Novel Model of Reversible Vestibular Syndrom Induced by Optogenetic Stimulation

Montardy Q. [1], Wei M. [1,2], Yi T. [1], Liu X. [1], Zhou Z. [1,3], Besnard S. [4,5], Tighilet B. [4,*], Chabbert C. [4,*] & Wang. L. [1,*]

1. Shenzhen Key Lab of Neuropsychiatric Modulation and Collaborative Innovation Center for Brain Science, Guangdong Provincial Key Laboratory of Brain Connectome and Behavior, CAS Center for Excellence in Brain Science and Intelligence Technology, the Brain Cognition and Brain Disease Institute (BCBDI), Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen-Hong Kong Institute of Brain Science-Shenzhen Fundamental Research Institutions, Shenzhen, 518055, China
2. University of Chinese Academy of Sciences, Beijing, 100049, China
3. McGovern Institute for Brain Research, Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA, 02139, USA
4. Aix Marseille University-CNRS, Laboratory of Sensory and Cognitive Neurosciences, UMR 7260. Team Pathophysiology and Therapy of Vestibular Disorders, Marseille, France. GDR Vertige (GDR#2074).
5. Université de Caen Normandie, CHU de Caen, Caen, France

* To whom correspondence should be addressed: BT, CC & LW

Abstract

Glutamatergic and GABAergic neurons represent the main component of the medial vestibular nuclei. We assessed the functional role of glutamatergic and GABAergic neuronal pathways arising from the vestibular nuclei in the maintenance of gate and balance, by optogenetically stimulating the vestibular nuclei of VGluT2-cre and GAD2-cre mice. We demonstrated the VN glutamatergic, but not GABAergic subpopulation are responsible for immediate and strong posturo-locomotor deficits, comparable to unilateral vestibular deafferentation models. During optogenetic stimulation, the support surface is also dramatically increased for VN-VGluT2+ animals, and rapidly come back to baseline level after stimulation, whilst it remained unchanged during all the experiment for VN-GAD2+ animals. This effect persists in condition where vestibular compensation is removed. Finally, posturolocomotor parameters such as support surface, immobility and velocity revealed the effect for VN-VGLuT2+ animals was still present

immediately after stimulation, while it disappears 1h after. Overall, these results indicate a fundamental role for VN-VGluT2+ neurons in balance and posturo-locomotor functions, but not for VN-GAD2+ neurons, at least at short term. This new optogenetic approach offers to better characterize the role of glutamatergic and gabaergic functions in vestibular compensation.

Introduction

Here we report the development of a new method of generating a vertigo syndrome in a rodent model as well as new findings of the glutamatergic versus gabaergic postural control from vestibular nuclei. For the first time, the optogenetic technique was used to map, but also stimulate some of the central circuits that support vestibular function. This approach is particularly noteworthy, since it constitutes a new step in understanding the vestibular pathology, by making it possible to modulate central neuronal pathways in a specific way, while/and controlling the stimulation pattern. This work opens up new avenues in terms of fundamental knowledge regarding to the anatomo-functional organization of the vestibular system and perspectives in the understanding of vestibular physiopathology and therapeutic.

If the first mentions of vertigo as a specific symptom date back to antiquity with Hippocrates writings (Adams 1849), it was not until the beginning of the 19th century that the study of vertigo and balance entered the experimental phases, with the works of Purkinje in humans (Purkinje 1819), and Flourens in the pigeon (Flourens 1842). These works allowed establishing a direct link, between damage of the inner ear sensors and the characteristic posturo-locomotor and vestibulo-ocular deficits encountered in the dizzy patient. A century later, Lorente de No brought the first anatomical descriptions of the central pathways that connect the peripheral vestibular sensors, to the oculomotor muscles (Lorente De No 1933).

Since then, the anatomical and functional organization of the vestibular system is now better understood, and its contribution to a wide range of functions, from postural and oculomotor reflexes to spatial representation and cognition, has been underscored (Angelaki and Cullen 2008; Borel et al. 2008). Indeed, type I and type II sensory hair cells of the semicircular canals, utricle and saccule within the vestibular endorgans, detect vestibular stimuli mainly related to linear,

rotational and gravity accelerations, transmit them to the vestibular nerve which carries them to the vestibular nuclei. Four vestibular nuclei (VN) are located in the brainstem under the floor of the IVth ventricle: the median (MVN), inferior VN (IVN), lateral (LVN) and superior (SVN) vestibular nuclei. At the VN level, this essentially sensory information is integrated and transformed into premotor signals, the transport of which is ensured by vestibulo-spinal and vestibulo-oculomotor pathways. The VN project, through the medial longitudinal fasciculus (MLF) on oculomotor nuclei whose motoneurons control gaze stabilization during head movements. The vestibular nuclei influence postural control via two descending pathways to the spinal cord, the lateral (mostly from ipsilateral NVL neurons) and medial (mostly from ipsi and contralateral NVM neurons) vestibulospinal tracts (Victor J Wilson and Jones 1979). Vestibular information is also transferred via vestibulo-thalamo-cortical pathways to different cortical areas providing perceptual and cognitive functions such as perception and orientation of the body in space (C. Lopez et al. 2015; Christophe Lopez 2016). Finally, the vestibular nuclei are interconnected to a multitude of neurovegetative centres in the brainstem(Balaban and Beryozkin 1994; Balaban and Porter 1998). Given this anatomo-functional organization of the vestibular system and its projection targets, unilateral alteration of peripheral vestibular inputs induces a quadruple syndrome: posturo-locomotor, oculomotor, vegetative and perceptivo-cognitive. Symptoms progressively decline, each with its own kinetics, generally leading to an almost full disappearance of the syndrome. This behavioral recovery phenomenon is referred to as "vestibular compensation" (Michel Lacour, Helmchen, and Vidal 2016).

