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RAPID REPORT

Modulation of the autonomic nervous system and behaviour by acute glial cell G_q protein-coupled receptor activation *in vivo*

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Key points

- The activation of glial G_q protein-coupled receptor (G_q-GPCR) signalling cascades broadly activates the autonomic nervous system
- The activation of glial G_q-GPCR signalling cascades affects activity-related behaviour.

Abstract: Glial fibrillary acidic protein (GFAP)-expressing cells ($GFAP^+$ glial cells) are the predominant cell type in the central and peripheral nervous systems. Our understanding of the role of $GFAP^+$ glial cells and their signalling systems *in vivo* is limited due to our inability to manipulate these cells and their receptors in a cell type-specific and non-invasive manner. To circumvent this limitation, we developed a transgenic mouse line (GFAP-hM3Dq mice) that expresses an engineered G_q protein-coupled receptor (G_q-GPCR) known as hM3Dq DREADD (designer receptor exclusively activated by designer drug) selectively in $GFAP^+$ glial cells. The hM3Dq receptor is activated solely by a pharmacologically inert, but bioavailable, ligand (clozapine-*N*-oxide; CNO), while being non-responsive to endogenous GPCR ligands. In GFAP-hM3Dq mice, CNO administration increased heart rate, blood pressure and saliva formation, as well as decreased body temperature, parameters that are controlled by the autonomic nervous system (ANS). Additionally, changes in activity-related behaviour and motor coordination were observed following CNO administration. Genetically blocking inositol 1,4,5-trisphosphate (IP_3)-dependent Ca^{2+} increases in astrocytes failed to interfere with CNO-mediated changes in ANS function, locomotor activity or motor coordination. Our findings reveal an unexpectedly broad role of $GFAP^+$ glial cells in modulating complex physiology and behaviour *in vivo* and suggest that these effects are not dependent on IP_3-dependent increases in astrocytic Ca^{2+}.

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Abbreviations ANS, autonomic nervous system; CNO, clozapine-*N*-oxide; DHPG, (*RS*)-3,5-dihydroxyphenylglycine; DREADD, designer receptor exclusively activated by designer drug; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; IP_3R2, inositol 1,4,5-trisphosphate receptor type 2; KO, knockout; LORR, loss of righting reflex; OGB-1, Oregon Green 488 BAPTA-1; PNS, peripheral nervous system; SR101, sulforhodamine101; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol.

C. Agulhon and K. M. Boyt contributed equally to this work.

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Introduction

Glial fibrillary acidic protein (GFAP)-expressing cells ($GFAP^+$ glial cells) comprise astrocytes in the CNS, and non-myelinating Schwann cells and satellite cells in the peripheral nervous system (PNS). They are closely associated with neuronal cell bodies, axons, synapses and microvasculature and express a large number of G protein-coupled receptors (GPCRs). Like other cell types, $GFAP^+$ glial cells express G_q, G_s and G_i-coupled GPCRs. Stimulation of $GFAP^+$ glial cell GPCRs activates a variety of signalling cascades; the most well studied is the elevation of intracellular Ca^{2+} through activation of G_q-GPCRs (Agulhon *et al.* 2008) and consequent stimulation of inositol 1,4,5-trisphosphate type 2 receptors (IP_3R2) (Petravicz *et al.* 2008). $GFAP^+$ glial cell G_q-GPCR activation in slice preparations has recently been implicated in several important neurophysiological functions, including the modulation of blood flow (Takano *et al.* 2006) and neuronal activity (Santello *et al.* 2012; Zorec *et al.* 2012). However, the roles of $GFAP^+$ glial cell G_q-GPCR signalling remain unresolved and controversial (Agulhon *et al.* 2008; Hamilton & Attwell, 2010; Nedergaard & Verkhratsky, 2012). A central limitation for addressing the role of these receptors has been the lack of tools to selectively activate $GFAP^+$ cell G_q-GPCR signalling cascades *in vivo*. We took advantage of an engineered G_q-coupled human M3 muscarinic receptor (hM3Dq) that does not respond to endogenous ligands, responds to an inert synthetic ligand (clozapine-*N*-oxide, CNO) that crosses the blood brain barrier and activates signalling cascades in a similar fashion to endogenous G_q-GPCRs (Armbruster *et al.* 2007). We created a transgenic mouse model expressing hM3Dq under the control of the GFAP promoter (GFAP-hM3Dq mice; Fig. 1*A*), and demonstrated that the selective activation of G_q-GPCR signalling cascades in $GFAP^+$ glial cells *in vivo* affects heart rate, blood pressure, saliva formation and body temperature, as well as activity-related behaviours and motor coordination. Interestingly, similar findings were observed in mice lacking IP_3-dependent Ca^{2+} responses in astrocytes. Overall, our findings demonstrate that $GFAP^+$ glial cell GPCR signalling has the potential to modulate complex physiology and behavior *in vivo*. The degree to which this occurs under physiological conditions will require conditional knockouts of $GFAP^+$ glial cell GPCR signalling in specific subsets of glia, experiments we plan to pursue in the near future.

Methods

Ethical approval

Protocols for animal care, behavioural studies and physiological measurements were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill. Isoflurane (1.5–2.0%) was used for anaesthesia and mice were killed by increasing the concentration of isoflurane to lethal levels and after respiratory failure performing cervical dislocation; this procedure was approved by our IACUC.

Maintenance and preparation of mouse lines

GFAP-hM3Dq mice were generated as follows: the coding sequence for the HA-tagged hM3Dq was cloned into the plasmid pTg1 (courtesy of R. Thresher, University of North Carolina at Chapel Hill (UNC-CH), USA). The 2.2kB human GFAP promoter (PGfa) from pGfa2Lac1 (courtesy of M. Brenner, University of Alabama at Birmingham, AL, USA) was placed upstream of the intron-HA-hM3Dq-pA sequence in pTg1 (Fig. 1*A*). This DNA construct was then injected into C3H/C57 hybrid embryos by the Animal Models Core Facility at UNC-CH. All tests were performed on N4 and N5 generational backcrossed C57BL/6J mice. GFAP-hM3Dq mice were back crossed with IP_3R2 knockout (KO) mice (Li *et al.* 2005) for at least two generations prior to testing. In all experiments, littermates were used for control mice. In the absence of treatment with CNO, there were no detectable differences between GFAP-hM3Dq, GFAP-hM3Dq::IP_3R2 KO mice and WT littermate mice in any parameter examined (grooming, exploration, social interactions, handling response, righting reflex, body weight or piloerection). Furthermore, GFAP-hM3Dq and GFAP-hM3Dq::IP_3R2 KO mice were born in the expected Mendelian ratios.

