bar, 500 μm and 100 μm (amplified images). All data are shown as the mean ± SEM, and were analyzed by two-way ANOVA of repeated measures and shown in **Table S1**. *$P < 0.05$, ****$P < 0.0001$. For more relevant information, see **Figures S2, S3,** and **Video S1**.

Figure 3. CB_1 receptors located in the ACC-BLA circuit are sufficient to promote water intake.

(A-B) Schematic representations of CB_1 receptor expression in the ACC-BLA circuit **(A)** and the viral approach used to specifically rescue CB_1 receptors in the ACC-BLA circuit **(B)**. **(C)** EBFP (pseudo red) and iCre-GFP (green) in ACC sections of ACC-BLA-*CB1*-SS (control). Scale bar, 100 µm. **(D)** FLIPo-EBFP (pseudo red) and iCre-GFP (green) in the ACC of ACC-BLA-*CB1*-RS (rescue). Arrows indicate colocalization of FLIPo and iCre. Scale bar, 100 µm. **(E)** CB_1 (red) immunostaining in BLA of ACC-BLA-*CB1*-SS and ACC-BLA-*CB1*-RS. Scale bar, 100 µm. **(F-G)** Cumulative water intake of ACC-BLA-*CB1*-SS (open squares) and ACC-BLA-*CB1*-RS (black squares) mice after 24-hour water deprivation **(F)** (ACC-BLA-*CB1*-SS n=10) and the i.p. administration of 1M NaCl (**G**) (ACC-BLA-*CB1*-SS n=12). All data are shown as the mean ± SEM, and were analyzed by two-way ANOVA of repeated measures and shown in **Table S1**. $*P < 0.05$, $**P < 0.01$. For more relevant information, see **Figure S4**.

Figure 4. Stimulation of the ACC-BLA circuit inhibits water intake.

(A) Schematic representation of the viral approach used to specifically express hM3D(Gq) in the ACC-BLA circuit. **(B)** Schematic behavioral diagram of the protocol used in this DREADD strategy. **(C)** mCherry (red) and c-Fos (green) in the ACC of ACC-BLA-mCherry. Scale bar, 500 μm. **(D)** hM3D(Gq) (red) and c-Fos (green) in the ACC of ACC-BLA-hM3D(Gq). Arrows indicate colocalization of hM3D(Gq) and c-Fos. Scale bar, 500 μm. **(E-F)** Cumulative water intake of ACC-BLA-mCherry **(E)** (n=7) and ACC-BLA-hM3D(Gq) **(F)** (n=7) mice treated by Saline (black circles) or CNO (2mg/kg, open circles) after 24-hour water deprivation. **(G-H)** Cumulative water intake of ACC-BLA-mCherry **(G)** (n=7) and ACC-BLA-hM3D(Gq) **(H)** (n=7) mice treated by Saline (black circles) or CNO (2mg/kg, open circles) after i.p. 1.5M NaCl. All data are shown as the mean ± SEM, and were analyzed by two-way ANOVA of repeated measures and shown in **Table S1**. *$*P$* < 0.05. For more relevant information, see **Figures S3 and S4**.

STAR ★ METHODS

RESOURCE AVAILABILITY

Lead Contact

Further information and requests should be directed to and will be fulfilled by the Lead Contact (Giovanni Marsicano; giovanni.marsicano@inserm.fr).

Materials Availability

This study did not generate new unique reagents. Materials used here are available from the Lead Contact upon reasonable request.

Data and Code Availability

Raw data supporting the current study (Figures 1-4 and S1-S4) have been deposited to Mendeley Data: http://dx.doi.org/10.17632/t8j2z6648f.2.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All experiments were approved by the Committee on Animal Health and Care of INSERM and the French Ministry of Agriculture and Forestry. The authorizing number from the ethical committee is 15493. Maximal efforts were made to reduce the suffering and the number of mice used. All behavioral experiments were performed during the light phase and animals were kept in individual cages under standard conditions in a day/night cycle of 12/12 hours (lights on at 7 am). Male wild-type C57BL/6 mice purchased from Janvier (France) were used for the pharmacological experiments. All mutant mice were generated and identified in previous studies, e.g. global CB_1 knockout (*CB_1*-KO) mice [32]; deletion of CB_1 receptors is specific in cortical glutamatergic Nex positive neurons (Glu-*CB_1*-KO) [34]; forebrain GABAergic

