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ORIGINAL ARTICLE

Balance of Go1α and Go2α expression regulates motor function via the striatal dopaminergic system

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Abstract

The heterotrimeric G-protein Go with its splice variants, Go1α and Go2α, seems to be involved in the regulation of motor function but isoform-specific effects are still unclear. We found that Go1α-/- knockouts performed worse on the rota-rod than Go2α-/- and wild-type (WT) mice. In Go1+2α-/- mice motor function was partially recovered. Furthermore, Go1+2α-/- mice showed an increased spontaneous motor activity. Compared to wild types or Go2α-/- mice, Go1+2α-/- mice developed increased behavioural sensitization following repetitive cocaine treatment, but failed to develop conditioned place preference. Analysis of dopamine concentration and expression of D1 and D2 receptors unravelled splice-variant-specific imbalances in the striatal dopaminergic system: In Go1α-/- mice dopamine concentration and vesicular monoamine uptake were increased compared to wild types. The expression of the D2 receptor was higher in Go1α-/- compared to wild type littermates, but unchanged in Go2α-/- mice. Deletion of both Go1α and Go2α re-established both dopamine and D2 receptor levels comparable to those in the wild-type. Cocaine treatment had no effect on the ratio of D1 receptor to D2 receptor in Go1+2α-/- mutants, but decreased this ratio in Go2α-/- mice. Finally, we observed that the deletion of Go1α led to a threefold higher striatal expression of Go2α. Taken together our data suggest that a balance in the expression of Go1α and Go2α sustains normal motor function. Deletion of either splice variant results in divergent behavioural and molecular alterations in the striatal dopaminergic system. Deletion of both splice variants partially restores the behavioural and molecular changes.

Keywords: behavioural sensitization, cocaine, conditioned place preference, D1-D2 receptor signalling, Goα splice variants, motor function.

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Dopaminergic neurotransmission is crucial for a variety of brain functions including drug-induced reward and sensitization, cognition and regulation of mood, as well as motor control. Dopamine and dopamine receptor-mediated signalling in the striatum play key roles in regulating motor activity (Calabresi *et al.* 2014; Keeler *et al.* 2014). Information processing in the basal ganglia occurs via the direct and indirect pathways. According to this model, projection neurons in the striatum can be divided into two subpopulations, depending on their neurochemical properties and

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Abbreviations used: CPP, conditioned place preference; D1R or D2R, dopamine 1 or 2 receptor; ko, knockout; MAO, monoamine oxidase; VMAT, vesicular monoamine transporter; wt, wild type.

projection targets. Medium spiny neurons (MSN) of the direct pathway are positively modulated by dopaminergic input via D1-receptors (D1R), MSN of the indirect pathway are negatively modulated via D2-receptors (D2R). The conceptualization of a movement promoting direct and a movement inhibiting indirect pathway is still common, though challenged by recent findings such as the finding that both pathways are concurrently active during movement (Keeler *et al.* 2014).

Besides the dopaminergic input, MSNs of both pathways inhibit each other with a stronger inhibitory connectivity from MSNs of the indirect pathway onto those of the direct pathway. This mutual inhibitory connectivity establishes a bistable competitive interaction between the two pathways, which can be shifted depending on dopamine concentrations in the striatum (Calabresi *et al.* 2014; Bahuguna *et al.* 2015). Because of the higher affinity of D2R, lower dopamine concentrations will suppress MSNs of the indirect pathway thereby resulting in preponderance of the direct pathway.

Dopaminergic neurotransmission and neuroplasticity within the cortico-basal ganglia-thalamic circuits is critical for drug-induced sensitization, reward processing and as such is relevant for the development of addictive behaviour (Spanagel and Weiss 1999; Lobo and Nestler 2011; Yager *et al.* 2015). Repeated intermittent exposure to psychostimulants leads to an augmented motor-stimulant response to these drugs. This phenomenon is called behavioural sensitization and can be assessed by monitoring motor activity. Beside involvement of brain areas responsible for movement initiation, the ventral tegmental area, nucleus accumbens and the medial prefrontal cortex have been implicated in behavioural sensitization (Steketee and Kalivas 2011). Drug reward can be measured by the conditioned place preference (CPP) paradigm (Tzschentke 2007). Both D1R- and D2R-mediated signalling have been reported to be important for the emergence of CPP (Steketee and Kalivas 2011).

Goα-subunits of the heterotrimeric G-protein class Go have been linked to the dopaminergic system and to the regulation of motor function. Go1α and Go2α are splice variants and are abundantly expressed throughout the brain where they couple to a variety of receptors like dopamine, muscarine, opioid and somatostatin receptors (Jiang and Bajpayee 2009). Previous studies revealed that Go1+2α-/- mice exhibit an impaired motor control and are hyperactive (Jiang *et al.* 1998). The ability of guanosine triphosphate (GTP) to promote a shift in the affinity of the D2R for dopamine is absent in brains of Go1+2α-/- mutants but unchanged in Go2α-/- mice (Jiang *et al.* 2001; Brunk *et al.* 2008; Calabresi *et al.* 2014) indicating that central nervous D2R preferentially couple to Go1α subunits. The splice variant Go2α has been shown to be a regulator of dopamine homeostasis. Deletion of Go2α selectively affects vesicular monoamine storage by directly sensing the vesicular monoamine content in neuroendocrine cells and neurons with no obvious involvement of heptahelical receptors (Ahnert-Hilger *et al.* 2003). In addition, motor activity following cocaine and amphetamine treatment, is changed by unbalancing signalling via D1R and D2R in Go2α-/- mice (Brunk *et al.* 2008, 2010). The striatal concentration of dopamine is lower in Go2α-/- compared to wild-type (WT) mice and these knockouts do not develop motor sensitization during repetitive applications of cocaine. These findings are accompanied by alterations in the expression of D1R and D2R and of other proteins like tyrosine hydroxylase and the vesicular monoamine transporter 2 involved in dopaminergic signalling (Brunk *et al.* 2008). So both Goα splice variants appear to be involved in the regulation of motor function but their interrelation and specific effects are still unclear.

In this study, we analysed the differential role of Go1α and Go2α in striatum with respect to monoamine concentrations, vesicular uptake and expression of D1R and D2R, in relation to motor function and cocaine-mediated effects. Our results indicate that Go1α and Go2α have antagonistic effects at the cellular and behavioural level in the striatal dopaminergic system.

Materials and methods

Antibodies

The following antibodies were used: Monoclonal mouse antibodies raised against both Goα splice variants (Synaptic Systems Cat# 271 111, RRID:AB_2619996), specifically recognizing Go2α (Synaptic Systems Cat# 271 011, RRID:AB_2619995), synaptophysin (Synaptic Systems Cat# 101 011C5, RRID:AB_887823), synaptobrevin (Synaptic Systems Cat# 104 211, RRID:AB_887811) (Winter *et al.* 2005; Brunk *et al.* 2010) and specifically detecting Go1α (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cat# sc-13532, RRID:AB_2111645) (Brunk *et al.* 2008). Further antibodies include rabbit antibodies against Gsα (Santa Cruz Biotechnology Cat# sc-823, RRID:AB_631538), Golfα (Santa Cruz Biotechnology Cat# sc-385, RRID:AB_10160576), tyrosine hydroxylase (Merck KGaA, Darmstadt, Germany; Cat# AB152, RRID:AB_390204), dopamine plasma membrane transporter (DAT, Merck KGaA Cat# AB2231, RRID:AB_1586991) and mouse monoclonal antibodies against the D2R (Santa Cruz Biotechnology Cat# sc-5303, RRID: AB_668816), the D1R (Merck KGaA Cat# MAB5290, RRID: AB_2094841) and actin (Sigma-Aldrich, St. Louis, MO, USA Cat# A5060, RRID:AB_476738).