Western blot detection and immunohistochemical studies

For description of the methods, see Supplemental Material file.

Brain slice preparation

Coronal brain slices (300 μm thick) were prepared from GFAP-hM3Dq and WT littermate mice (4–8 weeks old; >3 mice per group) as previously described (Agulhon *et al.* 2010). Sulforhodamine101 (SR101; 1 μM) was included in the incubation buffer to distinguish astrocytes ($SR101^+$ cells) from neurons ($SR101^-$ cells) (Kafitz *et al.* 2008). Additional details can be found in the Supplemental Material.

OGB-1 Ca^{2+} dye bolus loading

We recorded Ca^{2+} changes and discriminated between Ca^{2+} elevations occurring in astrocytes *vs.* neurons by SR101 bulk loading and subsequent bolus injection of Oregon Green 488 BAPTA-1 Ca^{2+} dye (OGB-1) in acute slices (Agulhon *et al.* 2010). Additional details can be found in the Supplemental Materials.

Drugs and chemicals

(*RS*)-3,5-Dihydroxyphenylglycine (DHPG), histamine, carbachol and 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP) were obtained from Tocris Bioscience (Bristol, UK). OGB-1 Ca^{2+} dye was obtained from Invitrogen (Carlsbad, CA, USA). CNO was obtained from the National Institutes for Health and administered through an I.P. injection at 0.25 mg kg^{-1}.

Cardiovascular measurements

Recordings were made in anaesthetized mice (2% isoflurane) by the UNC Mouse Cardiovascular Models Core Facility via catheter insertion into the carotid artery and down into the ascending aorta. Additional details can be found in the Supplemental Materials.

Saliva formation measurements

Mice were anaesthetized with avertin and placed on a warming pad to maintain body temperature. Following saline or CNO injection, a piece of weighed filter paper was placed between incisor teeth. After 15 min, the paper was removed and re-weighed.

Body temperature measurements

Mice were physically restrained and body temperature was measured using a rectal probe. Mice were then injected with saline or CNO and body temperature was measured again 30 min post-injection.

Behavioural testing

Mice were maintained on a 12 h light/dark cycle. Transgenic and littermate control mice were tested between the ages of 3–7 months. Both males and females were included, with no sex difference found. In testing with observer scoring (rotarod, loss of righting reflex (LORR)), the observer was blind to genotype. All testing was conducted between 09:00 and 18:00 h.

Open field measurements

Exploratory activity in a novel environment was assessed for 3 h in a photocell-equipped automated open field (41 cm × 41 cm × 30 cm; Versamax system, Accuscan Instruments, Columbus, OH, USA) (Moy *et al.* 2007). Mice were injected with saline or 0.25 mg kg^{-1} CNO immediately prior to placement in the chamber. Activity chambers were contained inside a sound-attenuating box, equipped with house lights and fans. Activity data were obtained in 5 min bins.

Rotarod measurements

Mice were assessed for balance and motor coordination on an accelerating rotarod as described by others (Moy *et al.* 2007). Speed (r.p.m.) was set at an initial value of 3, with a progressive increase to a maximum of 30 r.p.m. during the 5 min test session. Latency to fall, or to rotate fully around the turning barrel, was measured by the rotarod timer. On day 1, naïve mice were given a training session consisting of three trials, with 45 s between each trial. Mice were injected with saline or CNO (0.25 mg kg^{-1}) 20 min prior to trial 1. Forty-eight hours later, mice were given two trials with saline injection only (day 2). Mice were then retested 48 h later and given saline or CNO 20 min prior to testing (day 3). On day 3, GFAP-hM3Dq and GFAP-hM3Dq::IP_3R2 KO mice given saline on day 1 were given CNO and GFAP-hM3Dq and GFAP-hM3Dq::IP_3R2 KO mice given CNO on day 1 were given saline.

Loss of righting reflex measurements

Mice were injected with saline or CNO (0.25 mg kg^{-1}), followed by the $GABA_A$ agonist THIP (25 mg kg^{-1}) 5 min later. Once mice showed signs of reduced motor activity, they were placed in v-shaped troughs. Mice were considered to lose righting reflex if they stayed on their backs for more than 30 s. If a mouse righted itself, it was replaced on its back. If the mouse righted itself twice within 30 s, it was scored as regaining righting reflex.

Statistics

Data were analysed using two-way analysis of variance (ANOVA) with genotype and treatment as factors or repeated-measures ANOVA. When appropriate, group means were compared using Tukey–Kramer *post hoc* analysis. For all comparisons, significance was set at $P < 0.05$.

Results

hM3Dq is restricted to GFAP$^+$ cells and is functional

We performed Western blot experiments to examine the tissue distribution of hM3Dq and found that hM3Dq was restricted to neural tissues such as brain and spinal cord, with no detectable expression in peripheral organs except for salivary glands which include closely associated autonomic ganglia (Fig. 1*B*). We next examined the spatial and cellular expression of hM3Dq by immunohistochemistry and found that hM3Dq was detected throughout the CNS, with most intense levels of expression in the thalamus, amygdala, hypothalamus, brainstem, cerebellum and spinal cord (Fig. 1*C*, Supplementary Figs 1, 2 and 9; data not shown). Expression of hM3Dq was observed in astrocytes (Fig. 1*C*, Supplementary Figs 1 and 2) with no detectable expression in neurons (Fig. 1*D*,