Animal models of vestibular pathologies

Different animal models of vestibular disorders have been developed so far. The researchers set out either, on the basis of an epidemiological rationale, the pathogenic conditions likely to induce vestibular dysfunction and its functional consequences, or the neurophysiological mechanisms likely involved. The first case included: 1) ototoxic-based vestibular damages, reproducing inner ear-specific toxicity of drugs such as aminoglycosides or cisplatin, and food-born ototoxicity (Zhang et al. 2003; Hirvonen et al. 2005; Xia, Chen, and Yin 2012; Vignaux et al. 2012); 2)

excitotoxically-induced vestibular damages, reproducing the deleterious consequences on the first synapse of sensory cells suffering (O'Neill et al. 1999; Brugeaud et al. 2007; Desmadryl et al. 2012; Dyhrfjeld-Johnsen et al. 2013; Gaboyard-Niay et al. 2016); 3) destruction of peripheral receptors through surgical labyrintectomy (Smith and Curthoys 1989; Patk et al. 2003; Hitier et al. 2010); 4) vestibular neurectomy that relates to the full section of the vestibular nerve. This operation can be achieved either by severing the vestibular nerve peripherally (between the vestibular endorgans and the Scarpa's ganglion) (Li, Godfrey, and Rubin 1995; Hamann et al. 1998)or centrally (between the Scarpa's ganglion and the brainstem). Peripheral UVN leads to the loss of inputs from vestibular endorgans, while preserving those arising from vestibular ganglion neurons. Central UVN induces complete loss of inputs arising from both vestibular endorgans and Scarpa ganglion neurons. Several examples of central UVN have been documented previously in different species such as the monkey (M. Lacour, Roll, and Appaix 1976), the cat (Xerri and Lacour 1980) and the rat (Li, Godfrey, and Rubin 1995). Along with the lesional models mentioned above, there are models of reversible modulation of the vestibular input. The first model developed in this sense was the so-called "vestibular anesthesia" (Bárány 1936). Assuming that the episodes of vertigo attacks observed in the Menière patients resulted from transient unilateral hyperexcitability of primary vestibular neurons, this approach consisted of counteracting this hyperexcitability through transtympanic administration of neuron activity, such as lidocaine. Later similar modulatory effect was obtained through transtympanic administration of tetrodotoxin (TTX), selective blocker of voltage-gated calcium channels (Dutheil, Lacour, and Tighilet 2011). Under these conditions, the pharmacological modulation of peripheral sensory input allowed to either reproduce in animals, the vestibular syndrome encountered in patients with acute peripheral vestibulopathy, or conversely to alleviate when administrated in the ear opposite to the damaged ear.

Although these models allowed to better understand the conditions of generation of the vertigo syndrome, and its temporal evolution, number of questions related to the anatomical and functional organization of the vestibular central pathways remain. As an example, the target cerebral structures of neurons projecting from the VNs are not all identified so far, in particular at the cortical level. Likewise, the central pathways projecting from the cortical areas towards the

VNs are poorly documented. This lack of data prevents the development of studies allowing to understand the links between vestibular, cognitive and emotional functions. To answer these questions, we used optogenetics in mice, to map and stimulate some neural pathways originating in the VN. The principle of optogenetics is based on the insertion into defined cell populations (here glutamatergic or GABAergic neuron subpopulations), of genes encoding photoactivatable proteins, such as opsins. This method makes it possible on the one hand, to trace the neuronal pathways in which the opsins are selectively expressed, and on the other hand, to specifically stimulate these neurons. Our results demonstrate that it is possible to generate posturo-locomotor deficits by the technique of optogenetics. This posturo-locomotor deficits that is an essential component of the vestibular syndrome has been compared with that induced by classical chemical labyrintectomy.

Result

Validation of the optogenetic method in vestibular nuclei

Mice expressing either a cre recombinase driven by VGluT2+ (vesicular glutamate transporter 2) promoter in glutamatergic neurons (VGluT2-ires-cre), or a cre recombinase driven by GAD2 (glutamate decarboxylase 2) promoter in GABAergic neurons (GAD2-ires-Cre), were injected uniterally with the cre-dependent virus AAV9-DIO-ChR2-mCherry at the vestibular nuclei (VN) location under stereotaxic control. In parallel a control group of mice expressing VGluT2-cre or GAD2-cre received injection of AAV9-DIO-mCherry at the same location. 3 weeks after injection, the animals were implanted with an optical fiber above the injected VN, allowing to perform vestibular stimulation on awake and free-moving animals (Fig.1A,B). After completion of all experimental tests, brains were extracted and sectioned to verify if the virus expression and the optical fiber were properly located (VGluT2-cre: Fig.1C,D; GAD2-cre: Fig.1E,F). Animals presenting irregularities for one of these two parameters were removed from the experiment. In this respect, VN neurons co-expressed the mCherry viral fluorescence and the DAPI staining, indicating that VN neurons were locally infected in both VGluT2-cre (Fig.1C, right) and GAD2-

cre (Fig.1E, right) animals. We could also verify that the optical fibers were properly implanted in the upper parvicellular part of medial vestibular nucleus (Fig.1C,E). This positioning was on average respected in all animals, as shown by the location of the optical fibers across all experiments in VGluT2-cre (Fig.1D) and GAD2-cre (Fig.1F) mice.

Unilateral optogenetic stimulation of VN-VGluT2+ neurons, but not GAD2 neurons, triggers strong posturolocomotor deficits

Before starting the optogenetic stimulation protocol, we performed in a group of mice, a unilateral vestibular deafferentation using chemical labyrintectomy, in order to compare the functional consequences of optogenetic stimulation with characteristic acute vestibular syndrome, Transtympanic administration of sodium arsanilate was performed in WT mice as previously reported in rat (Vigneaux et al. 2012), and postural behavior of animals was recorded and analyzed in an open field using a set of parameters previously detailed (Pericat et al. 2017). These parameters included head tilt, bobbing, circling, retropulsion and tumbling (Fig. 2A). Posturolocomotor behavior was studied at several time points over 3 weeks, and the cumulative scores were reported on a posturolocomotor deficit graph (Fig. 2B, gray). Animals were strongly and significantly affected on the first post-lesion days, especially at Day 2 (Baseline = 0 vs D2 = 8.17, $P<0.05$, $t=2.69$, $df=8$), then partially recovered from D3 and over the 3 Weeks of survey (D2 vs W3, $P<0.05$, $t=2.94$, $df=5$, Fig. 2.B). Qualitatively analyzing the D2 post-lesion experiment, we can notice a relative heterogeneity of the deficits between animals, with some showing only head tilt, whilst others displaying all categories of symptoms, including tumbling (Fig. 2.C).