Secondary antibodies conjugated to horseradish peroxidase include horse anti-mouse (Vector Laboratories, Burlingame, CA, USA; Cat# PI-2000, RRID:AB_2336177) and goat anti-rabbit (Vector Laboratories Cat# PI-1000, RRID:AB_2336198).

G-protein deletion mutants

Go1+2α-/- (NIE/Birnb Goalpha 129SV/C57BJ/6) and Go1α-/- (NIE/Birnb Go1alpha 129SV/C57BJ/6) or Go2α-/- (NIE/Birnb Go2alpha 129SV/C57BJ/6) splice variant-specific mutants were

obtained from the lab of Lutz Birnbaumer, bred in the local animal facility (no RRID available) and genotyped as described (Jiang *et al.* 1998; Dhingra *et al.* 2002). For our studies, mutant and WT littermates were obtained by interbreeding of heterozygous parents. All experimental procedures were approved by the Committee on Animal Care and Use and were carried out in accordance with the local Animal Welfare Act taking into account the impairments of the individual mutants (internal numbers Go1α: 0896/1162; Go2α: 0949/1162; Go1+2α: 0897/1162; T0119/11). For biochemical studies, mice of both sex were included as we did not observe gender specific differences. All behavioural studies were obtained with male animals of the same age using the same or sister litters with no extra blinding.

Behavioural studies

Male WT and mutant mice (minimum 8 weeks old) were maintained on a light–dark cycle (12–12 h) under controlled temperature (21 ± 2°C) and humidity (50 ± 5%) conditions. The studies were not pre-registered. For all studies, mice were single housed and received *ad libitum* access to food and water. Behavioural testing was performed during the light phase of the light/dark cycle. All experiments were conducted in accordance with the EU guidelines for the care and use of laboratory animals and were approved by the local animal care committee (Regierungspräsidium Karlsruhe, Karlsruhe, Germany). The experimental flow for both tests is given in Fig. 1. No randomization was performed; animals were arbitrarily assigned to the rota-rod and to the motor sensitization/CPP experiments. As a pre-defined inclusion criterion, only animals older than 18 days were used.

Rota-rod performance test

WT and mutant mice, originating from the Go1α-/, Go2α-/- and Go1+2α-/- line (20–24 days old), were set on a rotating rod (Rozas *et al.* 1997). The body weights for WT were 10–11 g, for Go2α 11–12 g and for both Go1α and Go1+2α 6–7 g.

The rotating rod (home-made Charité) accelerates in speed from three to 50 rpm during a time course of 3 min. The performance in this test depends on motor activity, balance and coordination. Mice underwent one training round before being subjected to the final experiment. All mice managed to cling at least to the rod and this behaviour was included in the measurement until the end of the 3 min rotation or before the mice faa (see below). No animals were excluded. For the exact evaluation of the performance, mice were videotaped (digital-camcorder by Panasonic, Kadoma, Osaka, Japan). Three different parameters were analysed: The time mice spent on the rod while it is rotating, the time mice performed a first spin by clinging to the rotating rod and the overall number of spins mice performed on the rotating rod until they fall down or the test ended (Fig. 1).

Cocaine-induced behavioural sensitization and development of conditioned place preference (CPP)

The procedure of cocaine-induced CPP was adapted from our original description (Abarca *et al.* 2002; Brunk *et al.* 2008; Bilbao *et al.* 2014). The CPP paradigm consisted of three different phases: pre-conditioning, conditioning and drug-free test. The weight of the mice was as follows: Go1+2α 27.65 ± 2.18 g; WT 33.63 ± 0.95 g. For the pre-conditioning, the mice were injected with saline and immediately placed in the conditioning boxes for 20 min and allowed to explore the apparatus. During conditioning

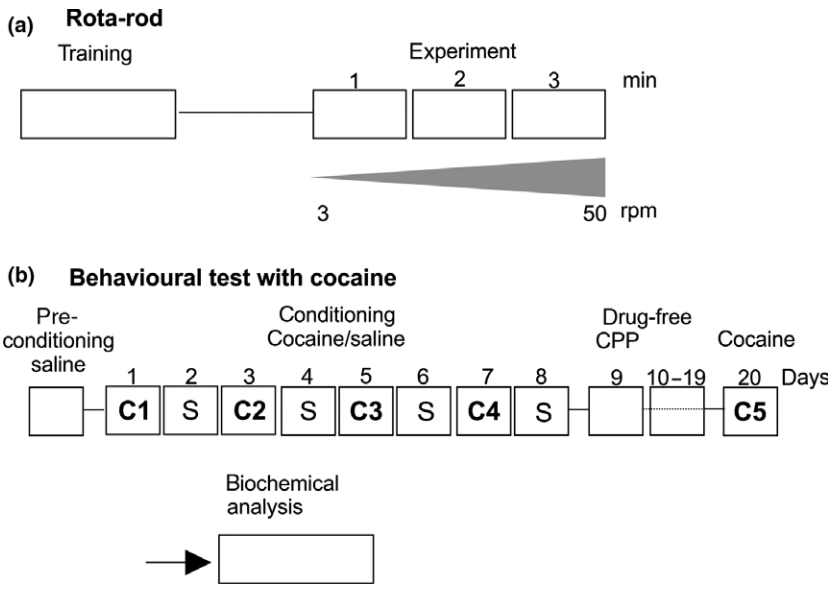

Fig. 1 Flowchart of rot rod or behavioural sensitization/ conditioned place preference (CPP) experiments. (a) The mice received a training at the day of the experiment before they were subjected to the rotating rod for maximal 3 min. Between 21 (WT) and 6–7 (mutants) mice were used see Fig. 2. (b) Following a pre-conditioning phase, the mice received alternating injections of either cocaine (days 1, 3, 5, 7, referred as C1 2, 3, 4) for 8 days. At day nine CPP was performed. Then the mice underwent a drug-free period of 11 days before at day 20 they received a final cocaine injection (C5). At the end of the test, the mice were killed and their brains were used for biochemical analyses see Fig. 6 and Table 3. Between 26 (WT) and 10 (mutants) mice were used see Fig. 3. For rota-rod, only animals of postnatal age of 20–24 days were used to allow for a comparison of all three mutants. Cocaine-induced behaviour was analysed by a second set of animals about 2–3 months old, which could not include Go1α-/- mice owing to their reduced life span.

phase, mice were treated for 8 days with daily alternating injections of cocaine (Cocaine hydrochloride, Sigma-Aldrich Chemie GmbH, Munich, Germany, 10 mg/kg, i.p.) or saline. The CPP apparatus consisted of a box with two equally sized compartments interconnected by a rectangular corridor. The compartments were differentiated by the motifs painted on the walls (dots or stripes), the colour (different shade of grey tones, light or dark) and texture (smooth or rough) of the floor (Panlab, Harvard Apparatus, Spain, boxes LE895 76-0728). All sessions were monitored by a video-tracking system (Ethovision 2.0, Noldus License number: EVPR20-99120008), which enabled us to determine locomotion and spatial placement of each mouse every 0.2 s across the whole session. Then, mice were confined into the corresponding compartment immediately after the injection for 30 min. For the expression or drug-free test, the mice were allowed to explore the whole apparatus without any treatment on day 9. The CPP score calculated represents the difference between the time spent (seconds) in the cocaine or saline-paired floor during the test day. During the CPP procedure, the effects of repeated cocaine injections on locomotion were assessed by comparing the distance travelled during the first and fourth drug-paired and the first non-drug-paired trials, and the expression of behavioural sensitization was assessed 11 days after the last cocaine injection. Mice were killed immediately after the end of the test (day 20) Their brains were collected, frozen on dry ice and stored at −80°C for further biochemical analyses (Fig. 1).