Dlx5/6 positive neurons (GABA-*CB$_1$*-KO)[34]; astrocytes (GFAP-*CB$_1$*-KO) [35]; dopamine receptor type 1 positive neurons (D_1-*CB$_1$*-KO) [36]; the Stop-*CB$_1$* mice (lack of CB_1) [43]; the global re-expression of CB_1 receptors (*CB$_1$*-RS) [43] and the re-expression of CB_1 receptors in forebrain GABAergic Dlx5/6 positive neurons (GABA-*CB$_1$*-RS) [44] or in cortical glutamatergic Nex positive neurons (Glu-*CB$_1$*-RS) [43]. All the mice used in this study were 7-10 weeks old at the beginning of the experiments and all the data was obtained by experimenters blind to the pharmacological or genetic conditions.

METHOD DETAILS

Water intake assays

Water intake was analyzed at 30, 60 and 120 minutes after 24-hour water deprivation and after the intraperitoneal (i.p.) injection of 1M sodium chloride (NaCl, VWRV0241, 10ml/kg body weight) **(Figure S1A)**. In the pharmacological experiments, Rimonabant (3mg/kg, 9000484, Cayman Chemical Company US) or vehicle (4% ethanol, 4% Cremophor, 92% saline) was injected half an hour prior to the water intake test. For the mice with intracerebroventricular (i.c.v.) injections **(Figure S1B)**, water intake was analyzed at 30 minutes after i.c.v. injection of Angiotensin II (Ang II, Bachem, H-1705.0025) and NaCl. In these mice we waited for 7 days after the i.c.v. cannula implantation for a full recovery of the surgery. In the progressive Ang II dose-response experiments, we made i.c.v. injections of saline, 5 ng, 15 ng, and 45 ng of Ang II (2μl/mouse) in successive days. Then, we made the dose-response of i.c.v. NaCl injections (0.15M, 0.3M, 0.6M, and 1.2M NaCl, 1μl/mouse), which started 3

days after the last i.c.v. Ang II injection. In the DREADDs hM3D(Gq) experiment, Clozapine-N-oxide (CNO, 2mg/kg, Tocris Bioscience) or the control saline were injected half an hour prior to the water intake test. In order to make sure that mice were drinking normally before the treatments, the daily water intake of each mouse was observed during the whole experiments.

Body water composition analysis

The basal body water composition test was performed in mice by using a mouse-specific nuclear magnetic resonance whole body composition analyzer (EchoMRITM-900, EchoMedical Systems, Houston, TX). Mice were placed in a specific chamber without strong movements and we obtained readouts every minute. Mice were put back to their home cage after the test.

Plasma osmolality analysis

We used facial veil blood collection and the blood was collected and put in the Micro Tube 1.3 ml K3E (SARSTEDT, 41.1395.005). By using a refrigerated centrifuge (VWR Micro Star 17R), blood samples were centrifuged at 4000 rpm for 15 minutes at 4℃. Following centrifugation, the plasma was immediately transferred to a clean Eppendorf tube and put on ice for the osmolality test. Plasma osmolality was analyzed by the Osmometer 3320 (Advanced Instruments, France).

Surgery and viral administration

Mice were anesthetized by isoflurane (5% for the induction and 2% during the surgery) and placed on a stereotaxic apparatus (Model 900, KOPF instruments, CA, USA) with a mouse adaptor and lateral ear bars. For viral vectors delivery, AAV vectors were