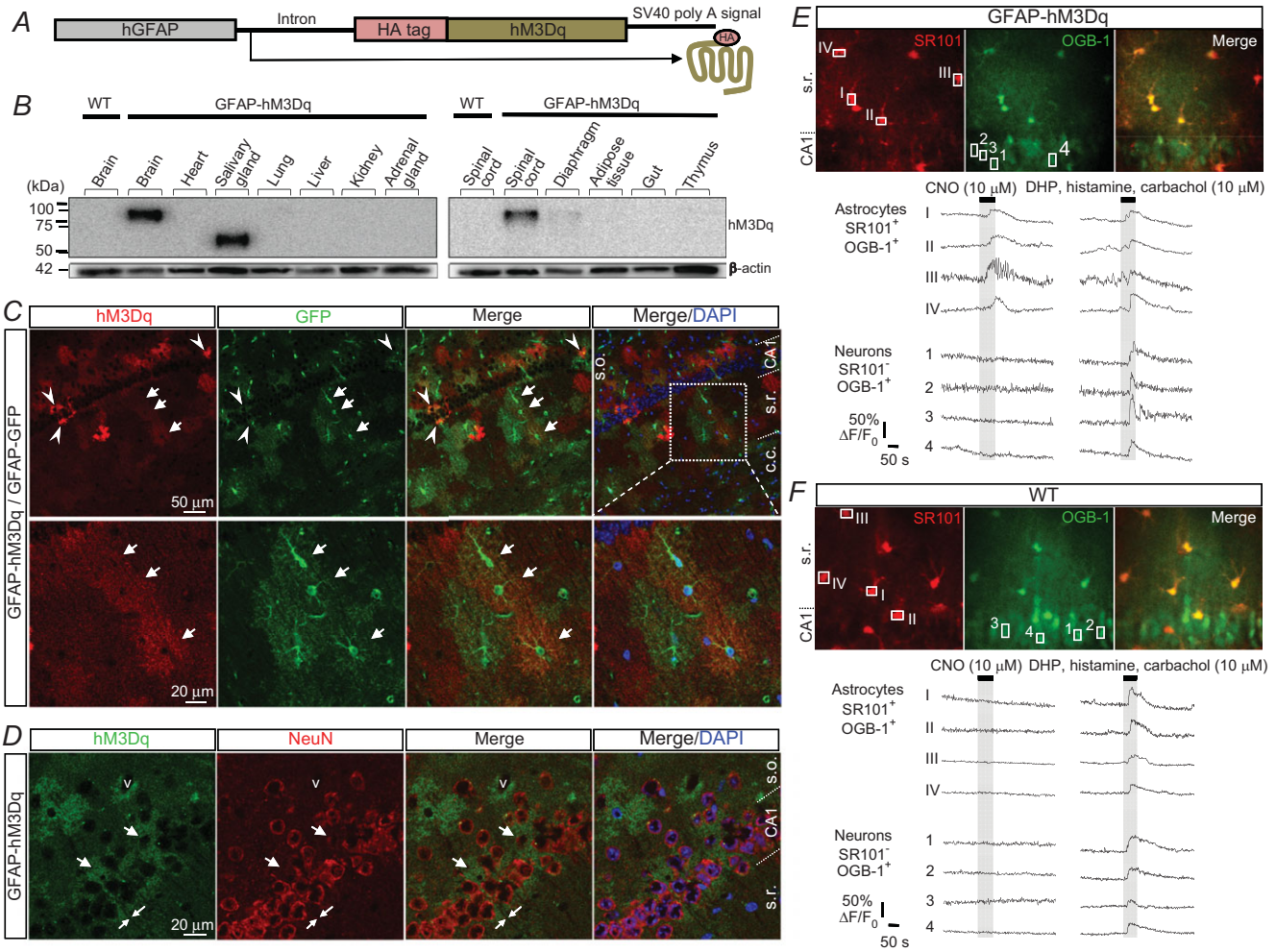


Figure 1. GFAP$^+$ glial cell-selective hM3Dq expression and function in GFAP-hM3Dq mice
A, schematic map of transgenic construct containing HA epitope tagged (HA)-hM3Dq driven by 2.2 kb human GFAP promoter (hGFAP). *B*, Western blots showing the pattern of expression of HA-tagged hM3Dq protein in neural and non-neural tissues of GFAP-hM3Dq *vs.* WT mice. Note that the salivary gland homogenate probably contained autonomic ganglia that are closely associated with salivary glands. The size of the hM3Dq in salivary glands is ~65 kDa, which is the expected size of this receptor (Armbruster *et al.* 2007). In brain and spinal cord, the size is larger, suggesting glycosylation of the receptor. *C*, immunohistochemistry for HA-tagged hM3Dq protein showing HA immunoreactivity (red) overlapping with green fluorescent protein (GFP) staining (green) in highly ramified astrocytic processes in CA1 region of hippocampus of GFAP-hM3Dq/GFAP-GFP double transgenic mice. Arrowheads denote dense astrocytic processes expressing higher levels of hM3Dq (red) in a subpopulation of astrocytes. Arrows point to astrocytes expressing moderate levels of hM3Dq. *C*, *lower panel*, higher magnification images of the region delimited by the white box (*upper panel*). *D*, HA-tagged hM3Dq immunoreactivity (green) was not detected in NeuN-expressing cell bodies of CA1 pyramidal neurons (red) in GFAP-hM3Dq mice. Small arrows denote hM3Dq staining (green) in ramified astrocytic processes that envelope an unstained CA1 neuron proximal dendrite. *E and F*, cells loaded with the astrocytic marker SR101 (red), Ca^{2+} indicator OGB-1 (green) and merged SR101 and OGB-1 (*upper*). Lower panels show time courses of CNO- and cocktail-mediated Ca^{2+} responses ($\Delta F/F_0$) in the astrocytes (I–IV) and neurons (1–4) outlined in the upper panels. Confocal Ca^{2+} imaging showed that hM3Dq is functional and gives Ca^{2+} increases following CNO application in astrocytes but not in neurons of GFAP-hM3Dq mice (*E*, *lower panel*). No responses were evoked in astrocytes or neurons of WT littermate control mice (*F*, *lower panel*). A cocktail (DHPG, histamine and carbachol) of ligands to endogenous G_q-GPCRs was used as a positive control to ensure cell viability. Abbreviations in *C–F*: CA1, CA1 pyramidal neurons; c.c., corpus callosum; s.o., stratum oriens; s.r., stratum radiatum; v, vessel.

Supplementary Fig. 3) or other glial cell types, such as nerve/glial 2 (NG2)-positive glia (Supplementary Fig. 4). In the PNS, hM3Dq was also observed in non-myelinating Schwann cells of peripheral nerves as well as satellite cells surrounding ganglionic neurons (Supplementary Figs 5–7), as expected for a GFAP-driven transgene.