Subcellular fractionation

To obtain synaptic vesicles (SVs), mouse brains were homogenized (Wheaton, Potter-Elvejhem, clearance 0.1–0.15 mm) in ice-cold buffer (320 mM sucrose, 4 mM HEPES/KOH, pH 7.4, 1 mM Phenylmethylsulfonyl fluoride (PMSF), and 1 µl/mL protease inhibitor cocktail (Pi, Sigma-Aldrich)) and centrifuged for 10 min at 4°C and 1300 *g* (Beckman rotor TLA-100.4). The resulting supernatant 1 was centrifuged again at 14 000 *g* for 15 min, yielding a synaptosome-containing pellet (P2).The synaptosomes were osmotically shocked by diluting them 1:10 in lysis buffer (10 mM HEPES/KOH, pH 7.4, 1 mM PMSF and 1 µl/mL protease inhibitors and mechanically disrupted by three strokes at 2000 rpm (Potter-Elvejhem, clearance 0.1–0.15_m). SVs were collected by two sequential centrifugation steps at 29 000 *g* (20 min) and the resulting supernatant then centrifuged at 350 000 *g* (30 min) (Huttner *et al.* 1983; Becher *et al.* 1999).

Immunoblot analysis

Samples from brain fractions were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and western blot techniques using the ECL detection system (GE Healthcare, Munich, Germany). For signal quantification, ECL-processed films were scanned and protein bands were densitometrically measured using the Labimage 1D program (KAPELAN, Halle, Germany) ensuring that signals were in the linear range of the ECL detection system. Comparative quantification of protein samples from WT mice and deletion mutants was performed in the same gel using actin, synaptophysin or synaptobrevin as internal controls.

HPLC analysis

Littermates of the Go1α-/- and Go1+2α-/- line were decapitated and their brains removed at the age of P20 and P15 respectively. After removal, the brains were immediately frozen on dry ice and kept at −80°C until use. The striatum was dissected on a cold plate (−15°C). The samples were then weighed and stored at −80°C again until they were used for homogenization. After adding 10–20 volumes of deionized water, frozen samples were homogenized by ultrasonication at 4°C. An equal volume of 0.2 N perchloric acid was added to the homogenates, which were then centrifuged for 10 min at 25 000 *g* and 4°C. Monoamines were determined in the supernatant using high performance liquid chromatography with electrochemical detection as given. Briefly, Purospher® RP-18 (5 µm) LiChroCART® 125-4 (Merck 150142) was used with a flow rate of 1 mL/min. Serotonin was analysed using a mobile phase of 0.1 M sodium acetate, 0.1 mM EDTA, 0.35 mM heptane sulphonic acid, 5% methanol at a pH at 4.5. Dopamine was analysed using a mobile phase of 0.1 M sodium phosphate, 1 mM EDTA, 0.75 mM octane sulphonic acid, 5% methanol adjusted to pH 5. The standards for DA and 5-HT were purchased from Sigma (Hortnagl *et al.* 1993).

Monoamine oxidase activity assay

The monoamine oxidase activity (type A and B) was determined with the Amplex® Red Monoamine oxidase assay Kit by Molecular Probes, Invitrogen, Germany.

Monoamine uptake

Monoamine uptake by synaptic vesicles derived from wt, Go1α-/- and Go1+2α-/- mice was performed using 5-Hydroxy-[3H] tryptamine trifluoroacetate (specific activity, 4.33 TBq/mmol) purchased from Amersham Biosciences. Briefly SVs were resuspended in potassium glutamate (KG) buffer (150 mM KG; 20 mM 1,4-piperazinediethanesulphonic acid; 4 mM EGTA; 2.9 mM $MgCl_2$, equivalent to 1 mM free Mg^{2+} and 2 mM ATP, adjusted to pH 7.0 with KOH). Uptake was started by adding KG-ATP buffer, supplemented with 1 mM ascorbic acid and 40 nM [^{3}H]serotonin or [^{3}H]dopamine supplemented with or without reserpine (6 µM) to determine non-specific uptake. Incubation (10 min at 25°C) was stopped by addition of 500 µl of ice-cold KG buffer followed by rapid centrifugation at 440 000 *g* for 10 min at 4°C. The pellets were lysed in 0.4% Triton X-100 to determine radioactivity by liquid scintillation counting and protein content using the bicinchoninic acid method (Brunk *et al.* 2008).

Data analysis

Samples were run in triplicates for biochemical experiments and experiments were repeated at least four times. The behavioural data were analysed by Student's *t* test (Excel) and one- or two-way ANOVAS, with a repeated measures factor when necessary, followed by Newman–Keul's post hoc tests, when appropriate (Graph Pad Prism). For the rota-rod data no assessment of normality has been performed. For biochemical data, the Kruskal–Wallis test and the Mann–Whitney *U*-test, (IBM SPSS Statistics) for independent samples were applied; both tests are valid also for data without normal distribution. All values are given as mean ± SEM or SD and statistical significance was set at $p < 0.05$. No sample calculation was performed to predetermine the sample size. No test for outliers was conducted on the data.

Results

Motor function of WT, Go2α-/-, Go1α-/- and Go1+2α-/- mice

To investigate the specific role of Goα and its splice variants, Go1α and Go2α, and their interrelation regarding motor function we first compared motor performance on the rota-rod of all deletion mutants and their WT littermates. In the rota-rod test, three different parameters were recorded: (i) total time spent on the rod, (ii) the time mice were running on the rod until the first spin occurred and (iii) number of spins while the mice were running on the rod during the 3 min course. There was no difference regarding any of the three parameters for Go2α-/- mice when compared to the WT mice (Fig. 2a–c), indicating an unaltered spontaneous motor activity of Go2α-/- mutants as described previously (Brunk *et al.* 2008). Performance of Go1α-/- and Go1+2α-/- mice was significantly worse regarding all three parameters. The average time WT and Go2α-/- mice stayed on the rotating rod was longer than for Go1α-/- and Go1+2α-/- mice, whereby Go1α-/- mice spent significantly less time on the rod when compared to Go1+2α-/- animals (Fig. 2a). The time until the first spin occurred was significantly shorter in Go1α-/- and Go1+2α-/- mice compared to both WT and Go2α-/- mutants. Again Go1α-/- mice spent significantly less time on the rod before a first spin occurred compared to Go1+2α-/- animals (Fig. 2b). Finally, both Go1α-/- and Go1+2α-/- genotypes showed a significant increase in the number of spins compared to WT animals and Go1α-/- again performed worst (Fig. 2c).

Taken together our results reveal that motor performance of both Goα mutants lacking Go1α is affected. Surprisingly, however, Go1α-/- mice with the Go2α isoform present show a stronger deficit than the Go1+2α-/- mutants. Thus, the additional elimination of the Go2α splice variant partially restores the motor deficits when compared with the lack of Go1α alone.

Cocaine-induced behaviours in WT and Go1+2α-/- mice

The data obtained from the rota-rod test revealed deficits in motor function only in Go1α-/- and Go1+2α-/- but not in Go2α-/- mice. As reported previously motor deficits in Go2α-/- mice become obvious when the motor system is challenged by either cocaine (Brunk *et al.* 2008) or amphetamine (Brunk *et al.* 2010). Therefore, we next analysed the impact of the deletion of both the Go1α and Go2α splice variant on motor response to psychostimulant treatment. As Go1α-/- mice have a very short life time (mean life span 24 days) we could only test the Go1+2α-/- mice and their WT littermates.

Mice were injected daily with cocaine (10 mg/kg, i.p.) or saline for eight alternating days to test the development of CPP and behavioural sensitization simultaneously (Fig. 3). In the Go1+2α-/- mutants basal motor activity determined after injection of saline (Fig. 3a, saline) was significantly higher compared to WT littermates confirming hyperactivity previously described (Jiang *et al.* 1998).