VGluT2-cre ChR2 and the control mCherry group were place in an open field and observed during 5 min in the environment during which animals were not showing any sign of posturolocomotor deficit. Then they received persistent 30s optogenetic stimulation (20Hz, 5ms, 473nm): during the 30s stimulation phase, the VGluT2-ChR2 group shown strong posturolocomotor deficit composed of head-tilt, bobbing, circling, retropulsion and tumbling (Fig. 2D, left). In particular, a major part of the VGluT2-cre (6 animals over 9) animals shown instantaneous tumbling behaviors immediately after the beginning of stimulation (Fig. 2D, 2F,

Sup. Video 1). In comparison, the control group of VGluT2-mCherry animals shown no deficit during the entire stimulation period (Fig. 2D, right). Quantitatively, the VGluT2-ChR2 group tended to present a higher deficit than the WT-lesioned animals, but not significantly compared with D2 post-lesion (D2 = 8.17 vs VGluT2-ChR2 = 11.67, $P>0.1$; Fig. 2B, red). We then performed the same experiment by using GAD2-cre animals, to stimulation VN GABAergic neurons (60Hz, 5ms, 473nm). VN-GAD2 neurons stimulation did not cause any postural deficits in the ChR2 or mCherry animals (Fig. 2E), as also demonstrated in the video examples (Fig. 2G). On average, the postural behavior in the GAD2-ChR2 animals was comparable to the non-lesioned animals (GAD2-ChR2 = 0 vs VGluT2-ChR2 = 11.67, $P<0.001$, $t=6.48$, $df=15$; Fig. 2B, blue). These data indicate that activation of VN glutamatergic neurons can provoke immediate, and strong posturolocomotor deficits, in average comparable to those observed during peak of vestibular unilaterally deafferentations. In contrast, activation of GABAergic neurons does not appear to have such a marked effect.

Tail-hanging landing test confirmed VN-VGluT2+ population is mainly responsible of posturolocomotor deficits, compared with VN-GAD2+ population

Our previous results revealed that only VN-VGluT2+ and not VN-GAD2+ neurons could provoke posturolocomotor deficits, whilst the animals were standing on a static ground. To verify whether this observation persists in condition where vestibular compensation is removed, we performed a tail-hanging landing test and scored the three mains following parameters: landing, twirl and posture after landing (Fig. 3A). First, WT animals following arsanilate injection shown strong and homogeneous deficits, in particular when observing twirls and posture after landing, whilst landings were only moderately affected (scored 1 for 4 animals over 6; Fig. 3C). Over the testing, the effects were especially marked at D2 post-lesion (Baseline = 0 vs D2 = 7.33, $P<0.0001$, $t=27.8$, $df=8$), but did not recover even 3 weeks after surgery (D2 vs W3, $P<0.005$, $t=4.83$, $df=5$, Fig 3B, gray). Next, the VN-VGluT2+ and VN-GAD2+ were lifted similarly to WT group, and were then subject to 30s continuous optogenetic stimulation. Confirming previous results, all VGlut2-ChR2 animals shown the full range of postural deficits, some stronger than

lesioned group; apart for one animal only showing moderate symptoms and no deficit during landing (Fig. 3D, left, Fig. 3F). When facing the same experiment, control VGluT2-mCherry animals did not show any deficit (Fig. 3D, right), confirming viral injection surgery did not damage the VN. Overall, stimulation of VN-VGluT2+ provoked postural deficits similar to those with arsanilate at D2 post-lesion (D2 = 7.33 vs W3 = 7.78, P>0.1; Fig. 3B, red). Next GAD2-ChR2 animals were tested in the same condition. When delivering the stimulation, we could not detect any variation of postural behavior in GAD2-ChR2, which remained indistinguishable from control GAD2-mCherry (Fig. 3E,G). GAD2-ChR2 stimulation was, overall, not similar to arsanilate animals before lesion. Together, these data indicated that brief activation of VN-VGluT2+ is sufficient to provoke strong postural imbalance, whilst transient activation of VN-GAD2+ neurons do not seem to participate in equilibration, at least at very short term.

Optogenetic stimulation of VN-VGLuT2+ induced reversible postural instability

To asses in more thoroughly static postural instability caused by VN stimulation, we monitored the support surface area (Marouane et al. 2020) of the animals before, during and 5 minutes after optogenetic stimulation, as well as with animals lesioned with arsanilate. The area was calculated by measuring the surface delimited by the four legs of animals while they were static during for at least one second. Before stimulation or lesion, VGluT2-ChR2, GAD2-ChR2, and WT-lesioned animals displayed similar scores (Fig. 4A, Pre). Then during optogenetic stimulation, VGluT2+ mice drastically increased their support surface area compared to before stimulation (VGluT2-Pre = 0.006 % vs VGluT2-Stim = 0.0145 %, P<0.0001, t=13.66, df=7), and to WT-lesioned at D2 (VGluT2-Stim = 0.0145 % vs D2 = 0.0113 %, P<0.05, t=2.124, df=12; Fig. 4A,B,D). In line with previous results, GAD2-ChR2 stimulation did not cause any significant modification of the area, and was statistically lower from VGluT2-ChR2 stimulation (VGluT2-Stim = 0.0145 % vs GAD2 = 0.0055 %, P<0.0001, t=10.76, df=14, Fig. 4A, Stim; Fig. 4C). 5 minutes after the stimulation, VGluT2-ChR2 animals were still higher than GAD2-ChR2 (VGluT2-Stim = 0.0068 % vs GAD2 = 0.0054 %, P<0.05, t=2.49, df=14; Fig. 4A,C), but were not significantly different from the pre-stimulation baseline state. These results confirmed stimulation of VN-VGLuT2+, but not VN-

GAD2+, artificially provoked continuous instability during the whole duration of stimulation, comparable and even stronger than unilateral vestibular deafferentation model. Importantly, this effect was reversible and seemed to fade some minutes after the end of the stimulation.

VN-VGluT2+ activation provokes short term disturbances of locomotor functions

To assess the functional consequences of unilateral optogenetic stimulation of VN on locomotor functions, we next studied several parameters previously identified as biomarkers of locomotor dysfunctions in rodents (Rastoldo et al. 2020). These parameters were monitored on animals placed in an open field before, 5 minutes after, and 1 hour after optogenetic stimulation. Before stimulation, VGluT2-ChR2 and VGluT2-mCherry animals walked a similar distance, but right after stimulation VGluT2-ChR2 drastically decreased their displacements ($P<0.05$, $t=3.86$, $df=5$), especially compared to control animals (ChR2 = 2.04 vs mCherry = 5.87, $P<0.001$, $t=4.7$, $df=11$, Fig. 5A). After 1h, when animals were placed again in the apparatus, VGluT2-ChR2 displacement did not significantly differed anymore compared to the VGluT2-mCherry group. In parallel, the velocity of the VGluT2-ChR2 right after stimulation was significantly lower than the control VGluT2-mCherry group (ChR2 = 0.012 vs mCherry = 0.019, $P<0.001$, $t=4.6$, $df=11$). This difference disappeared 1h after stimulation, although again VGluT2-ChR2 tended to be slightly lower (Fig. 5B). Also, after optogenetic stimulation VGluT2-ChR2 spent significantly more time immobile (freezing time) compared with control VGluT2-mCherry animals (ChR2 = 86.46% vs mCherry = 53.15%, $P<0.005$, $t=3.69$, $df=11$), being still higher 1h after stimulation but not significantly (Fig. 5C). Overall, these results indicated stimulation of VN-VGluT2+ significantly altered animals locomotion even 5 minutes after stimulation, but this effect disappeared after at least 1 hour. In comparison, we could not find any significant difference or trend for GAD2-ChR2 animals compared with GAD2-mCherry, either for the distanced moved (Fig. 5D), the mean velocity (Fig. 5E) or the immobility time (Fig. 5F), at any time point of the experiment. Together these results corroborated previous data, and indicated that stimulation of VN-VGluT2+ neurons, but not VN-GAD2+ continued to have an effect on locomotion for a short period of time after stimulation.