To assess whether hM3Dq was functional and coupled to G_q in GFAP$^+$ glial cells, we performed confocal Ca^{2+} imaging experiments in acute CA1 hippocampal slices, a well-characterized model system that has been used extensively in neurophysiology. Slices were loaded with the specific astrocytic marker SR101 (Nimmerjahn *et al.* 2004) and Ca^{2+} indicator OGB-1 to simultaneously monitor Ca^{2+} elevations in astrocytes *vs.* neurons. In slices obtained from GFAP-hM3Dq mice, the vast majority (95.51%) of SR101$^+$ astrocytes responded to bath applied 10 μM CNO with Ca^{2+} increases ($n = 156$ cells, $n = 25$ slices, $n = 9$ mice, Fig. 1*E*). Importantly, no Ca^{2+} increases were detected in 99.4% of CA1 pyramidal neurons ($n = 319$ cells, Fig. 1*E*). Furthermore, no CNO-mediated Ca^{2+} responses were observed in astrocytes ($n = 90$ cells) or CA1 neurons ($n = 88$ cells) of WT littermate control mice, while a G_q-GPCR cocktail reliably triggered robust Ca^{2+} increases in 100% of these cells ($n = 10$ slices, $n = 4$ mice, Fig. 1*F*). Importantly, both the amplitude and the time course of Ca^{2+} responses following activation of hM3Dq or endogenous G_q-GPCRs were similar following treatment with CNO or neuroligand cocktail, respectively. The immunohistochemical studies showed only a moderate level of hM3Dq expression in the hippocampus with a number of cells exhibiting no detectable hM3Dq expression (Fig. 1C and Supplementary Fig. 1); the observation that 95.5% of hippocampal astrocytes responded to CNO indicates that almost all of the astrocytes in this region expressed hM3Dq, albeit at levels marginally detectable by immunohistochemical methods. Conversely, the fact that no CNO-induced Ca^{2+} increases were observed in CA1 pyramidal neurons (Fig. 1*E*) effectively rules out the possibility that CA1 neurons express hM3Dq. These results confirm that CNO does not activate any endogenous astrocytic or neuronal G_q-GPCRs, and that hM3Dq is not expressed in neurons of GFAP-hM3Dq mice (Fig. 1*D* and *E*, Supplementary Figs 3, 6B and 9). Taken together, the anatomical and functional results validate the use of GFAP-hM3Dq mice for examining the role of GFAP$^+$ glial cell G_q-GPCR signalling cascades in physiology and behaviour.

Stimulation of hM3Dq in GFAP$^+$ glial cells *in vivo* affects autonomic nervous system (ANS) activity

The activation of GFAP$^+$ glial cell G_q-GPCR signalling *in vivo* via I.P. injection of CNO resulted in the modulation of several functions controlled by the ANS. First, cardiovascular function in GFAP-hM3Dq mice was markedly affected by CNO administration, resulting in an approximately 22% increase in heart rate (genotype: $F_{3,12} = 11.71$, $P = 0.0009$; genotype over time: $F_{45,180} = 3.08$, $P < 0.0001$) as well as ∼33 and ∼31% increases in systolic blood pressure (genotype: $F_{3,12} = 9.63$, $P = 0.0016$; genotype over time: $F_{48,192} = 7.57$, $P < 0.0001$) and diastolic blood pressure (genotype: $F_{3,12} = 6.92$, $P = 0.0069$; genotype over time: $F_{48,192} = 4.77$, $P < 0.0001$), respectively (Fig. 2*A*–*C*). Heart rate and blood pressure were not continuously monitored for longer than 30 min to avoid the confounding effects associated with prolonged surgical procedures under anaesthesia, according to the UNC Mouse Cardiovascular Models Core Facility's standard protocol. However, using a non-invasive system to monitor heart rate and pulse distension, we found that the effects on the cardiovascular system persisted for at least 3 h (Supplemental Fig. 10). Because mice were anaesthetized and blood oxygen levels maintained above 95%, increases in blood pressure and heart rate were probably not mediated by changes in oxygenation due to respiration. Second, a large increase in saliva secretion from 0.17 ± 0.05 to 22.5 ± 1.6 mg over a 15 min period (Fig. 2*D*) was observed ($P < 0.05$). Third, while housed at room temperature (∼23°C), the body temperature of GFAP-hM3Dq mice dropped from 38.4 ± 0.3 to 35.6 ± 0.5°C within 30 min of CNO injection ($P < 0.001$; Fig. 2*E*). These effects were long lasting, only returning to baseline after ∼3 h (data not shown). Previous work from our laboratory (Petravicz *et al.* 2008) and others (Di Castro *et al.* 2011; Takata *et al.* 2011) has demonstrated that astrocytic G_q-GPCR-dependent Ca^{2+} responses are obliterated via a KO of IP_3R2. Experiments were performed to confirm that the absence of IP_3R2 also ablated CNO-mediated increases in astrocytic Ca^{2+}. Neither 10 μM CNO nor a cocktail of G_q-GPCR agonists evoked detectable astrocytic Ca^{2+} responses when brain slices were obtained from GFAP-hM3Dq::IP_3R2 KO mice (0/60 astrocytes from 7 hippocampal brain slices, 2 mice). In contrast, 90.3% (56/62 astrocytes) and 93.6% (58/62 astrocytes) of hippocampal astrocytes responded to 10 μM CNO or a cocktail of G_q-GPCR agonists, respectively, when derived from GFAP-hM3Dq positive littermate control mice (8 slices, 2 mice). Experiments were performed to determine if IP_3R2-dependent Ca^{2+} responses in astrocytes were required for the responses observed following CNO administration to GFAP-hM3Dq mice. Aside from heart rate, GFAP-hM3Dq::IP_3R2 KO mice exhibited similar autonomic responses to CNO as GFAP-hM3Dq mice (Fig. 2). The reason that activating glial signalling in GFAP-hM3Dq::IP_3R2 KO mice led to a greater increase in heart rate relative to GFAP-hM3Dq mice is currently under investigation.