loaded in a glass pipette and infused by a pump (UMP3-1, World Precision Instruments, FL, USA). AAV-CAG-GFP (Hybrid AAV1/2, 5.18 x 10E10 vg/ml) and AAV-CAG-Cre-GFP (Hybrid AAV1/2, 4.2 x 10E10 vg/ml) were injected into the insula (IC) (200nl/side, 100nl/min). The coordinates for the anterior IC injection are AP +1.2mm, ML ± 3.0mm, DV 3.5mm, and the coordinates for the posterior IC injection are AP -0.3mm, ML ± 3.7mm, DV 4.0mm. AAV-CaMKIIα-GFP (Hybrid AAV1/2, 6.73 x 10E10 vg/ml) or AAV-CaMKIIα-Cre-HA (Hybrid AAV1/2, 1.05 x 10E11 vg/ml, provided by Karl Deisseroth from Stanford University (Standford, CA) were injected into the anterior cingulate cortex (ACC) (200nl/side, 100nl/min). The coordinates for the ACC injection are AP +0.6mm, ML ± 0.3mm, DV 2.0mm. For the ACC-BLA-*CB*$_1$-RS or ACC-BLA-*CB*$_1$-SS mice, the AAV-hEF1α-FRT-iCre-GFP (Addgene #24593, ZNZ VVF v245, 6.3 x 10E12 vg/ml) was injected into the ACC with the coordinates mentioned above in both group of mice (200nl/side, 100nl/min). The rAAV2-retro-hSyn1-chI-FLIPo-EBFP (Addgene #60663, ZNZ VVF v151, 6.4 x 10E12 vg/ml) or rAAV2-retro-hSyn1-chI-EBFP (ZNZ VVF v140, 4.1 x 10E12 vg/ml) were injected into the BLA (150nl/side, 100nl/min, the coordinate is AP -1.6mm, ML ±3.3mm, DV 4.9 mm, 150nl/side, 100nl/min). AAV-hEF1α-FRT-iCre-GFP, rAAV2-retro-hSyn1-chI-FLIPo-EBFP and rAAV2-retro-hSyn1-chI-EBFP were produced by the Viral Vector Facility (VVF) of the Neuroscience Center Zurich (ZNZ). The re-expression of CB_1 receptors was verified by immunohistochemistry in all the mice used in the behavioral experiments. For the stimulation of the ACC-BLA circuit by DREADDs, the AAV-hSyn-DIO-hM3D(Gq)-mCherry (Addgene AAV8,

44361-AAV8, 2.2 x 10E13 GC/ml) or AAV-hSyn-DIO-mCherry (Addgene AAV8, 50459-AAV8, 2.3 x 10E13 GC/ml) were injected into the ACC in combination with the injection of rAAV2-retro-hSyn1-chI-iCre-EBFP (Addgene #25493, ZNZ VVF v148, 6.7 x 10E12 vg/ml) into BLA with the coordinates mentioned above. The coordinates used were decided according to the mouse brain atlas (Paxinos and Franklin, 2001, Second edition) [61].

Immunohistochemistry

After the behavioral experiments, mice were anesthetized with pentobarbital (Exagon, 400 mg/kg body weight), transcardially perfused first with the phosphate-buffered solution (PBS, 0.1M, pH 7.4) and then fixed by 4% formaldehyde (Sigma-Aldrich, HT501128) [18, 45]. Serial brain coronal sections were cut at 40 μm and collected in PBS at room temperature (RT). Sections were permeabilized in a blocking solution of 4% donkey serum, 0.3% Triton X-100 and 0.02% sodium azide prepared in PBS for 1 hour at RT. For the CB_1 immunohistochemistry, free-floating sections were incubated with goat CB_1 receptors polyclonal primary antibodies (CB_1-Go-Af450-1; 1:2000, Frontier Science Co. ShinKO-nishi, Ishikari, Hokkaido, Japan) for 48 hours at 4℃. The antibody was prepared in the blocking solution. After three washes, the sections were incubated with a secondary antibody anti-goat Alexa Fluor 555 (A21432, 1:500, Fisher Scientific) for 2 hours at RT and then washed in PBS at RT. For the HA immunohistochemistry (IHC), the procedure is similar to the one of CB_1 IHC. Sections were incubated in anti-HA tag monoclonal antibody (1:1000, Fisher Scientific, 2-2.2.14) for 18 hours at 4℃ and in secondary antibody anti-mouse Alexa Fluor 488

(A21202, 1:500, Fisher Scientific) for 2 hours at RT. For the IHC of c-Fos, sections were incubated in guinea pig anti-c-Fos antibody (226004, 1:1000, Synaptic Systems) for 18 hours at RT and in secondary antibody goat anti-guinea pig Alexa Fluor 488 (11073, 1:500, Fisher Scientific) for 2 hours at RT. All sections were mounted, dried and coverslipped. Images of these sections were taken by a Nanozoomer microscope (Hamamatsu, Japan) and Leica SP8 confocal microscope (Leica, Germany) and analyzed by Image J (NIH). For the mouse brain reconstruction, images were collected by Nanazoomer, Z-stack images were made by Image J and the 3D reconstruction and video were made by Imaris software (Imaris, Oxford instrument, UK).