Discussion

This study demonstrates for the first time that it is possible to reproduce the posturo-locomotor deficits characteristic of vestibular syndrome, by optogenetic stimulation of the vestibular nuclei area in rodents. By authorizing the selective stimulation of the different types of nerve bundles originating from the vestibular nuclei (VN), this approach brings new opportunities to identify the projection zones of central vestibular efferents, and to better understand their functional relevance under normal or pathological conditions.

Technical considerations

Among the four main vestibular nuclei, we choose to stimulate specifically the medial vestibular nuclei (MVN) for different reasons: The medial vestibular nucleus is the one most extensively studied in animals anatomo-functional studies; for review, see (Paterson et al. 2004). It the largest of the nuclei form the vestibular nuclear complex (VNC) in most mammals (Alvarez et al. 1998). This nucleus is known to receive convergent semicircular and otolithic afferents, and to be involved in both oculomotor and postural functions (Victor J Wilson and Jones 1979). The MVN contains a wide diversity of neuron classes which project to the oculomotor nuclei, spinal cord, cerebellum, thalamus, contralateral vestibular nuclei, or function as interneurons (Straka et al. 2005; Highstein and Holstein 2006). It contains both glutamatergic and GABAergic neurons (Gliddon, Darlington, and Smith 2005; de Waele, Mühlethaler, and Vidal 1995), which can themselves be divided in subgroups depending of their morphology, their respective networks, or their molecular background. In this context, AAV virus injection in VGluT2-cre and GAD2-cre only allow to stimulate glutamatergic or GABAergic neurons as if it was homogeneous neuronal populations. This additional layer of complexity cannot be targeted by using this method, and will necessitate future investigation to elucidate.

Functional considerations

To better appreciate the expected functional consequences of the stimulation of these respective sub-nuclei zones, it is worthy to remind the organization and projections of the VNs. The medial

vestibulospinal tract is primarily composed of axons from the MVN neurons, but fibers from the lateral and descending vestibular nuclei also contribute to the tract (Carpenter 1991). Axons of the medial tract travel in the medial longitudinal fasciculus and principally terminate in upper cervical regions of the spinal cord that innervate upper-body musculature, particularly neck musculature (the extent to which the medial tract innervates lower segments of the spinal in humans is unknown). Thus, the MVN and medial vestibulospinal tract provide the circuitry necessary to rapidly control vestibulocollic reflexes and neck postural changes associated with alterations in head orientation. The lateral vestibulospinal tract is the largest of the two tracts and is primarily composed of axons from neurons in the lateral vestibular nucleus (LVN, also referred as Dieter's nucleus), with some contribution from the descending (inferior) nucleus. The fibers descend ipsilaterally in the ventrolateral columns and have branches that innervate multiple levels of the spinal cord, thus providing the capacity to modulate spinal motoneuron activity across segments (Abzug et al. 1974). The primary sites of termination of vestibulospinal projections are on to interneurons in Rexed's laminae VII and VIII, thus this pathway indirectly influences spinal motoneuron activity via di- or polysynaptic connections. Rexed's lamina VII is also a major site of termination proprioceptive afferents and descending projections from reticulospinal and corticospinal pathways. Thus, vestibulospinal pathways are well positioned to modulate the excitability of reflex responses to anticipated or imposed postural displacements. Experiments in animals have shown that electric stimulation of the LVN evokes a net increase in the excitability of extensor motoneurons and inhibitory effects on flexor motoneurons (V J Wilson and Yoshida 1969; Grillner, Hongo, and Lund 1970; McCall, Miller, and Yates 2017). The facilitatory effect of vestibulospinal inputs on extensor motoneuron excitability suggests that the predominant function of this pathway is to provide appropriate levels of extensor tone, to achieve vertical support against gravity. In present study, the characteristic behavior observed in optogenetically-stimulated mice, such as tumbling, circling, and retropulsion could be attributed to the specific stimulation of the MVN, while the enlargement of the support surface, and head tilt (observed but not measured in present study) resulted from stimulation of the LVN.

It is interesting to mention that the posturo-locomotor syndrome induced by unilateral optogenetic stimulation of glutamatergic MVN neurons is the mirror image of the typical

posturo-locomotor behavior evoked by unilateral lesion of the vestibular system with arsanilate. In fact, during the optogenetic stimulation of MVN glutamatergic neurons, the animals start to rotate on the opposite side of the simulation, while they perform a circling towards the side ipsilateral to the lesion in the arsanilate model. These typical postural behaviors generated by both optogenetic stimulation and arsanilate lesion result from the electrophysiological asymmetry between ipsi and contralateral MVNs.

Indeed, a large number of studies have shown that immediately after unilateral vestibular lesion, the resting discharge rate of the MVN neurons on the ipsilesional side is abolished, while the activity of MVN neurons on the contralesional side is either normal or increased (Smith and Curthoys, 1988 a, b; Newlands and Perachio, 1990 ; Ris et Godeaux, 1998). In this situation, the vestibular deficit always concerns the side of the body corresponding to the MVN which has lost its electrical activity. Muscle hypotension, falls, head tilt and circling affect the side in which the MVN is electrically deficient. Furthermore, the decline of the posturo-locomotor symptoms coincides with a recovery of the resting activity of ipsilesional MVN (Lacour and Tighilet , 2010). The posturo-locomotor deficits consecutive to optogenetic stimulation of MVN Glutamatergic neurons likely result from an electrophysiological imbalance with a highly increased activity on the stimulated side. A moderate activation of the non-stimulated side may also take place through the stimulation of the excitatory commissural neurons that are also glutamatergic. Indeed, most of the commissural inhibition is provided by type I excitatory neurons on one side acting on type II inhibitory neurons on the other side, thus releasing GABA in tonic type I neurons on the opposite side (Kasahara et al. 1968; Mano, Oshima, and Shimazu 1968; Precht, Schwindt, and Baker 1973).