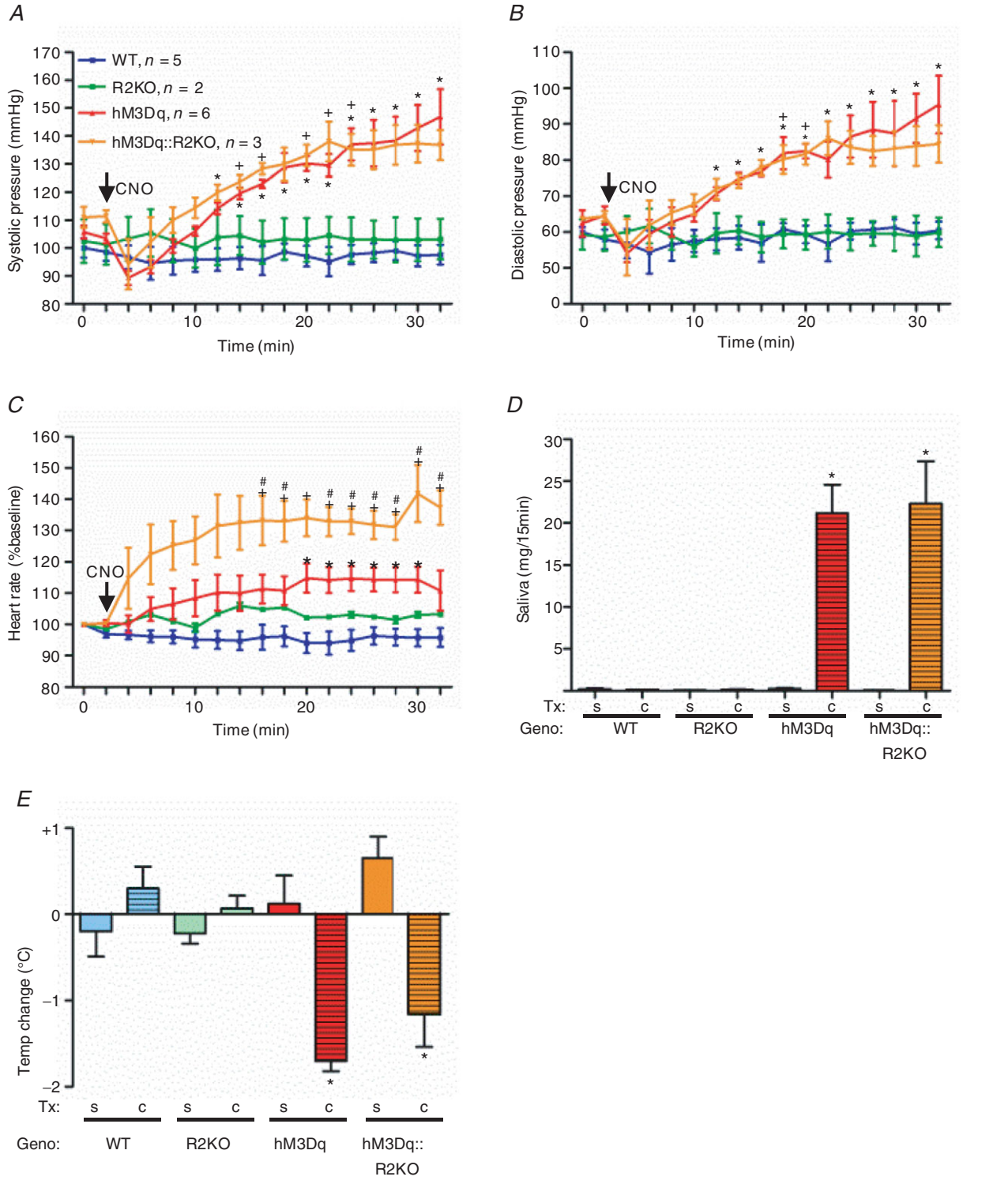


Figure 2. Effects of hM3Dq activation on cardiovascular function, saliva production and thermal homeostasis

A–C, significant increases in heart rate and blood pressure in CNO-treated GFAP-hM3Dq and GFAP-hM3Dq::IP_3R2 KO mice relative to littermate controls. $^*P < 0.05$ GFAP-hM3Dq *vs.* WT; $+P < 0.05$ GFAP-hM3Dq::R2 KO *vs.* R2 KO; $\#P < 0.05$ GFAP-hM3Dq *vs.* GFAP-hM3Dq::R2 KO. *D*, saliva produced over 15 min measured in GFAP-hM3Dq, GFAP-hM3Dq::IP_3R2 KO and littermate controls treated with saline (S) or CNO (C). *E*, body temperature changes measured 30 min after treatment with CNO (C) or saline (S) in GFAP-hM3Dq, GFAP-hM3Dq::IP_3R2 KO and littermate controls. hM3Dq refers to GFAP-hM3Dq mice and R2KO refers to IP_3R2 KO mice. Error bars, SEM. $^*P < 0.05$ compared to control groups.

Stimulation of hM3Dq in GFAP$^+$ glial cells *in vivo* affects activity-related behaviour

We next examined whether activation of GFAP$^+$ glial cells affected locomotor activity. Mice were placed into an open field activity chamber immediately after an I.P. injection of saline or CNO and spontaneous locomotor activity was monitored over a 3 h period. Compared to control treatment groups, CNO-treated GFAP-hM3Dq mice exhibited a decrease in horizontal activity, total distance travelled, distance travelled in the centre area and time spent in the centre area during the initial ∼40 min of testing (Fig. 3*A*, *C*, *E* and *G*). During the remainder of the testing period (60–180 min after CNO administration), there was a small increase in horizontal activity and centre distance travelled, and a striking increase in centre time (Fig. 3*A*, *E* and *G*). The difference in locomotor activity was significant based on genotype and treatment over time (horizontal activity: $F_{105,3010} = 5.46$, $P < 0.0001$; total distance: $F_{105,3010} = 2.86$, $P < 0.0001$; centre distance: $F_{105,3010} = 5.79$, $P < 0.0001$; centre time: $F_{105,3010} = 7.13$, $P < 0.0001$). In addition, there was a significant effect of genotype and treatment on centre time ($F_{3,86} = 15.19$, $P < 0.0001$). CNO elicited similar changes in GFAP-hM3Dq mice and GFAP-hM3Dq::IP$_3$R2 KO mice where astrocytic Ca^{2+} responses to G_q-GPCRs are obliterated (genotype and treatment over time: horizontal activity $F_{105,2345} = 6.61$, $P < 0.0001$; total distance $F_{105,2345} = 4.93$, $P < 0.0001$; centre distance $F_{105,2345} = 5.14$, $P < 0.0001$; centre time $F_{105,2345} = 2.92$, $P < 0.0001$; genotype and treatment effect: total distance $F_{3,67} = 2.83$, $P < 0.05$; centre time $F_{3,67} = 11.56$, $P < 0.0001$; Fig. 3*B*, *D*, *F* and *H*). Interestingly, while the effects of CNO on activity returned to baseline in most activity tests after 3 h, in GFAP-hM3Dq::IP$_3$R2 KO mice centre time remained markedly elevated 3 h after CNO injection. Overall, these data provide behavioural evidence that the activation of G_q-GPCR signalling in GFAP$^+$ glia can lead to biphasic and relatively long-lasting changes in locomotor activity.