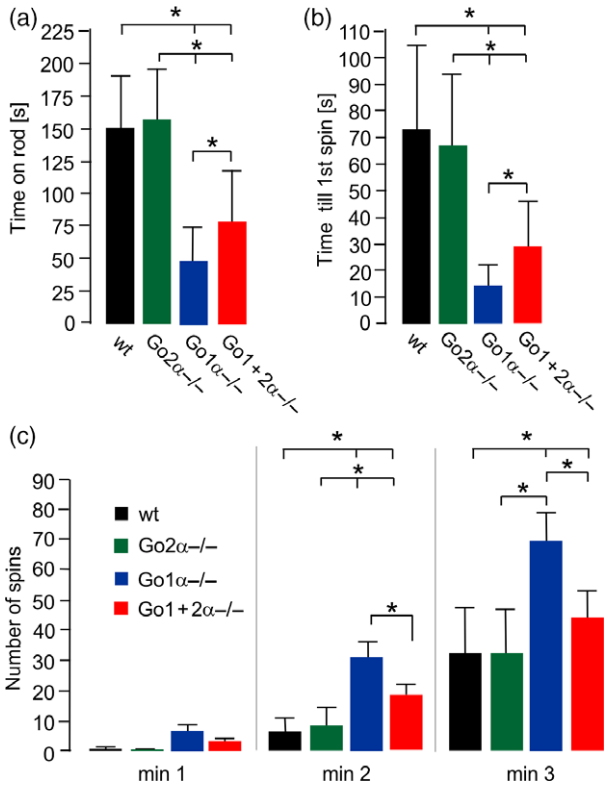

Fig. 2 Motor function of WT (n = 21), Go2α-/-(n = 6), Go1α-/- (n = 7) and Go1+2α-/- (n = 8) mice. WT mice and mutants originating from the Go1α-/-, Go2α-/- and Go1+2α-/-line were investigated using the rota-rod performance test. Mice were set on a rotating rod, which accelerates in speed from three to 50 rpm in 3 min challenging the mice's performance regarding motor activity, balance, coordination and strength. Three different parameters were recorded: (a) Total time mice spent on the rod. (b) Time mice were running on the rod till a first spin occurs. (c) Number of spins after mice were running on the rod during the 3 min course. The body weights were for WT 10–11 g, for Go2α 11–12 g and for both Go1α and Go1+2α 6–7 g. There was no difference regarding all three parameters comparing WT and Goα2-/- mice. Go1+2α-/- mice were significantly less impaired compared to Go1α-/- animals. (a) The average time mice spent on the rotating rod was for WT 150.8 ± 39.2s and for Go2α-/- mice 157.3 ± 37.9s. Go1α-/- mice spent 49.6 ± 25.4s and significantly less time on the rod till a first spin occurred then Go1+2α-/- animals (77.9 + 38.9s; $p < 0.01$). (b) The time until the first spin occurred was significantly shorter in Go1α-/- (14.5 ± 7.5s) compared to Go1+2α-/- mice (28.5 ± 16.9s; $p < 0.0005$) and dramatically reduced compared to WT (73.2 ± 30.4s) and Go2α-/- animals (66.9 ± 26.6s). (c) Go1α-/- mice showed a significant increase in the number of spins during the 1st, 2nd and 3rd min of the course compared to Go1+2α-/- mice (after 1 min Go1α-/-: 6.8 ± 2.3; Go1+2α-/-: 3.1 ± 1.2; 1st min $p < 0.0005$; 2nd $p < 0.0001$; 3rd $p < 0.005$), whereas WT and Go2α-/- animals reached only 0.4 ± 0.7. Student's t-test was used and values are expressed as means ± SD.

After the first, acute administration, cocaine induced a higher increase in locomotor activity in the mutant mice compared to WT animals (Fig. 3a Coc-1). Following repeated injections, the locomotor response to cocaine was further increased only in the mutants indicative of development of behavioural sensitization (Fig. 3a Coc-4). In the WT, behavioural sensitization did not develop to a significant degree (because of the mixed 129SvxC57BL/6J background) and was less pronounced than in our previous studies (Brunk *et al.* 2008). Furthermore, in contrast to the WT, after a drug-free period of 11 days, Go1+2α-/- mice further increased their sensitized response to cocaine as the mice exhibited a more robust response than WT mice (Fig. 3a, Coc-5). Deletion of only Go2α had been shown to attenuate development of motor sensitization induced by cocaine (Brunk *et al.* 2008) and amphetamine (Brunk *et al.* 2010). The enhancement of motor sensitization in the Go1+2α-/- mice indicates the importance of a balanced expression of both splice variants for adequate motor function.

Repeated exposures to the cocaine-associated compartment induced a CPP in WT mice, whereas Go1+2α-/- mice failed to develop cocaine preference (Fig. 3b). As we demonstrated previously, deletion of Go2α alone did not impair the development of cocaine-induced CPP (Brunk *et al.* 2008), indicating that the absence of CPP in Go1+2α-/- mice can be attributed to the deletion of Go1α.

Monoamine content and expression of Goα splice variants in striata of WT, Go2α-/-, Go1α-/- and Go1+2α-/- mice

In our previous analysis of Go2α-/- mice, we found that striatal dopamine levels are decreased in comparison to WT animals and this is accompanied by a reduced monoamine uptake by synaptic vesicles (Brunk *et al.* 2008). Therefore, we investigated if altered motor function in Go1α- and Go1+2α deletion mutants is also associated with changes in striatal dopamine levels. Indeed, HPLC analyses revealed increased dopamine as well as serotonin levels in the striatum of Go1α-/- mice compared to the corresponding WT. In contrast, in the striata of Go1+2α-/- mice (lacking both isoforms) dopamine and serotonin levels remained comparable (Fig. 4a).

Furthermore, we found an increased vesicular uptake of monoamines in striatal SV preparations of Go1α-/- mice (59% total uptake) when compared with WT littermates (41% total uptake). This difference was attenuated when analysing littermate pairs of WT (45.0% total uptake) and Go1+2α-/- mice (55.0% total uptake) (Fig. 4b).

These data complement the behavioural experiments indicating that lack of Go1α results in changes in striatal monoamine levels and vesicular uptake. Removal of both splice variants appears to restore partially the WT situation. These results suggest that imbalance in the expression of Goα isoforms leads to opposite effects depending on the splice variant deleted.