When the optogenetic stimulation is interrupted, a restoration of normal postural behavior is observed as the electrophysiological balance is restored between opposing MVNs. The advantage of the optogenetic stimulation protocol is that we instantly appreciate the posturolocomotor recovery phenomenon. The kinetics of vestibular compensation at a behavioral and electrophysiological levels is later in the cases of vestibular lesion models. The disappearance of

the behavioral deficit as well as the rebalancing of spontaneous activity in the homologous NVM is observed around one week after unilateral vestibular lesion of the vestibular system in the guinea pig (Ris et al. 1997).

Unlike the glutamatergic neural network, unilateral optogenetic stimulation of GABAergic neurons in the MVN did not produce a posturo-locomotor syndrome. Optogenetic stimulation of the GABAergic neural network should have induced ipsilateral inhibition of the MVN, thus generating an ipsilateral postural syndrome similar to that observed after arsanilate injury. One can ask the question of what types of MVN GABAergic neurons were activated by the optogenetic stimulation? Answering this question is a challenge considering the complex architectural organization of the MVN GABAergic neural network. Indeed, the GABAergic neurons within the vestibular nuclear complex (VNC) have been classified into five functionally distinct groups (Highstein and Holstein 2006). Group one consists of commissural GABAergic neurons. Group two consists of GABAergic neurons that project to the oculomotor motoneurons that are involved in the vestibulo-ocular reflex (VOR); for review see (Beitz and Anderson 2000). These two groups of neurons are located predominantly in the superior vestibular nucleus (SVN) and the rostral medial vestibular nucleus (MVN) and send projections through the ipsilateral medial longitudinal fasciculus. Group three consists of GABAergic neurons within the MVN, prepositus hypoglossi (PH), and inferior vestibular nucleus (IVN) that project to the nucleus of the inferior olive. Group four consists of GABAergic neurons within the rostral MVN and lateral vestibular nucleus (LVN) that project to the medial vestibulo-spinal tract. Finally, group five consists of local interneurons. To answer the above question, it would be necessary to know which type of GABAergic neurons was the target of this optogenetic stimulation. We can possibly refer to a morphological criterion size. Indeed, it can be proposed that the size can indicate whether it is a local GABAergic neuron or on the conversely a projection neuron (Tighilet et al. 2007). However, the size parameter is probably not sufficient per se to establish the real nature of the GABAergic neurons targeted by the optogenetic stimulation. Neuroanatomical tracing and electrophysiological experiments should clarify the nature of these neurons.

Additional explanation for the absence of behavioral effect under optogenetic stimulation of GABAergic neurons is related to the low ratio of GABAergic neurons in the VNC. Indeed, the number of GABAergic neurons was estimated to be less than 10% of the VNC neurons(Walberg, Ottersen, and Rinvik 1990). It is therefore likely that the optogenetic stimulation did not recruit a sufficient number of neurons to generate an inhibition of the NVM. Finally, it is possible that optogenetic stimulation targeted vestibulospinal GABAergic neurons with bilateral projections, thus canceling any effect. The vestibular nuclei influence postural control via the medial (mostly from ipsi and contralateral NVM neurons) vestibulospinal tracts (Victor J Wilson and Jones 1979).

Taken that efferences arising from the MVePC also project onto oculomotor muscles, as well as in multiple cortical areas; for review see (Christophe Lopez 2016; Christophe Lopez and Blanke 2011), it is likely that VN unilateral optogenetics stimulation also triggers other functional alterations through modulating the vestibulo-ocular reflex (nystagmus and oscillopsia), vestibulo-sympathetic reflex (increase heart rhythm, drop of body temperature) (Balaban 1999; Yates, Bolton, and Macefield 2014), and vestibulo-cortical pathways (spatial disorientation). Further studies will be necessary to decipher whether these functional consequences reported upon unilateral labyrinthectomy and UVN, also take place after optogenetics VN stimulation. The optogenetic approach, which makes it possible to specifically stimulate neurons in different areas of the brain, is suitable for identifying downstream networks originating from the VNs, but also the upstream pathways heading to these same VNs and which can control their functioning.

Materials & Methods

Animals

All procedures were approved by Animal Care and Use Committees in the Shenzhen Institute of Advanced Technology (SIAT), Chinese Academy of Sciences (CAS). Wild type C57BL/6 (6–8 weeks) mice were purchased from Beijing Vital River Laboratory Animal

Technology Co. Ltd, Beijing, China). Adult (6-8 weeks old) male VGluT2-ires-cre (Jax No. 016963) and male GAD2-ires-cre (Jax No. 010802) transgenic mice were used in this study. All mice were maintained on a 12/12-h light/dark cycle at 25°C. Food and water were available ad libitum.

Viral preparation

For optogenetic experiments, we used one of the following viruses depending of the protocol: AAV9-EF1a-DIO–hChR2(H134R)–mCherry, AAV9-EF1a-DIO–mCherry,. Virus titer were approximately of 2-6x10^{12} vg/ml. Viruses were purchased from Brain VTA Co., Ltd., Wuhan or Shanghai Taitool Bioscience Co.Ltd. .

Viral injections

VGluT2-ires-cre and GAD2-ires-cre mice were anesthetized with pentobarbital (i.p., 80 mg/kg) and fixed on stereotaxic apparatus (RWD, Shenzhen, China). During the surgery, mice were kept anesthetized with isoflurane (1%) and placed on a heating pad to keep the body temperature at 35°C. A 10 μL micro-syringe with a 33-Ga needle (Neuros; Hamilton, Reno, USA) was connected to a microliter syringe pump (UMP3/Micro4; WPI, USA) and slowly placed for virus injection into VN (coordinates: AP:–6.0 mm, ML: –1.2 mm and DV:–4.5 mm). Then virus was injected (120nl at the rate of 50nl/min). After the injection, the needle was kept in the place for an additional 5-10 min to facilitate the diffusion of the virus after which it was slowly withdrawn.

Implantation of optical fibers

Optical fibers (200um in diameter, NA : 0.37) were chronically implanted in the VN 3 weeks following virus expression. For optogenetic experiments, the optical fiber was unilaterally implanted above VN（AP:–6.0 mm, ML: –1.2 mm and DV:–3.8 mm). After surgery all animals were allowed to recover at least during 1 week.