Stimulation of hM3Dq in GFAP$^+$ glial cells *in vivo* affects motor coordination without affecting motor learning or memory

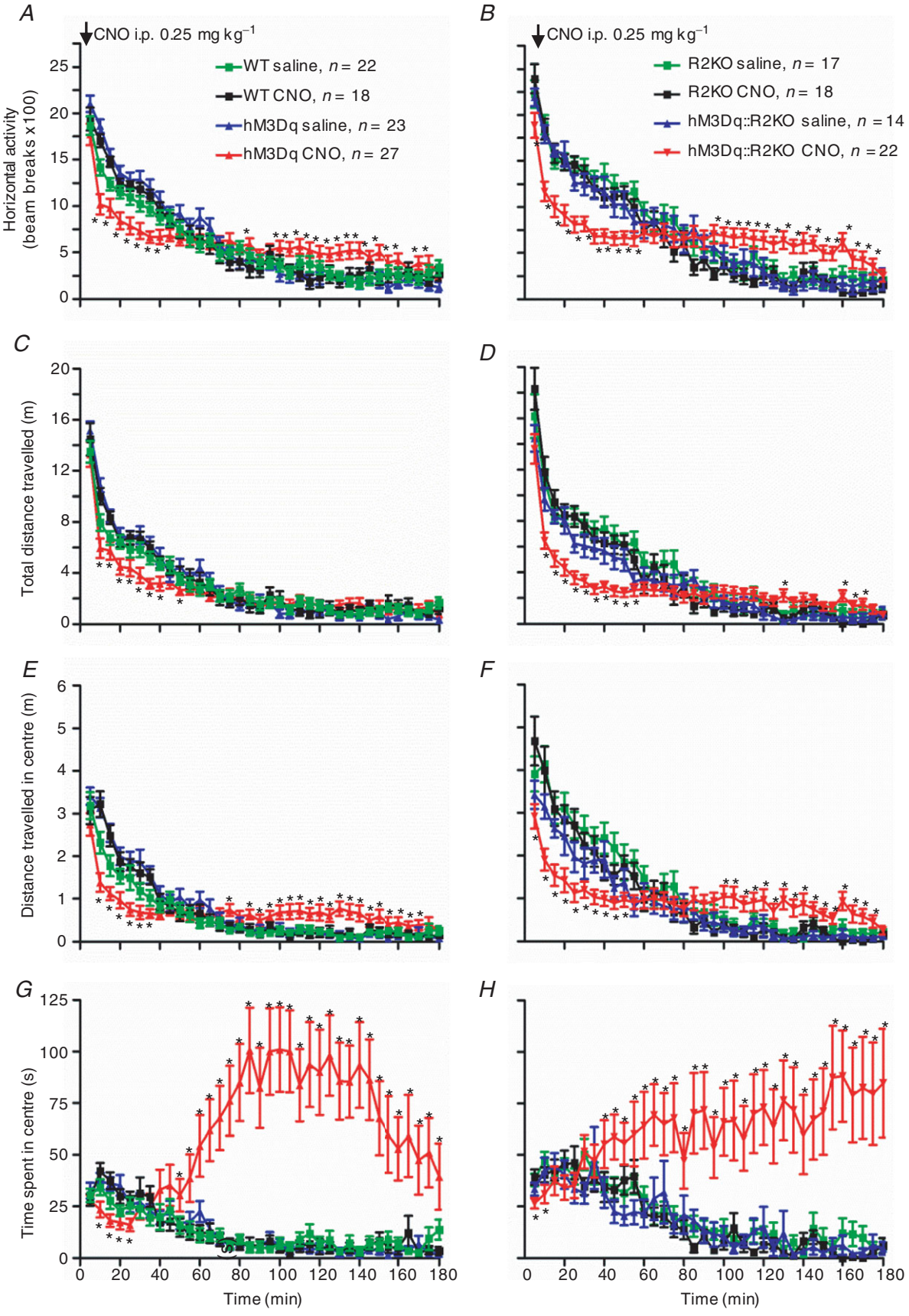
To further assess the influence of G_q-GPCR signalling in GFAP$^+$ glial cells in motor function, motor coordination was measured by performance on an accelerating rotarod. On day 1, mice were injected with either CNO or saline 20 min prior to start of training. Although CNO-treated GFAP-hM3Dq mice performed similarly to control groups on trial 1, they failed to significantly improve their performance over trials 2 and 3 relative to control groups (Fig. 4*A*), suggesting defects in motor coordination or motor learning (treatment effect for hM3Dq $F_{1,31} = 13.29$, $P = 0.001$). On day 2, all groups received saline treatment and showed a similar high level of performance including the GFAP-hM3Dq mice initially treated with CNO on day 1 (Fig. 4*B*). These results support the interpretation that the reduced performance on day 1 of CNO-treated GFAP-hM3Dq mice was due to altered motor coordination rather than a deficit in motor learning or memory. This was confirmed on day 3 by a decrease in motor performance toward baseline level when GFAP-hM3Dq mice were given CNO (Fig. 4*C*). GFAP-hM3Dq::IP$_3$R2 KO mice responded similarly to GFAP-hM3Dq mice, indicating that responses to CNO were not dependent on IP$_3$-dependent increases in astrocytic Ca^{2+} (Fig. 4*A*–*C*; day 1 treatment effect for hM3Dq::R2 KO $F_{1,20} = 58.49$, $P < 0.0001$; day 3 genotype effect $F_{3,83} = 6.97$, $P = 0.0003$; treatment effect $F_{1,83} = 14.80$, $P = 0.0002$; genotype by treatment effect $F_{3,83} = 4.78$, $P = 0.004$).