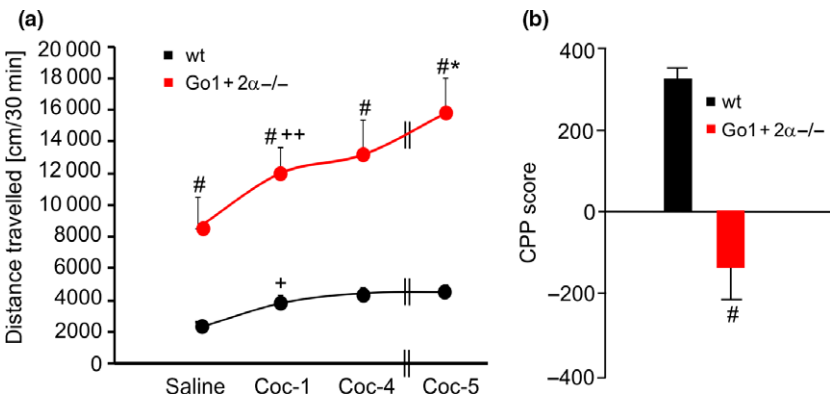

Fig. 3 Cocaine-induced behaviours in WT (n = 26) and Go1+2α-/- (n = 10) mice. (a) In the Go1+2α-/-mutants basal motor activity (saline) was significantly higher (8519.1 ± 1976.6 cm/30 min) compared to WT littermates (2539.7 ± 152 cm/30 min; $p < 0.0005$). Cocaine induced a higher acute increase in locomotor activity in the mutant mice (Coc-1, WT (+) $p < 0.05$; Go1+2α-/-(++), $p < 0.0005$). Following repeated injections behavioural sensitization further increased in the mutants (Coc-4, Sensitization effect: $F_{(3,102)} = 38.4$, $p < 0.0001$). After a drug-free period of 11 days, Go1+2α-/- mice further increased their sensitized response to cocaine as the mice exhibited a more robust response than WT mice (Coc-5, Gen effect: $F_{(1,34)} = 61$, $p < 0.0001$; Gen x Sensitization interaction effect: $F_{(3,102)} = 11$, $p < 0.0001$). The strain of the mice has a mixed 129SvxC57BL/6J background, known to have weaker locomotor responses to cocaine when compared to other strains like the C57BL/6J. The WT mice showed a significant increase in the locomotor response on day 4 respect day 1, indicating development of behavioural sensitization ($t(25) = -2.2$, $p = 0.03$). However, when performing statistics together with the mutants, this significance disappeared. (b) In contrast to WT mice, Go1+2α-/- mice failed to develop cocaine-induced conditioned place preference (CPP) as indicated by the CPP score ($t_{(34)} = 6.57$; $p < 0.0001$). Two-way ANOVA test was used and values are expressed as means ± SEM. The average weight of the mice was: Go1+2α 27.65 ± 2.18 g; WT 33.63 ± 0.95 g.

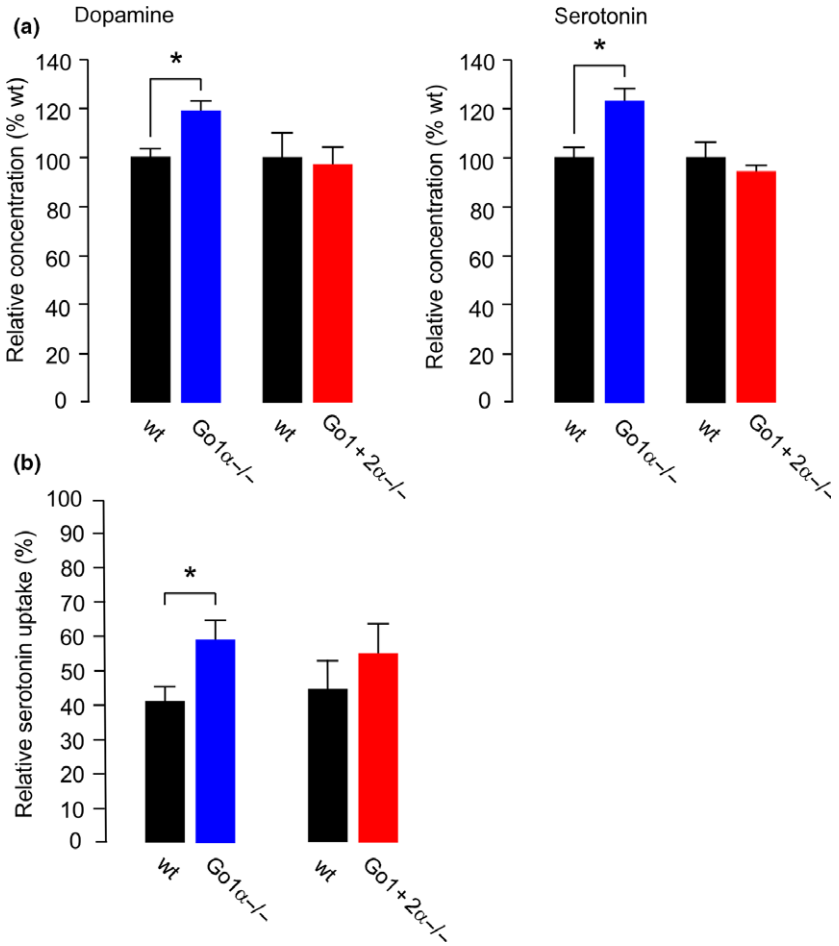

Fig. 4 Striatal monoamine levels and monoamine uptake by synaptic vesicles of Go1α-/- and Go1+2α-/- mice. (a) The striatal monoamine levels in WT, Go1α-/- and Go1+2α-/- mice were quantified by HPLC analysis. Concentrations of dopamine (9034.4 ± 257.9 pg/mg wet weight) and serotonin (420.3 + 20.7 pg/mg wet weight) were increased in Goα1-/- mice compared to their WT littermates (dopamine: 7538.0 ± 279.1 pg/mg wet weight; serotonin: 343.4 ± 13.1 pg/mg wet weight; dopamine $p < 0.01$; serotonin: $p < 0.05$,). In contrast, Goα1+2-/- mice showed no difference in comparison to their WT littermates (dopamine WT: 3321.1 ± 319.5 pg/mg wet weight; Go1+2α-/-: 3210.0 ± 229.3 pg/mg wet weight; serotonin WT: 232.5 ± 14.8 pg/mg wet weight; Go1+2α-/-: 219.0 ± 11.6 pg/mg wet weight). Data in the graph are expressed as % of WT control. (b) monoamine uptake by synaptic vesicles of Go1α-/- mice was significantly higher than uptake by vesicles of WT mice ($p < 0.01$). In contrast monoamine uptake by synaptic vesicles of Go1+2α-/- mice did not differ significantly from vesicular uptake by WT mice ($p = 0.166$). Results are the mean of four different experiments. Data were normalized to the total uptake in each experiment. Kruskal–Wallis and Mann–Whitney U-tests were used and values are expressed as means ± SD.

To test if the deletion of one splice variant induces an increased expression of the other splice variant, we next analysed the striatal amounts of the respective splice variants using the recently characterized Goα splice variant-specific antibodies (Brunk *et al.* 2010). Go1+2α-/- mice served as negative controls. Expression of Go2α was found to be increased three-fold in the striatum of Go1α-/- mice compared to WT littermates, whereas the expression of Go1α was not altered in the striatum of Go2α-/- mice (Fig. 5). This increased amounts of Go2α in the striatum of Go1α-/- mice could not be resolved previously when using synaptosomes from whole brain (Brunk *et al.* 2008). The striatal imbalance in Goα splice variant expression is more severe in Go1α-/- mutants compared to Go2α-/- mutants since besides the lack of Go1α expression of Go2α is increased. An increase in Go2α, as found in the Go1α mutants, may explain their pronounced poor performance in the rota-rod test (compared to the absence of both splice variants).