Histology, immunohistochemistry, and microscopy

Mice were euthanized with overdose of chloral hydrate (300mg/kg, i.p) and transcardially perfused with ice-cold 1 x PBS and then with ice-cold 4% paraformaldehyde (PFA, sigma) in 1 x PBS. The brains were extracted from the skull and submerged in 4% PFA at 4°C overnight and then switched to 30% sucrose in 1 x PBS to equilibrate. Brains were cut into 40 μm thick coronal sections with cryostat microtome (Lecia CM1950, Germany). Finally, slices were mounted and cover-slipped in anti-fade aqueous mounting reagent with DAPI (ProLong Gold Antifade Reagent with DAPI, life technologies). The brain sections were then photographed and analyzed with an Olympus VS120 virtual microscopy slide scanning system and ImageJ, Photoshop software.

Arsanilate injection

Once the mice were deeply anesthetized using pentobarbital (i.p., 80 mg/kg), we created a hole in the eardrum using a micro needle (30 gauge) and infused 0.150 ml of a 10% solution of p-arsanilic acid (Abcam ab120100, dissolved in PBS). Once the transtympanic instillation was done, we kept the mouse on its side for 30 min (the lesion side was on the top).

Behavior

For the arsanilate animals, we assessed behavioral capacities of the mice at 8 times points. Base line (before surgery), 1 day after surgery (24 h), 2 days after surgery (48 h), 3 days after surgery (72 h), 1 week after surgery (1W), 2 weeks after surgery (2W) and 3 weeks (3W). The animals performing optogenetic stimulations were exposed to the same apparatus as the arsanilate group, and could habituate to the open field a day before the experiment. During the stimulation day, animals were first placed in the field for a first phase of 5 min without stimulation , then the optogenetic stimulation (473-nm blue laser; Aurora-220-473, NEWDOON, Hangzhou) was delivered for 30 seconds (VGluT2: 20Hz, 5ms, 473nm; GAD2: 60Hz, 5ms, 473nm), finally followed by another 5 minutes without stimulation.

The locomotion test was performed in an open field (40cmx40cmx40cm) to which mice were habituated the day before starting of experiment. During the tests, mice were lifted by their tails

for 5 quick vertical movements, then were immediately placed in the open-field. Behaviors recording were made through 2 cameras (one top-view, one side-view) for a duration of 1 min, to asses the vestibular symptoms, which includes: (i) Tumbling: spontaneous or evoked rotations of the animals along their body axis, rated 5 in our evaluation scale; (ii) Retropulsion: characterizes backwards movements of the animals. rated 4; (iii) Circling: circular movements of the rats in the horizontal plane, rated 3; (iv) Bobbing: relates to rapid head tilts to the rear and is assimilated to cephalic nystagmus, rated 2; (v) Head tilt, rated 1. When none of these behaviors were observed, animals received a score of 0.

For the tail-hanging landing paradigm (THL), mice were lifted by their tails for 5 quick vertical movements. This test normally induced a forelimb extension as the animals reach the ground. Unilateral vestibular deficit resulted in trouble in the landing process. Such deficits were quoted from 0 (perfect preparation of the two front paws before reaching the ground) to 3 (absence of preparation for landing). The landing process was accompanied by axial rotation of the body, i.e. twirl item that we quantified from 0 (no rotation) to 3 (continuous twisting). We also monitored the intensity of the syndrome reactivation after the mice landed, from 0 (no sign) to 3 (max expression/accentuation of circling, tumbling, muscle dystonia, bobbing or head tilt). Mice receiving optogenetic stimulation during THL test were first exposed to the same protocol as arsanilate group (lifted by the tail without additional stimulation). Then they received optogenetic stimulation continuously during 30s, whilst being tested for lifting and landing. Two cameras recorded,1 from the side,1 from the bottom.

Data analysis

For speed, immobility, and distance, data were analyzed by using Anymaze software (Stoelting Co.). For support surface area, video frames were first analyzed using Kinovea software (https://www.kinovea.org/), and data were MATLAB to compute the size of the surface. Significance was determined using two-tailed Student's t-tests with a significance level of $p<0.05$.

Acknowledgements

This work was supported by Key-Area Research and Development Program of Guangdong Province 2018B030331001; National Natural Science Foundation of China (NSFC) 31930047(to L.W.); NSFC 31630031 (to L.W.); NSFC 91732304 (to L.W.); the Strategic Priority Research Program of Chinese Academy of Science XDB32030100; Key Laboratory of SIAT 2019DP173024; International Partnership Program of Chinese Academy of Sciences 172644KYS820170004 (to L.W.); Shenzhen Key Science and Technology Infrastructure Planning Project ZDKJ20190204002; Guangdong Province Basic Research Grant 2019A1515110870 (to X.L)

Author contributions

Quentin Montardy: Conceptualization, methodology, validation, formal analysis, data curation, writing – original draft, Writing - Review & Editing, visualization, project administration **Mengxia Wei:** Investigation (virus injection, fiber implantation, arsanilate injection and behavior, immunohistochemistry and microscopy), data curation and analyzes, writing – original draft, Writing **Tian Yi:** Investigation (virus injection and fiber implantation, arsanilate injection and behavior), data curation and analyzes, writing – original draft, Writing **Xuemei Liu:** Validation, investigation (performed immunohistochemistry and quantitative analyzed of the tracing data), , manuscript review and editing **Zhou Zheng:** Methodology (setup injection protocol), conceptualization, , manuscript review and editing **Stephane Besnard:** Conceptualization, manuscript review and editing **Brahim Tighilet:** Conceptualization, original draft writing, manuscript review and editing **Christian Chabbert:** Conceptualization, supervision, original draft writing, manuscript review and editing **Liping Wang:** Conceptualization, supervision & Project administration, manuscript review and editing

Figure Legends

Figure 1 - VN virus injection and optical fiber implantation. A. Experimental protocol used in the study. AAV9-DIO-ChR2-mCherry was injected under surgery in VN of VGluT2-ires-cre or GAD2-ires-cre. Three weeks later, the optical fiber was positioned in the vicinity of the VN, and the behavior tests and brain extraction were performed 1 week thereafter. **B.** Schematic representation of virus injection and optical fiber location in VN. **C.** Representative picture of virus expression in VN of VGluT2+ animals. Red: ChR2-mCherry virus; Blue: DAPI; Scale bar, 100 µm (left), and 50 µm (right). MVePC (parvicellular medial vestibular nucleus) MVeMC (magnoellular medial vestibular nucleus), SPVe (superior vestibular nucleus), LVe (lateral vestibular nucleus) **D.** Representation of fiber tip position for individual VGluT2+ animals (in red) **E.** Representative picture of virus expression in VN of GAD2+ animals. **F.** Representation of fiber tip position for individual VGluT2+ animals (in blue).