Fluorescent *in situ* hybridization

The detailed procedure referred to previous publications [17, 18, 62]. Mice were sacrificed by cervical dislocation, then their brains were rapidly extracted and placed on dry ice. The frozen brains were stored at 80℃ for sections by a cryostat (14 μm, CM1950, Leica). For the probes, fluorescein (FITC)-labeled riboprobes against mouse CB_1 receptor and digoxigenin (DIG)-labeled riboprobes against mouse VGLUT1 were made by our lab [62]. After hybridization overnight at 60℃ with the mixture of probes, the slides were washed with different stringency wash buffers at 65℃. Then, the slides were blocked with a blocking buffer prepared according to the manufacturer's protocol. Anti-DIG or anti-FITC antibodies conjugated to horseradish peroxidase (HRP) (Roche; 1:2000) were applied 2 hours at RT or overnight at 4℃ to detect respectively VGLUT1-DIG or CB_1-FITC probes. Probes hybridization was

revealed by a tyramide signal amplification (TSA) reaction using Cyanine 3-labeled tyramide (Perkin Elmer; 1:100 for 10 minutes) to detect VGLUT1 signal or FITC-conjugated tyramide (Perkin Elmer; 1:80 for 12 minutes) to amplify the signal of CB_1. The slides were incubated in 4',6-diamidino-2-phenylindole (DAPI; 1:20,000; Fisher Scientific) for 5 minutes. Then, slides were mounted with coverslips, visualized by Leica SP8 confocal microscope (Leica, Germany), and images were analyzed by Image J (NIH).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data collection and statistical analysis were performed using Microsoft Excel and GraphPad Prism 6 software. The dose-response experiments of i.c.v. administration of NaCl or Ang II and the body water composition data were analyzed by two-way analysis of variance (ANOVA). For the water intake tests with several time points (Figures 1-4), data were statistically analyzed by the two-way ANOVA of repeated measures. The data of the i.p. administration of different doses of NaCl and the plasma osmolality were analyzed by two-tailed Student's t-test. P values of ≤0.05 were considered statistically significant at a confidence interval of 95%. For detailed statistical analysis, see statistical table **(Table S1)**.