Striatal expression of dopamine receptors, D1R and D2R, in WT, Go1α-/- and Go1+2α-/- mice

Previous findings indicated that most central nervous D2R are coupled to Go1α (Jiang *et al.* 2001; Brunk *et al.* 2010). Cocaine-induced motor-sensitization and CPP are based on

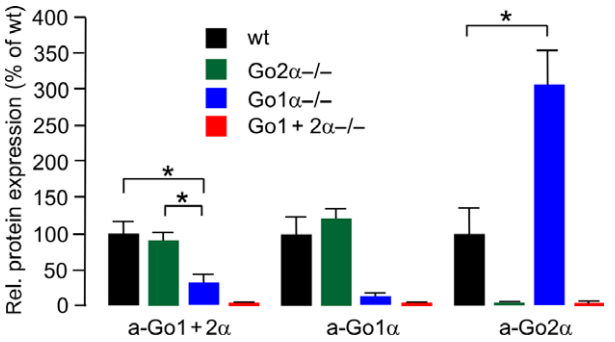

Fig. 5 Striatal expression of Goα-subunits in WT, Go2α-/-, Go1α-/- and Go1+2α-/- mice. To quantify the striatal Goα protein expression in WT, Go1α-/-, Go2α-/- and Go1+2α-/- mice an antibody detecting both Goα splice isoforms (clone 101.1) and splice isoform specific antibodies (clone 101.4 and sc-13532) have been applied in western blots of striatal synaptosomes from each genotype. The expression of splice isoforms in WT preparations of the striatum was set to 100% for each antibody. Only a small amount of total Goα accounts for Go2α and Go1α is the major splice isoform in the striatum (ab clone 101.1). The expression of Go2α was significantly increased in Go1α-/- mice by about 200% compared to WT littermates (ab clone 101.4). In contrast, the expression of Go1α was not altered in Go2α-/- mice compared to their WT littermates (ab sc-13532). The Go1α-antibody exhibited a negligible cross-reactivity towards Go2α evident in the Go1α animals. Four animals per mouse line were included, values represent the mean ± SD (Kruskal–Wallis test and the Mann–Whitney *U*-test). The star (*) denotes significance $p < 0.05$ (relative OD Go2α: WT 0.25 ± 0.03; Go1α-/- 0.79 ± 0.12; relative OD Go1α: WT 0.25 ± 0.03; Go2α-/- 0.29 ± 0.04).

changes in D1R and D2R signalling in various brain areas (ventral tegmental area, nucleus accumbens and medial prefrontal cortex) (Steketee and Kalivas 2011). Thus, deletion of Go1α, the main coupling Gα-subunit of D2R, may cause the lack of cocaine-induced CPP observed in Go1+2α-/- mutants observed here (see Fig. 3).

Analysis of striatal expression of the D1R revealed no difference between WT animals and Go1α-/- mutants. In contrast, expression of the D2R was higher in the Go1α-/- mutants compared to their WT littermates (Fig. 6a). In Go1+2α-/- mutants, striatal expression of both D1R and D2R was not altered in comparison to WT mice, both without (Fig. 6b) or after (Fig. 6c) repetitive application of cocaine. Taken together the ratio of D1R and D2R expression is lowered in striata of Go1α-/- mice (45.0 ± 5.7% of WT) and resembles the situation in cocaine treated Go2α-/- mice (41.2 + 15.5% of WT). In Go1α-/- mice the expression of D2R in the striatum was increased (Fig. 6a). However, an increase in the striatal D2R content was also observed in cocaine treated Go2α-/- mutants in the presence of Go1α (Brunk *et al.* 2008). Although other possibilities cannot be excluded, the data support the idea that both Goα subunits may couple to D2R but may differentially respond to cocaine. In this line, no change in the D2R amount was seen in Go1+2α-/- mutants, irrespective of cocaine treatment (Fig. 6b and c). The D1R-/D2R ratio was not altered significantly in striata of untreated and cocaine treated Go1+2α-/- mutants compared to WT animals, though the analyses tended to result in a higher ratio in the mutants (Fig. 6d, Table 1). These findings are in line with development of motor sensitization after repetitive cocaine treatment in Go1+2α-/- mice and with lack of motor sensitization in Go2α-/- mice, but they do not account for higher motor activity of Go1+2α-/- mutants compared to their WT littermates.

The amounts of D1R, Gsα, TH and VMAT2 did not differ in striata of all mutants tested here. There was also no difference in the striatal expression of calbindin, TH, VMAT2 and Gsα and of monoamine oxidase activity (Table 2) in between the different genotypes. Amounts of Golfα were increased in Go1α-/- mutants in contrast to the decreased expression in Go2α-/- mutants (Table 3; (Brunk *et al.* 2008)). Gsα, Golfα, TH and VMAT2 were comparably expressed in striata of WT mice and of Go1+2α-/- mutants after repetitive treatment with cocaine (Table 3).

Discussion

This study reveals that Go1α and Go2α, the two major Goα splice variants, affect the dopaminergic system in a complementary manner. Motor function tests revealed that Go1α-/- mice perform considerably worse in comparison to both WT and Go1+2α-/- mice lacking both splice variants. Furthermore, in Go1α-/- mice dopamine concentration and vesicular monoamine uptake was higher compared to WT littermates (this paper), whereas in Go2α-/- mice dopamine uptake and content was reduced (Brunk *et al.* 2008). As both Goα-splice variants reside on secretory vesicles (Takamori *et al.* 2002) they may fine tune antagonistically vesicular monoamine content. Besides the dopamine content, the expression of the D2R receptor was higher compared to WT littermates or Go2α-/- mice. Deletion of both Go1α and Go2α re-established both dopamine levels and D2R amounts comparable to those in the wt. As we found no evidence for changes in the leading enzymes for dopamine synthesis of degradation it is not clear yet whether the dopamine imbalances are owing to the changes at the vesicular level, the receptor level or both.

Distinct opposing effects of Go1α and Go2α on basal motor function

Goα comprises about 1% of total membrane protein content of the brain with Go1α expression exceeding that of Go2α in mature neurons (Jiang and Bajpayee 2009). In mouse brain, we have previously measured an overall ratio of 2:1 for Go1α expression over Go2α (Brunk *et al.* 2008). Here we show that the two splice variants exert opposing effects on the striatal dopaminergic system. Unexpectedly, when tested