Figure 2 - Optogenetic stimulation revealed VN-VGluT2+ neurons, but not GAD2 neurons, could provoke strong posture-locomotor deficits. A. Illustration of the evaluation grid used when animals are placed in the open field. A first state in which all symptoms are expressed is rated 15. A second stage in which the tumbling has gone is rated 10. A third state in which both the tumbling and the retropulsion behaviors are absent is rated 6. Then, two states (rated 3 and 1 respectively) related to states in which both bobbing and head tilt, or head tilt alone remained. **B.** Illustration of the time course of the behavioral evaluation score across time for WT animals lesioned with arsanilate (gray), compared with VN-VGluT2+ (red) and VN-GAD2+ animals during optogenetic stimulation. **C.** Behavioral evaluation score of WT-lesioned animals, at D2 post-lesion, whilst animals were placed on a static ground. **D.** Diagram representation of the behavioral evaluation score of VGluT2-ChR2 animals (left), VGluT2-mCherry animals (right). **E.** GAD-ChR2 (left) and GAD2-mCherry (right) animals during a 30s stimulation whilst animals were on the ground. GAD-ChR2 and mCherry do not show any deficit. **F.** Pictures showing an example of posturo-locomotor behavior of a VGluT2-ChR2 animal (essentially tumbling here) and **G.** Illustration of a GAD-ChR2 animal during stimulation. Time in second.

Figure 3 - Tail-hanging landing test confirmed VN-VGluT2+ population is mainly responsible of posture-locomotor deficits, compared with VN-GAD2+ population. A.

Illustration of the evaluation grid used during tail-hanging landing test. **B.** Illustration of the time course of the behavioral evaluation score across time for WT animals lesioned with arsanilate (gray), compared with VN-VGluT2+ (red) and VN-GAD2+ animals during optogenetic stimulation. **C.** Behavioral evaluation score of WT-lesioned animals, at D2 post-lesion, whilst animals underwent tail-hanging landing test. **D.** Behavioral evaluation score of VGluT2-ChR2 animals (left), VGluT2-mCherry animals (right) **E.** GAD-ChR2 (left) and GAD2-mCherry (right) animals during a 30s stimulation during tail-hanging landing test. GAD-ChR2 and mCherry do not show any deficit. **F.** Pictures showing an example of posturo-locomotor behavior of a VGluT2-ChR2 animal. **G.** Illustration of a GAD-ChR2 animal during stimulation. Time in frame; left: before stimulation; right: during stimulation.

Figure 4 - Illustration of reversible postural instability upon optogenetic stimulation of VN-VGLuT2+ neurons. A. Surface area in percentage, averaged between all VN-VGluT2+ (red), VN-GAD2+ (blue) and WT-lesioned animals (gray). Three time points were investigated, of 5 minutes duration each: before stimulation, 5 minutes after stimulation, 1h after stimulation. **B.** Example of the support surface area computed on one individual VN-VGluT2+ animal and **C.** a VN-GAD2+ animal, before, right after, and 1h after stimulation, as well as **D.** Illustration of a WT-lesioned animal before and after lesion.

Figure 5 - VN-VGluT2+ activation provokes short term disturbances of locomotor functions.

Averaged locomotor behaviors for animals which received in VN either VGluT2-ChR2 (dark red) or VGluT2-mCherry injection (light red), whilst freely moving in an open field. Three time points were investigated, of 5 minutes duration each: before stimulation, 5 minutes after stimulation, 1h after stimulation. **A.** Distance moved by the animals, in meter. **B.** Mean velocity of the animals during movement, in m/s. **C.** Time animals spent immobile whilst in the apparatus, in percentage of total experimental time. Averaged locomotor behaviors for GAD2-ChR2 (dark blue) or GAD2-mCherry injection (light blue), computed as previously described **D.** Distance moved (m). **E.** Mean velocity (m/s). **F.** Time animals spent immobile (%).