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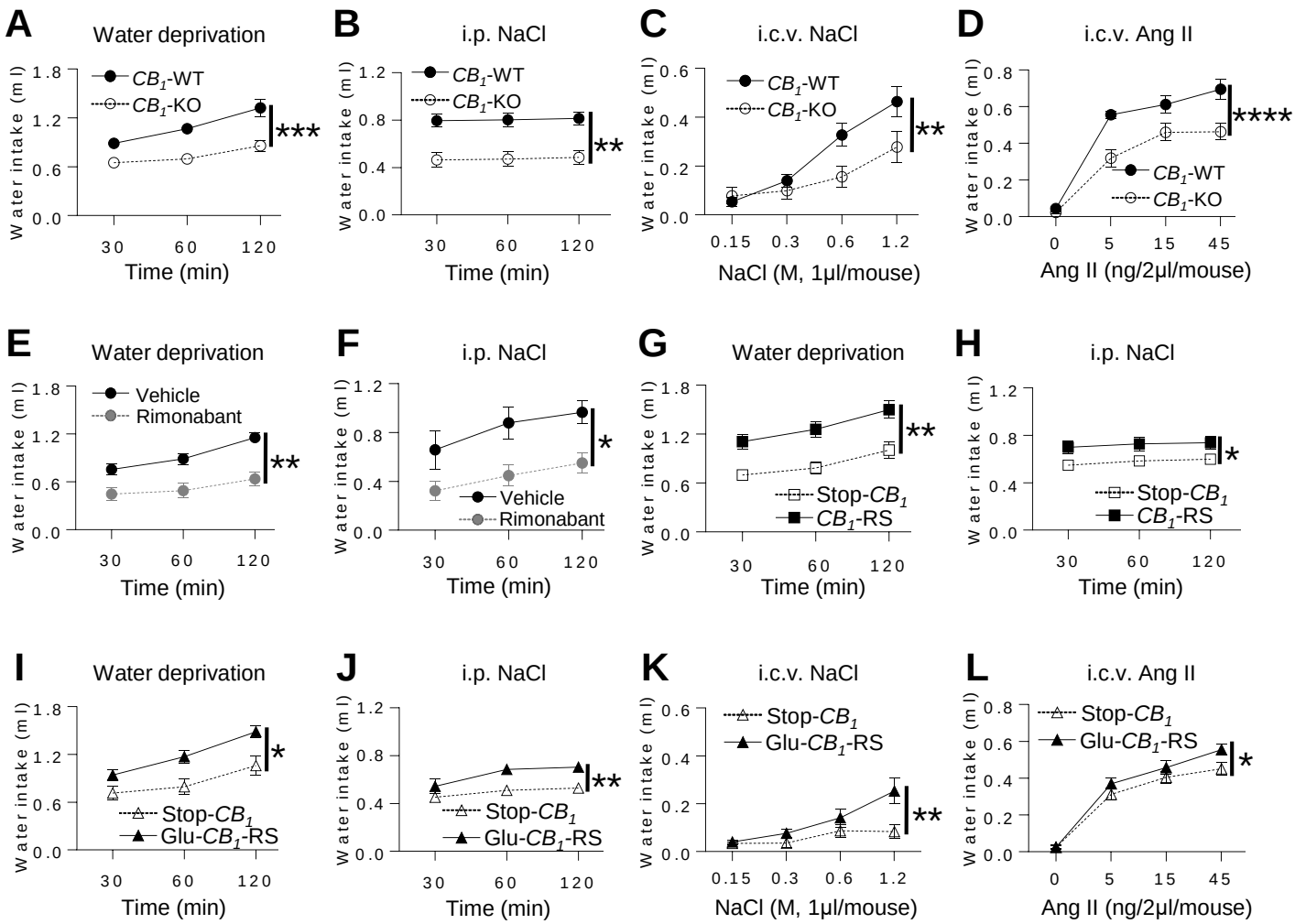