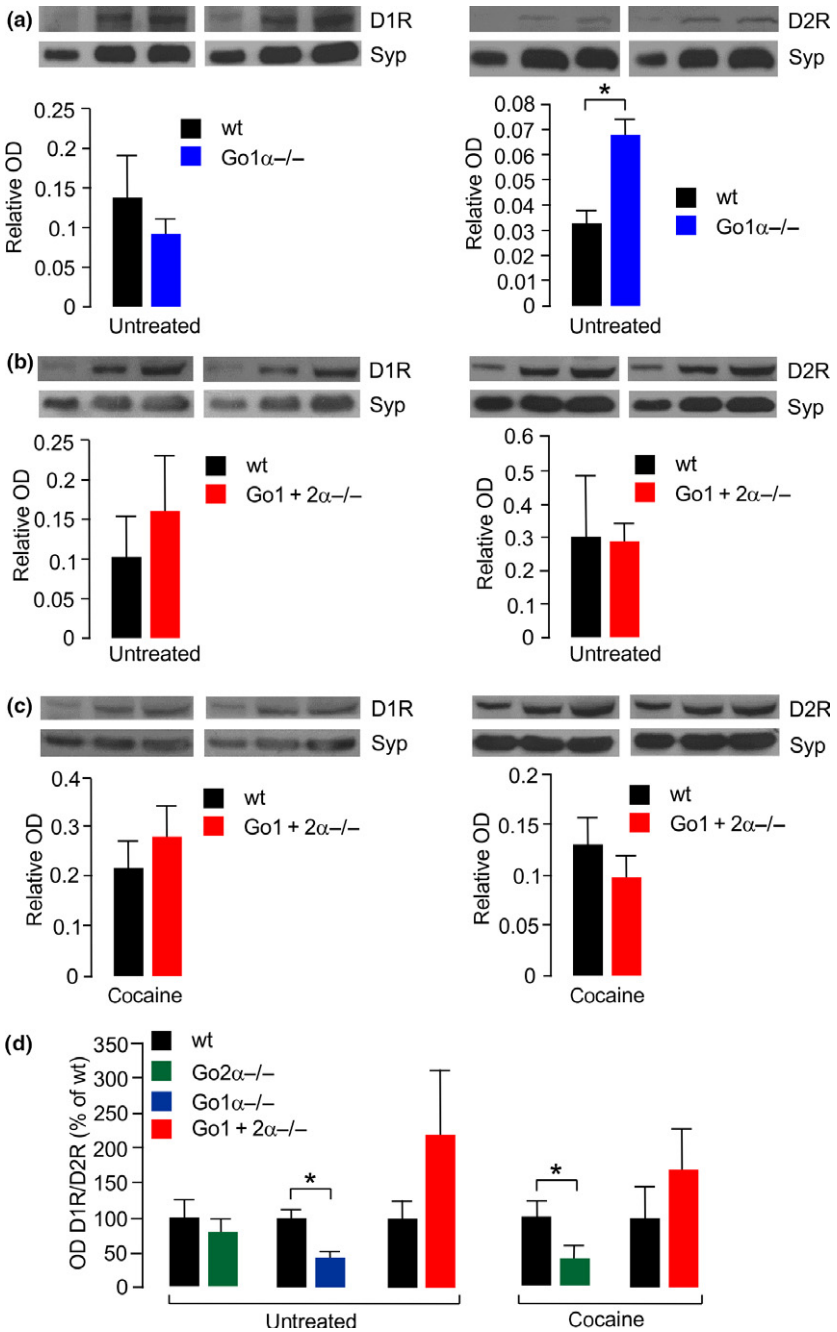

Fig 6 Striatal expression of DR in WT, Go1α-/- and Go1+2α-/- mice. To quantify the striatal protein expression of D1R and D2R the respective antibodies were applied to western blots of striatal synaptosomes prepared from each genotype. (a) D1R was comparably expressed in striata of WT and Go1α-/- mice, expression of D2R was increased in deletion mutants compared to WT littermates (relative OD D1R: WT 0.14 ± 0.05; Go1α-/- 0.09 ± 0.02; relative OD D2R WT 0.03 ± 0.01 Go1α-/- 0.07 ± 0.01 $p < 0.05$). (b) D1R and D2R were expressed in comparable amounts in striata of WT and Go1+2α-/- mice (relative OD D1R: WT 0.10 ± 0.05; Go1+2α-/- 0.16 ± 0.07; relative OD D2R: WT 0.39 ± 0.10; Go1+2α-/- 0.29 ± 0.06). (c) D1R and D2R amounts were comparable in striata of cocaine-treated WT and Go1+2α-/- mice (relative OD D1R: WT 0.22 + 0.05; Go1+2α-/- 0.28+0.06; relative OD D2R: WT 0.13 + 0.03; Go1+2α-/- 0.10 ± 0.02). (d) The ratio of D1R to D2R expression was lower in Go1α-/- striata compared to WT (45.0 ± 5.7% of WT), resembling the situation in Go2α-/- mice after repetitive treatment with cocaine (41.2 + 15.5% of WT) (*$p < 0.05$). There was no difference between untreated WT mice and Go2α-/- or Go1+2α-/- mutants (214.7 ± 94.6% of WT) as well as between cocaine treated WT mice and Go1+2α-/- mutants (135.1 + 41.2% of WT). Values are obtained from four animals per mouse line and genotype and are expressed as percent of the respective WT and as means ± SD (Kruskal–Wallis test and the Mann–Whitney *U*-test) using synaptophysin or synaptobrevin as internal controls.

on a rota-rod, motor function was not affected in Go2α-/- mutants but was severely impaired in Go1α-/- mutants. Moreover, motor activity was improved in mutants deficient of both splice variants when compared with Go1α-/- mice.

The three different behavioural tests (rota-rod performance test, cocaine-induced sensitization and CPP) used in this study address different aspects of the role of dopamine in the striatum. The rota-rod performance test examines motor coordination, overall activity as well as muscle strength. In this test both Go1α-/- and Go1+2α-/- mutants perform worse compared to the Go2α-/- mice and the corresponding WT littermates. The poor performance of the Go1α-/- and the Go1+2α-/- mice compared to both WT and Go2α-/- can be attributed not only to deficits in motor control (Jiang *et al.* 1998) but also to their overall weakness. Indeed, in the Go1+2α-/- mutants a weight reduction by about 40% has been observed earlier (Jiang *et al.* 1998). Surprisingly, however, the Go1α-/- mutants perform even worse compared to the Go1+2α-/- mice despite the fact that the postnatal weight development is comparable in both genotypes.

Table 1 Relations of D1R and D2R expression in striata of Go2α-/-, Go1α-/-, Go1+2α-/- mice compared to WT littermates

Parameter of interest	Go2α-/-	Go1α-/-	Go1+2α-/-
D1R (OD ko/OD wt)	0.765 ± 0.095	0.772 ± 0.284	1.603 ± 0.691
D2R (OD ko/OD wt)	0.986 ± 0.096	1.777 ± 0.745*	0.763 ± 0.127

Expression levels of the indicated proteins were determined by western blot analysis of four animals from each genotype. D1R and D2R expression and D1R/D2R ratio was set 1 for WT animals (*$p < 0.05$). For statistic Kruskal–Wallis test and the Mann–Whitney *U*-test were used and values are expressed as means ± SD.

Although Go1+2α-/- mutants are impaired with respect to their body weight and muscle strength, motor performance appears to be functional besides the effects directly influenced by strength.

Opposing effects of Go1α and Go2α on the striatal dopaminergic system

Consistent with these divergent effects on motor functions, striatal dopamine concentration as well as the D2R expression were differentially affected in Go1α-/- vs Go2α-/- mutants with the former showing increased and the latter decreased levels, respectively, compared to their WT littermates. Recently it has been shown that the two splice variants of D2R differentially affect the dopaminergic system. D2RL mediates post-synaptic effects including motor activity following cocaine treatment, whereas D2RS pre-synaptically modulates dopamine synthesis (Radl *et al.* 2018). The comparable increase in total D2R amounts observed in Go1α-/- and Go2α-/- following cocaine treatment could be therefore because of differential Goα-splice variant-specific changes in the expression of the D2R splice variants.

In contrast, the presence of only Go2α, whose expression is increased in whole brain and striatum in Go1α-/- mutants, further deteriorates motor activity and coordination. This observation may be related to the fact that the striatal amount of D2R is increased and the ratio of D1R to D2R expression is reduced in Go1α-/- animals. So, in Go1α-/- mutants the indirect pathway via D2R signalling could be enforced leading to more severe deficits in motor control than the complete absence of both splice variants, which does not affect the ratio of D1R to D2R. Especially D2R is linked to Go proteins in the central nervous system (Jiang *et al.* 2001). Absence of Go2α has no impact on motor control in the rotarod performance test indicating that Go1α is more important for successful motor control under physiological conditions than Go2α. The additional involvement of Go2α becomes obvious after challenging the dopaminergic control of motor activity by treatment with psychostimulants (Brunk *et al.* 2008, 2010). In Go1+2α-/- mutants D2R signal transduction should be impaired because of the loss of Goα signalling. Interestingly these mice are still able to perform motor tasks and skills. This is reminiscent of the situation observed in D2R deletion mutants, which, nevertheless, are able to perform motor coordination tasks, although impaired in comparison to WT mice (Kelly *et al.* 1998; Clifford *et al.* 2000). In addition, the lack of D2R autoreceptors (corresponding to D2RS,) (Radl *et al.* 2018) leads to locomotor

Table 2 Key proteins of the monoaminergic system in brains of WT, Go1α-/- mice and Go1+2α-/- mice