Stimulation of hM3Dq in GFAP$^+$ glial cells potentiates GABA-induced sedation

The decrease in locomotor activity observed in the activity chambers during the first 40 min following hM3Dq stimulation as well as the decrease in motor coordination observed in the rotarod performance study suggests that the activation of G_q-GPCR signalling in GFAP$^+$ glia may have mild sedative effects. To test this hypothesis, we measured LORR, a test that has been widely used to assess the sedative/hypnotic effects of neuroactive compounds (Barbaccia *et al.* 2005). Administration of CNO alone failed to induce LORR in GFAP-hM3Dq mice (data not shown). However, injection of the GABA$_A$ agonist THIP at a concentration that failed to induce LORR in combination with either saline in GFAP-hM3Dq mice or CNO in WT mice led to a large increase in LORR when co-administered with CNO in GFAP-hM3Dq mice (51.1 ± 4.8 min, Fig. 4*D*) relative to controls (0.38 ± 0.3 min, Fig. 4*D*). Similar findings were observed following injection of CNO into GFAP-hM3Dq::IP$_3$R2 KO mice (Fig. 4*D*; genotype effect $F_{3,42} = 22.74$, $P < 0.0001$; treatment effect $F_{1,42} = 57.87$, $P < 0.0001$; genotype by treatment effect $F_{3,42} = 20.39$, $P < 0.0001$).

Discussion

In this study, we have created a new mouse model that enables the selective activation of G_q-GPCR signalling in CNS GFAP$^+$ glial cells (astrocytes), and PNS GFAP$^+$ glial cells (non-myelinating Schwann cells and satellite cells). To make this mouse, an engineered receptor (hM3Dq DREADD (designer receptor exclusively activated by designer drug)) was expressed under the control of the GFAP promoter; this receptor is activated only by



an otherwise inert ligand, CNO. Extensive immunohistochemical screening indicated that the expression of hM3Dq is restricted to astrocytes within the CNS, and non-myelinating Schwann cells and satellite cells within the PNS, with no detectable expression in other types of glial cells and neurons. Using confocal Ca^{2+} imaging, we verified (i) that hM3Dq is functional in CNS astrocytes and (ii) that no Ca^{2+} increases in neighbouring neurons were induced by CNO. This second observation confirms our immunohistological data, and rules out the possibility that neurons express hM3Dq, as the Ca^{2+} imaging is much more sensitive than immunohistological staining for detection of receptor expression. CNO-induced activation of G_q-coupled pathways in $GFAP^+$ glial cells of CNS and PNS resulted in striking modulation of ANS, motor and righting reflex functions. Our study broadens the conventional view by showing that $GFAP^+$ glial cells may be implicated in autonomic and motor functions upon acute stimulation, and provides insight into potential roles of $GFAP^+$ glial cell G_q-GPCR signalling cascades.

Activating hM3Dq in $GFAP^+$ glial cells induced large changes in autonomic activity, as well as biphasic changes in activity-related behaviour and defects in motor coordination. Although the full extent of each of these phenotypes is probably due to the combined activation of $GFAP^+$ glial cells in several areas of the CNS and/or PNS, hM3Dq in some specific regions might be primarily responsible for the phenotypes observed. Remarkably, the highest levels of hM3Dq expression were found in certain areas of the ANS, such as regions of the brainstem (Supplementary Fig. 8), pons and hypothalamus in the CNS, and the sympathetic and parasympathetic ganglia and nerves in the PNS. This supports the idea that stimulating $GFAP^+$ glial cells of the ANS may contribute to modulation of heart rate, blood pressure, salivation and thermal homeostasis, functions known to be controlled by the ANS (Guyenet, 2006). Even more specifically, the high expression level of hM3Dq in the satellite cells of the parasympathetic ganglia in the salivary glands provides evidence of a potential role of these cells in salivation.

The activation of astrocytic signalling led to prolonged effects not generally predicted following the activation of G_q-GPCR signalling cascades: changes in heart rate, blood pressure, saliva formation, body temperature, and locomotor activity lasted ∼2–3 h; and LORR lasted ∼ 1 h. This is interesting in light of reports indicating that following a single injection, CNO is rapidly removed from the brain (Bender *et al.* 1994). These findings, together with the observation that the responses appear to be Ca^{2+} independent, suggest that brief activation of astrocytes leads to prolonged modulation of neuronal activity unlike that typically described for GPCR-mediated astrocyte-neuronal interactions (Martineau *et al.* 2006; D'Ascenzo *et al.* 2007; Santello *et al.* 2011). Prolonged astrocyte–neuronal interactions could result from the activation of processes affected by protein kinase C-dependent phosphorylation (e.g. glutamate transporters (Kalandadze *et al.* 2002)) or $\beta\gamma$-dependent activation of signalling cascades (Smrcka, 2008). The GFAP promoter drives transgene expression in astrocytes as well as peripheral, non-myelinating glia. Consequently, we cannot rule out the possibility that certain of the changes observed following the activation of G_q-GPCR signaling cascades are due to the activation of signalling in peripheral $GFAP^+$ glia. However, trospium chloride, an impermeable muscarinic acetylcholine receptor antagonist, blocked CNO-induced saliva formation without affecting CNO-induced LORR, suggesting that CNO-induced LORR is mediated by central mechanisms (Supplemental Fig. 11).

Currently, there is controversy in the literature concerning whether astrocytes release gliotransmitters in response to GPCR-mediated increases in intracellular Ca^{2+} (Agulhon *et al.* 2010; Hamilton & Attwell, 2010; Nedergaard & Verkhratsky, 2012). The findings presented here indicate that the activation of G_q-GPCR signalling cascades in $GFAP^+$ glia modulate neuronal activity in IP_3R2 KO mice which lack G_q-GPCR dependent increases in astrocytic Ca^{2+} (Petravicz *et al.* 2008). Most studies to date suggest that gliotransmission is initiated by increases in astrocytic Ca^{2+} (Verkhratsky *et al.* 2012; Zorec *et al.* 2012). These findings coupled with the long duration of physiological and behavioural changes observed following the activation of hM3Dq DREADD in $GFAP^+$ glia suggest that alternative modes of glial modulation of neuronal activity need to be considered.