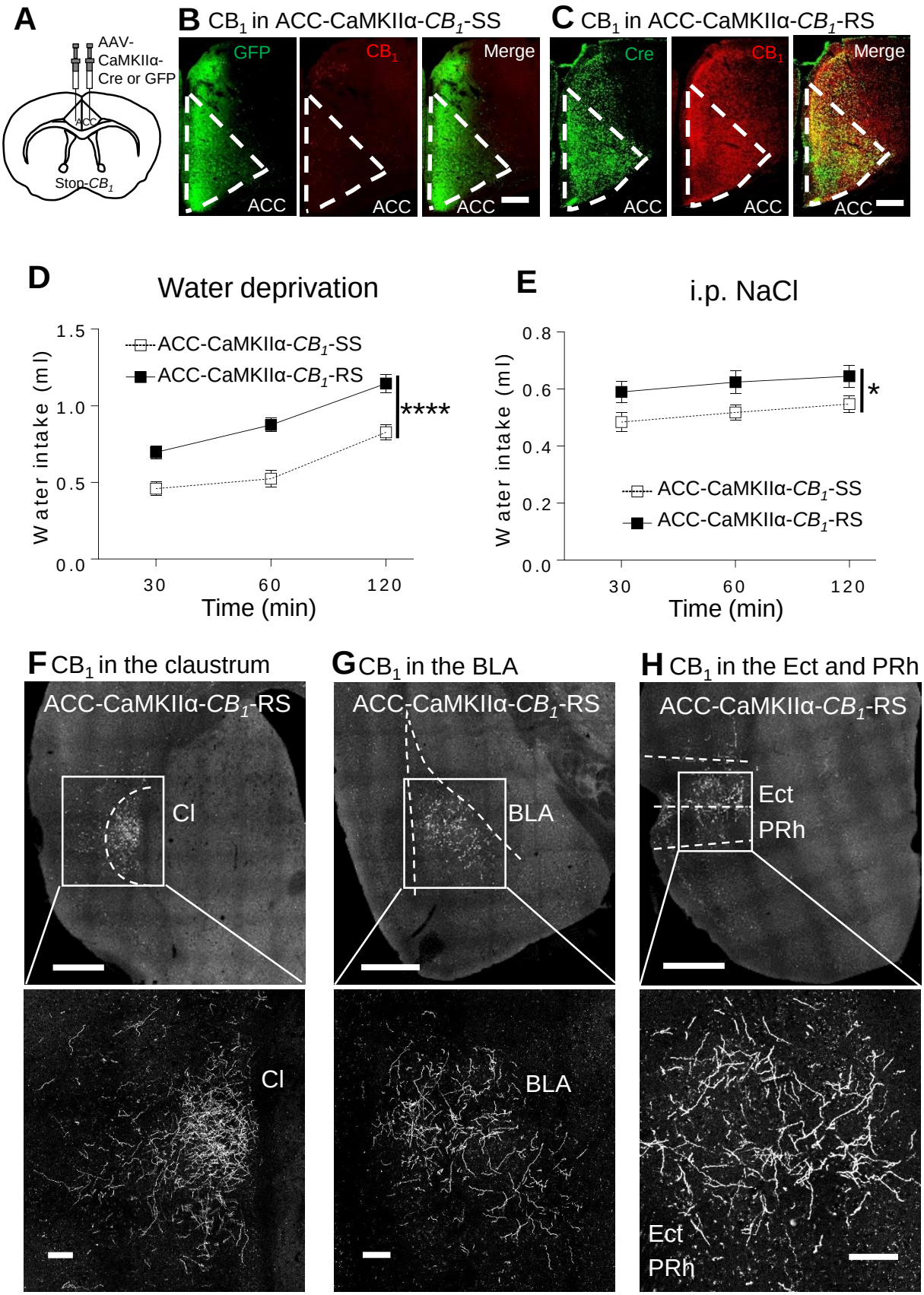
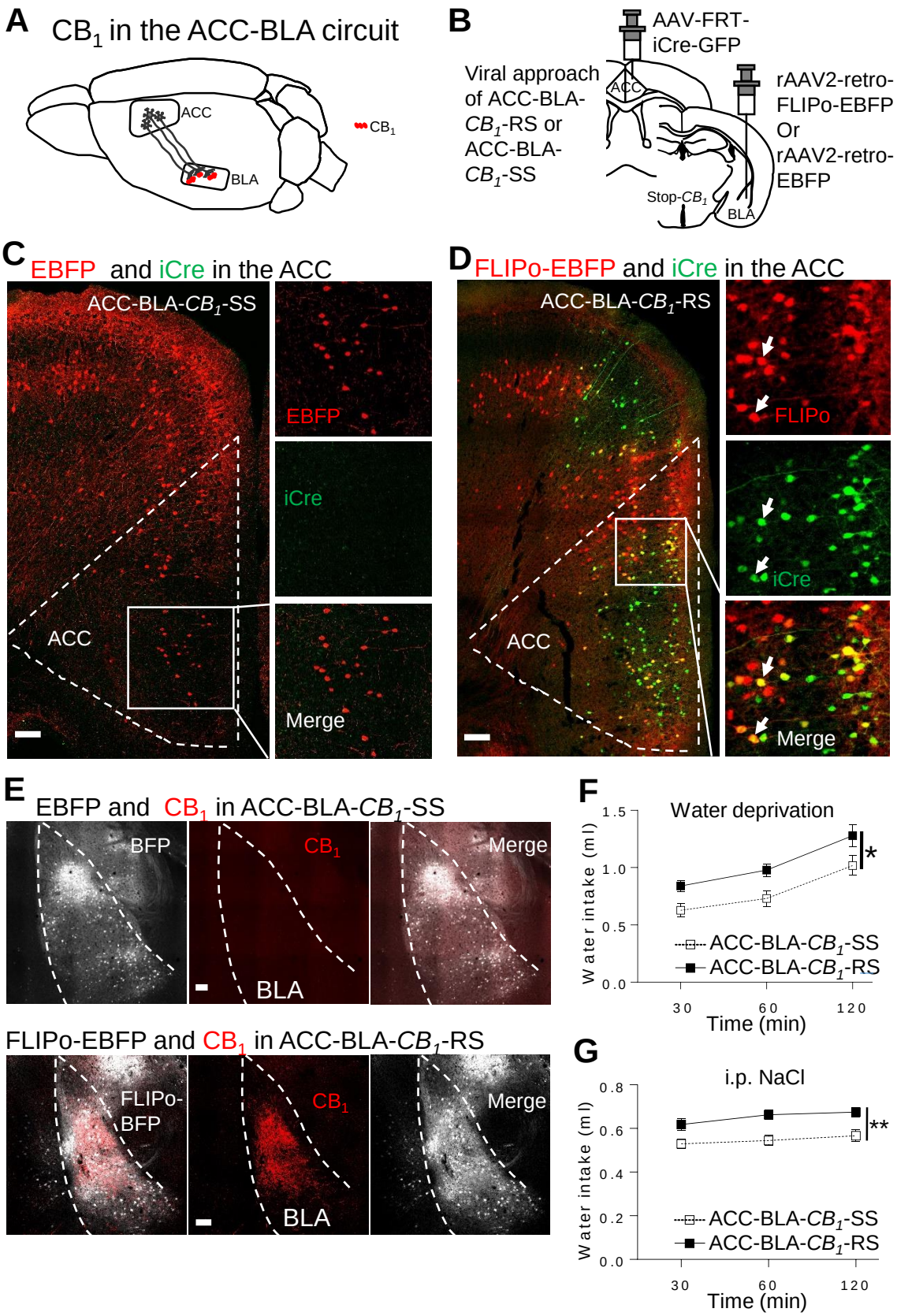
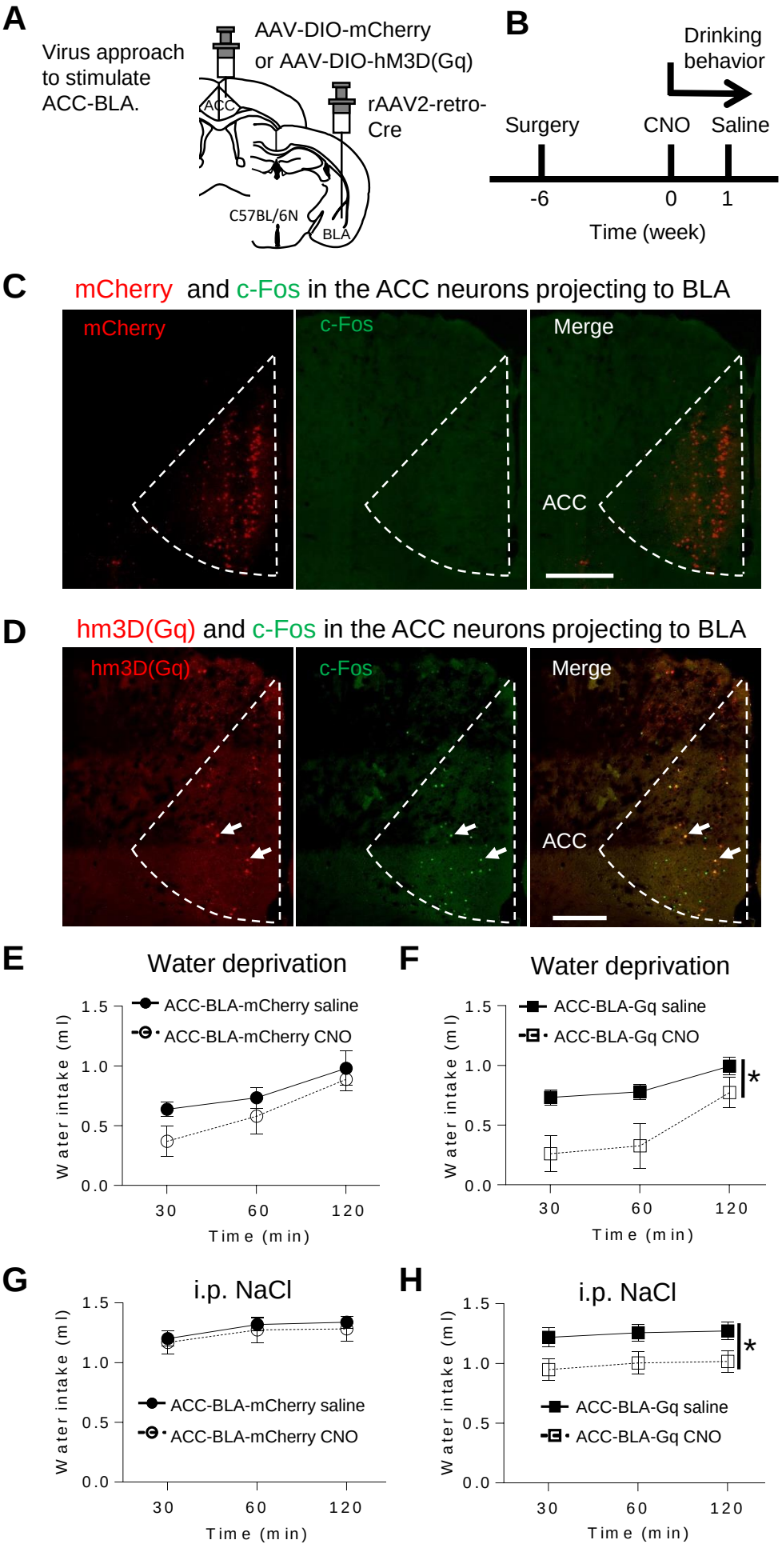