		Go1α-/-		Go1+2α-/-	
Expression of	Region	wt	ko	wt	ko
Calbindin	Striatum	0.923 ± 0.255	0.756 ± 0.092	0.902 ± 0.085	0.720 ± 0.218
D1R	Striatum	0.138 ± 0.053	0.092 ± 0.017	0.103 ± 0.048	0.160 ± 0.070
D2R	Striatum	0.033 ± 0.005	0.068 ± 0.006*	0.387 ± 0.010	0.289 ± 0.056
Golfα	Striatum	0.045 ± 0.018	0.131 ± 0.042*	0.177 ± 0.192	0.215 ± 0.149
Gsα	Striatum	0.056 ± 0.033	0.079 ± 0.034	0.080 ± 0.028	0.060 ± 0.019
TH	Striatum	0.718 ± 0.123	0.659 ± 0.135	1.099 ± 0.248	0.837 ± 0.322
VMAT2	Striatum	0.076 ± 0.016	0.041 ± 0.020	0.441 ± 0.079	0.333 ± 0.079
Activity of MAO	Whole brain				
V_{max}		6493 ± 355.4	6685 ± 371.2	8138 ± 714.0	8301 ± 713.1
Km		31.8 ± 5.102	29.02 ± 4914	40.26 ± 9.374	34.22 ± 8.349

Expression levels of the indicated proteins were determined by western blot analysis using striata from four different animals of each genotype. Quantification is given as the mean of the relative optical densities ± SD. and was performed using synaptophysin or synaptobrevin as internal control. Overall activity of MAO was measured using the Amplex Red MAO Assay Kit with tyramine as substrate. V_{max} (fluorescence intensity) and Km (min) values are expressed as means ± SD. Expression levels of D2R and Golfα were lower in striata of WT mice compared to Go1α-/- mutants. For all other parameters, no differences between WT mice and mutants could be observed (*$p < 0.05$). For statistic Kruskal–Wallis test and the Mann–Whitney *U*-test were used.

Table 3 Influence of repetitive cocaine treatment on expression of proteins related to the monoaminergic system in striata of WT and Go1+2α-/- mice

Protein of interest	wt	ko
D1R	0.216 ± 0.053	0.278 ± 0.064
D2R	0.130 ± 0.025	0.097 ± 0.020
Gsα	0.245 ± 0.136	0.385 ± 0.133
Golfα	0.100 ± 0.065	0.107 ± 0.060
TH	0.450 ± 0.095	0.467 ± 0.125
VMAT2	0.404 ± 0.238	0.440 ± 0.226

Expression levels of the indicated proteins were determined by western blot analysis of brains from four animals of each genotype following cocaine treatment (see Fig. 2). No difference between WT mice and deletion mutants could be observed. Quantification is given as relative optical densities expressed as means ± SD using synaptophysin or synaptobrevin as internal control. For statistic Kruskal–Wallis test and the Mann–Whitney *U*-test were used.

hyperactivity. (Bello *et al.* 2011) This is reminiscent to the hyperactivity observed in the Go1+2α-/- mice (see Fig. 2 this paper). As Go2α-/- mice do not show an increased but a reduced motor activity, the hyperactive phenotype in the Go1+2α-/- mice may be mainly because of the loss of Go1α (Brunk *et al.* 2008). When the D2R is ablated during adulthood motor control is severely disturbed. This indicates that reduced motor control as a result of deletion of D2R before birth can be partially overcome during postnatal development (Bello *et al.* 2016).

Differing effects of Go1α and Go2α on psychostimulant-induced behaviour

Cocaine-induced behavioural sensitization could only be tested in Go1+2α-/- mice. Go1α-/- mutants were not tested because of their reduced life span (mean 24 days). Similar to the observations on the rota-rod test, Go2α-/- mutants exhibited reduced cocaine-induced behavioural sensitization (Brunk *et al.* 2008), whereas Go1+2α-/- mice strongly responded in this test (this paper). All three parameters addressed, basal motor activity, motor response to first cocaine injection and behavioural sensitization following repeated cocaine injections were increased in Go1+2α-/- mutants compared to WT mice. Finally, development of CPP towards cocaine was almost completely abolished in Go1+2α-/- mutants (this paper), whereas it was unaltered in the Go2α-/- mice (Brunk *et al.* 2008). The absence of both Goα proteins boosts behavioural sensitization, which is under these conditions probably mediated by D1R and Golfα. Golfα is highly expressed in the striatum. Deletion of Golfα abolishes cocaine-induced increase in motor activity, whereas the basal motor activity of the mutants is increased (Zhuang *et al.* 2000). Recently we found behavioural sensitization following repetitive cocaine administration in Go2α-/- mice to be decreased (Brunk *et al.* 2008). In this context Goα proteins seem to have complementary effects with Go1α down-regulating behavioural sensitization. On the other hand, the D2R also appears to be relevant for cocaine-induced behavioural sensitization as sensitization is diminished in D2R knockout mice (Welter *et al.* 2007). Thus, behavioural sensitization may be sustained depending on either receptor equipment (D1R or D2R) or signal transducing G-proteins (Go, Gs or Golf). A partially restored balance of D1R and D2R expression in Go1+2α-/- mice and the signal transduction by either Golf or Gs with both G-proteins present at amounts comparable to the WT situation may sustain the development of behavioural sensitization.

The CPP test addresses development of addiction but has to be distinguished from behavioural sensitization according to our observations in the Go1+2α-/- mutants. This is in accordance with our previous observation that behavioural sensitization following cocaine or amphetamine treatment is reduced in Go2α-/- mice, whereas CPP is not changed (Brunk *et al.* 2008, 2010). This is in some respects contradictory to the literature, where sensitization and CPP are described to be interrelated, though not obligatory connected (Steketee and Kalivas 2011). The complete deletion of both Goα splice variants almost abolishes CPP indicating that signal transduction via Go1α is essential for the development of this behaviour. Go has been mainly linked to D2R activation (Jiang *et al.* 2001). The ability of GTP to promote a shift in the affinity of D2R for dopamine is absent in brains of Go1+2α-/- mutants but unchanged in Go2α-/- mice indicating that central nervous D2R preferentially couples to Go1α subunits (Jiang *et al.* 2001; Brunk *et al.* 2010). In this line, CPP is nearly abolished in D2R knock out animals (Welter *et al.* 2007). However, CPP is also decreased in animals lacking D1R (Abraham *et al.* 2016). Results obtained with constitutive Go1+2α-/- mice suggest that CPP requires Go1α to develop, probably using either D1R or D2R. Indeed, cocaine-induced sensitization and reinstatement is based on changes in D2R and D1R signalling in the ventral tegmental area (Steketee and Kalivas 2011).

Taken together our data indicate that a balance in the expression of Go1α and Go2α maintains normal motor function signalling via striatal dopamine receptors. Deletion of one of the splice variants leads to an imbalance affecting motor performance of the individual mutant. Balance is partially restored when both splice variants are deleted, because the expression of D1R and D2R is not affected in Go1+2α-/- mice, and signalling can occur via Gsα and Golfα, which are still present in the mutants. It has to bear in mind that the actual data were obtained with conventional mutants and therefore an impact on various cell types and brain regions besides dopaminergic neurons in the striatum could not be discriminated. This also implies that besides dopamine receptors other receptors like M2, opioid or

somatostatin receptors that couple to Go proteins (Jiang and Bajpayee 2009) may be involved. So far, Go2α-specific effects have been described for somatostatin-receptor-mediated effects in pancreatic islets (Wang *et al.* 2011). In addition, there may be redundancy of G-protein signalling via different receptors. In this line it has been recently described that D1R differentially couple to either Golfα or Gsα depending on agonist and brain region (Yano *et al.* 2018). Whether Go1α may be also linked to D1R under certain conditions is not known so far. The opposing effects of Go1α and Go2α shown here using global knockouts will pave the way for future studies using conditional knockouts for either Goα splice variant to improve our understanding how motor function is controlled.

Acknowledgement and conflict of interest disclosure

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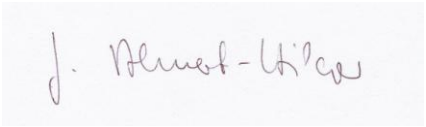

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