One of the strengths of the GFAP-hM3Dq mouse model is that it allows global stimulation of hM3Dq in all $GFAP^+$ glial cell types, providing an idea of the breadth of physiology and behaviour potentially modulated through the activation of G_q-GPCR signalling cascades in these cells. Teasing apart the areas of the nervous system as well as the cellular effectors and mechanisms acting downstream of $GFAP^+$ glial cell G_q-GPCR to modulate ANS and motor functions will be an important next step. Although this is beyond the scope of the present work, further studies using local infusions of CNO in specific areas of the CNS and PNS *in vivo* will provide answers

Figure 3. Activation of hM3Dq signalling affects activity-related behaviour
Locomotor activity measured in the open field chamber. Mice were injected with saline or CNO immediately before placing in the chamber. hM3Dq refers to GFAP-hM3Dq mice and R2KO refers to IP_3R2 KO mice. $^*P < 0.05$ for GFAP-hM3Dq or GFAP-hM3Dq::R2 KO injected with CNO *vs*. littermate controls.

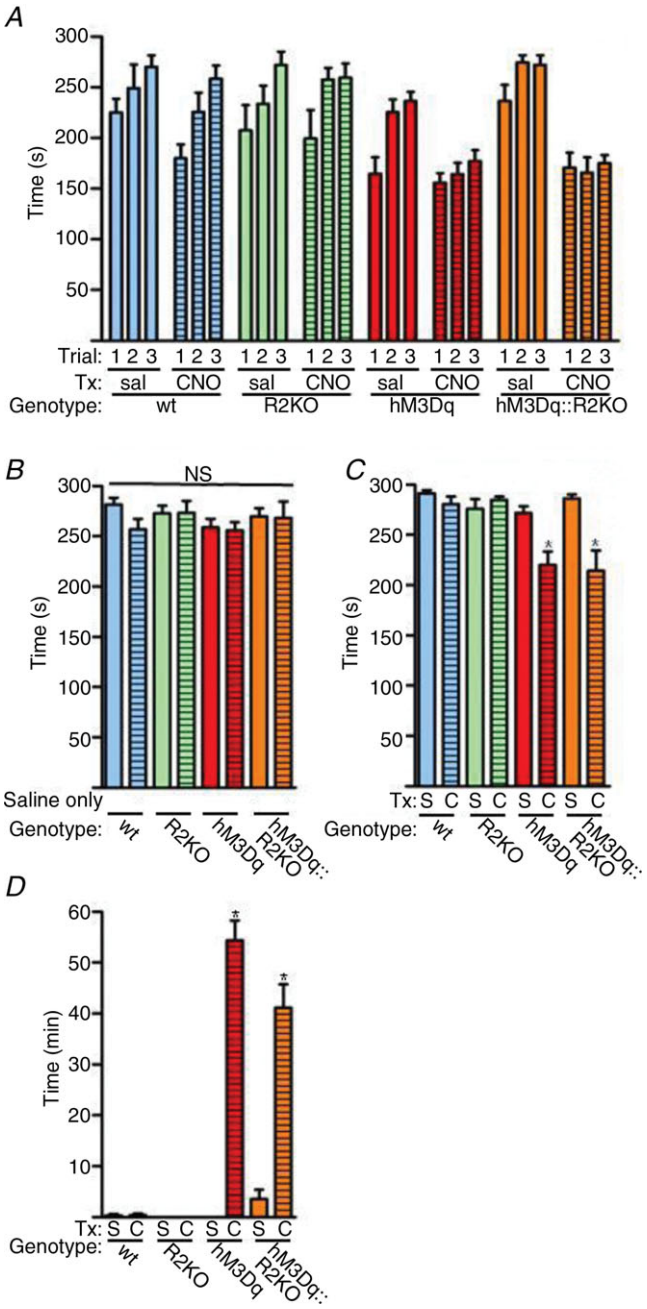


Figure 4. Activation of hM3Dq signalling in GFAP$^+$ cells affects motor coordination, but not motor learning, and induces sedation
A, in contrast to controls, GFAP-hM3Dq and GFAP-hM3Dq::IP$_3$R2 KO mice injected with CNO failed to improve their rotarod scores between trials 1 and 3 on day 1 of testing. *B*, on day 2 of testing, in the absence of CNO, GFAP-hM3Dq and GFAP-hM3Dq::IP$_3$R2 KO mice performed similarly to control mice. *C*, on day 3 of testing, CNO depressed the rotarod performance of GFAP-hM3Dq and GFAP-hM3Dq::IP$_3$R2 KO mice that were given saline on day 1 (S = saline; C = CNO). *D*, LORR was measured in GFAP-hM3Dq, GFAP-hM3Dq::IP$_3$R2 KO and littermate control mice after CNO or saline injection in the presence of the GABA$_A$ agonist THIP (25 mg kg^{-1}; a dose that alone failed to induce LORR) administration. $^*P < 0.05$.

to these questions, and pave the way for dissecting the specific GFAP$^+$ glial cell types that are implicated in the modulation of autonomic or motor functions. The use of the DREADD technology has recently led to several important discoveries in neuronal functions, demonstrating the power of this technology *in vivo* (Alexander *et al.* 2009; Ferguson *et al.* 2011; Krashes *et al.* 2011; Atasoy *et al.* 2012; Garner *et al.* 2012; Kozorovitskiy *et al.* 2012; Vrontou *et al.* 2013). To our knowledge, this is the first application of this technology to study the role of GFAP$^+$ glial cell signalling *in vivo*. The phenotypes observed in the CNO-treated GFAP-hM3Dq mouse model open doors to a myriad of new investigations, which have the potential to significantly impact our understanding of the role of glia in physiology and behaviour.

In conclusion, our findings demonstrate potential roles for GFAP$^+$ glial cells in autonomic and motor functions. Although caution should be exercised in extrapolation of our findings to other systems and human physiology or pathology, they provide evidence for an active role of GFAP$^+$ glial cells *in vivo*, and a new framework for elucidating the key glial G$_q$-GPCR-mediated signal transduction pathways involved in physiology and behaviour. GPCRs are a principal target of many therapeutic agents, and it is conceivable that certain medications act, at least in part, through the modulation of GFAP$^+$ glial cell GPCR activity. Consequently, our findings might also be of significance for understanding and improving the action of medications that may act through the modulation of GFAP$^+$ glial cell GPCRs.

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Additional Information

Competing interests

None.

Author contributions

K.B., A.X.X. and K.D.M. designed and performed physiology and behavioural experiments. C.A., F.F. and A.X.X. designed and performed Western blot, immunocytochemical and calcium imaging experiments. C.A., K.B., A.X.X. and K.D.M. analysed the data. B.L.R. provided the hM3Dq construct and advice on DREADD technology. C.A., K.B. and K.D.M. wrote the paper. All authors discussed the results and commented on the manuscript.

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