A
Water deprivation
Water intake (ml)
CB1-WT
CB1-KO
***
Time (min)
B
i.p. NaCl
**
C
i.c.v. NaCl
NaCl (M, 1µl/mouse)
D
i.c.v. Ang II
****
Ang II (ng/2µl/mouse)
E
Vehicle
Rimonabant
F
G
Stop-CB1
CB1-RS
H
I
Glu-CB1-RS
J
K
L


A
AAV-
CaMKIIα-
Cre or GFP
ACC
Stop-CB1
B CB1 in ACC-CaMKIIα-CB1-SS
GFP
CB1
Merge
ACC
C CB1 in ACC-CaMKIIα-CB1-RS
Cre
CB1
Merge
ACC
D
Water deprivation
ACC-CaMKIIα-CB1-SS
ACC-CaMKIIα-CB1-RS
Water intake (ml)
1.5
1.0
0.5
0.0
****
30
60
120
Time (min)
E
i.p. NaCl
ACC-CaMKIIα-CB1-SS
ACC-CaMKIIα-CB1-RS
Water intake (ml)
0.8
0.6
0.4
0.2
0.0
*
30
60
120
Time (min)
F CB1 in the claustrum
ACC-CaMKIIα-CB1-RS
Cl
Cl
G CB1 in the BLA
ACC-CaMKIIα-CB1-RS
BLA
BLA
H CB1 in the Ect and PRh
ACC-CaMKIIα-CB1-RS
Ect
PRh
Ect
PRh


A
CB1 in the ACC-BLA circuit
ACC
BLA
CB1
B
Viral approach of ACC-BLA-CB1-RS or ACC-BLA-CB1-SS
AAV-FRT-iCre-GFP
rAAV2-retro-FLIPo-EBFP Or rAAV2-retro-EBFP
ACC
Stop-CB1
BLA
C
EBFP and iCre in the ACC
ACC-BLA-CB1-SS
EBFP
iCre
Merge
ACC
D
FLIPo-EBFP and iCre in the ACC
ACC-BLA-CB1-RS
FLIPo
iCre
Merge
ACC
E
EBFP and CB1 in ACC-BLA-CB1-SS
BFP
CB1
Merge
BLA
FLIPo-EBFP and CB1 in ACC-BLA-CB1-RS
FLIPo-BFP
CB1
Merge
BLA
F
Water deprivation
Water intake (ml)
1.5
1.0
0.5
0.0
*
ACC-BLA-CB1-SS
ACC-BLA-CB1-RS
30
60
120
Time (min)
G
i.p. NaCl
Water intake (ml)
0.8
0.6
0.4
0.2
0.0
**
ACC-BLA-CB1-SS
ACC-BLA-CB1-RS
30
60
120
Time (min)

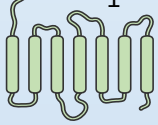

A
Virus approach to stimulate ACC-BLA.
AAV-DIO-mCherry
or AAV-DIO-hM3D(Gq)
rAAV2-retro-Cre
ACC
C57BL/6N
BLA
B
Drinking behavior
Surgery
CNO
Saline
-6
0
1
Time (week)
C
mCherry and c-Fos in the ACC neurons projecting to BLA
mCherry
c-Fos
Merge
ACC
D
hm3D(Gq) and c-Fos in the ACC neurons projecting to BLA
hm3D(Gq)
c-Fos
Merge
ACC
E
Water deprivation
ACC-BLA-mCherry saline
ACC-BLA-mCherry CNO
Water intake (ml)
1.5
1.0
0.5
0.0
30
60
120
Time (min)
F
Water deprivation
ACC-BLA-Gq saline
ACC-BLA-Gq CNO
*
G
i.p. NaCl
H
i.p. NaCl

Water restriction

Hypertonic saline

Water Intake

CB_1



Lack of CB_1

Re-expression of CB_1

Inhibition

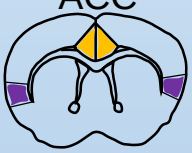
Facilitation

Cell types

Presence of CB_1 in Cortical Glutamatergic Neurons

Facilitation

ACC

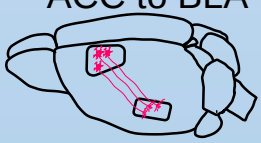


IC

Presence of CB_1 in ACC Glutamatergic Neurons

Facilitation

ACC to BLA



Presence of CB_1 in ACC to BLA Neurons

Stimulation of ACC to BLA Neurons

Facilitation

Inhibition