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Fig. 1

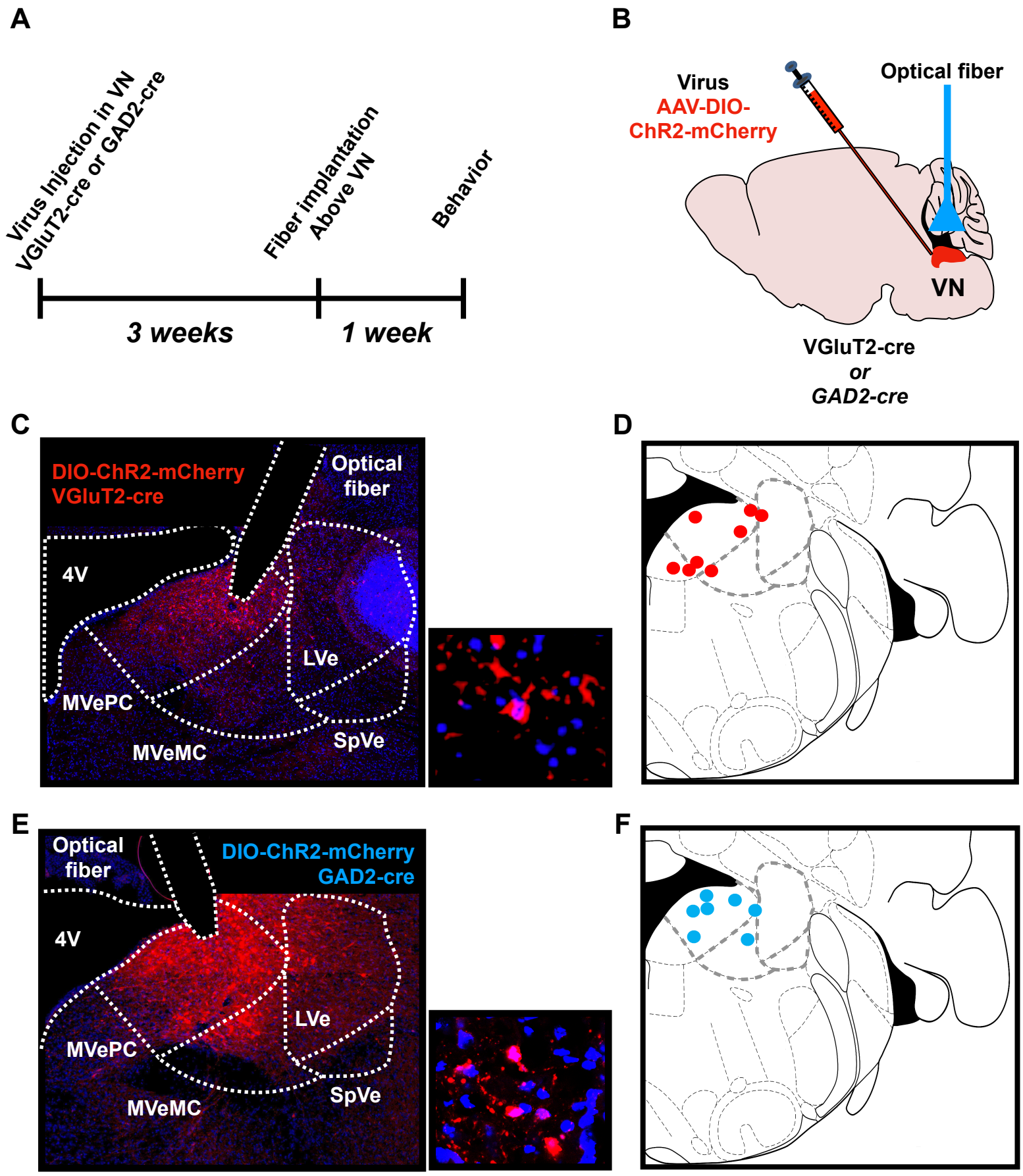
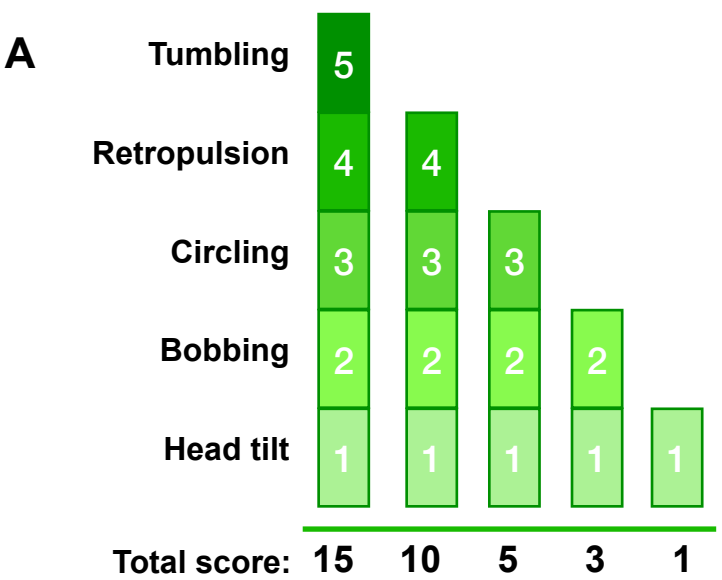
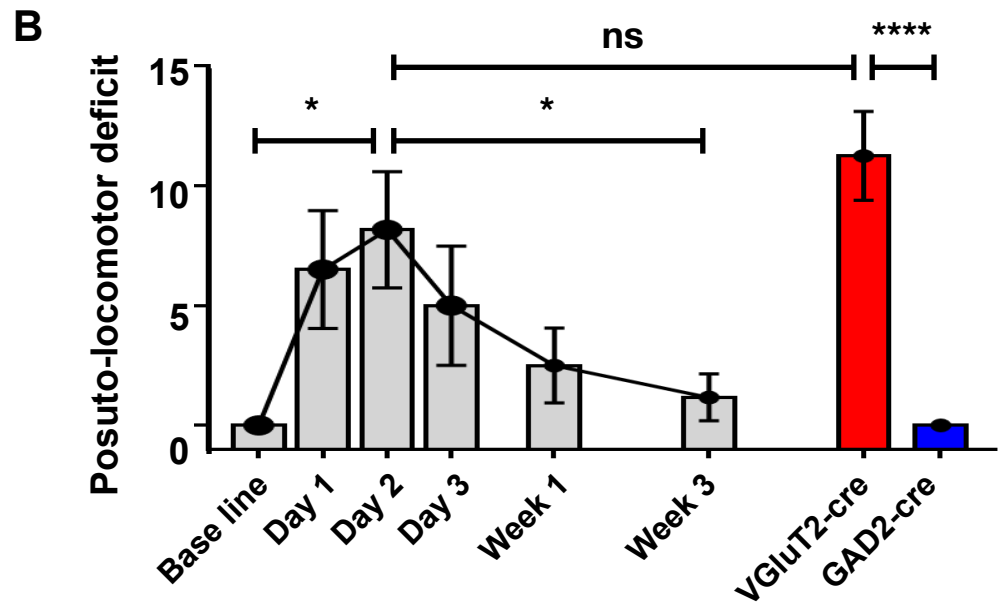

A
Virus Injection in VN
VGluT2-cre or GAD2-cre
Fiber implantation
Above VN
Behavior
3 weeks
1 week
B
Virus
AAV-DIO-
ChR2-mCherry
Optical fiber
VN
VGluT2-cre
or
GAD2-cre
C
DIO-ChR2-mCherry
VGluT2-cre
Optical
fiber
4V
LVe
MVePC
MVeMC
SpVe
D
E
Optical
fiber
DIO-ChR2-mCherry
GAD2-cre
4V
LVe
MVePC
MVeMC
SpVe
F

Fig. 2


A
Tumbling
Retropulsion
Circling
Bobbing
Head tilt
Total score: 15 10 5 3 1


B
Posuto-locomotor deficit
ns
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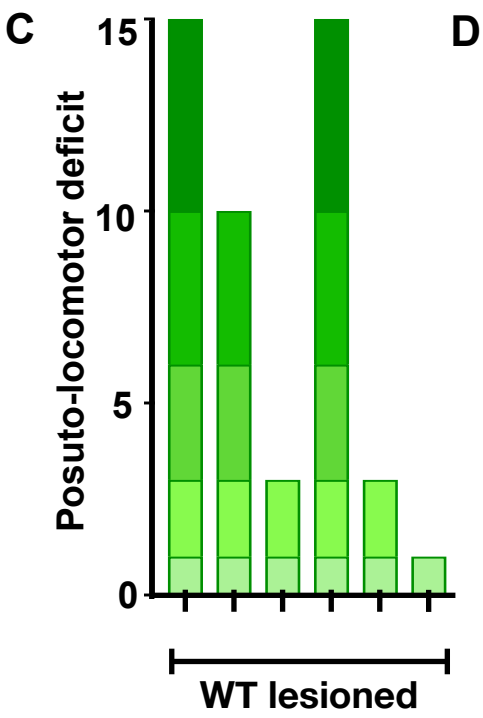
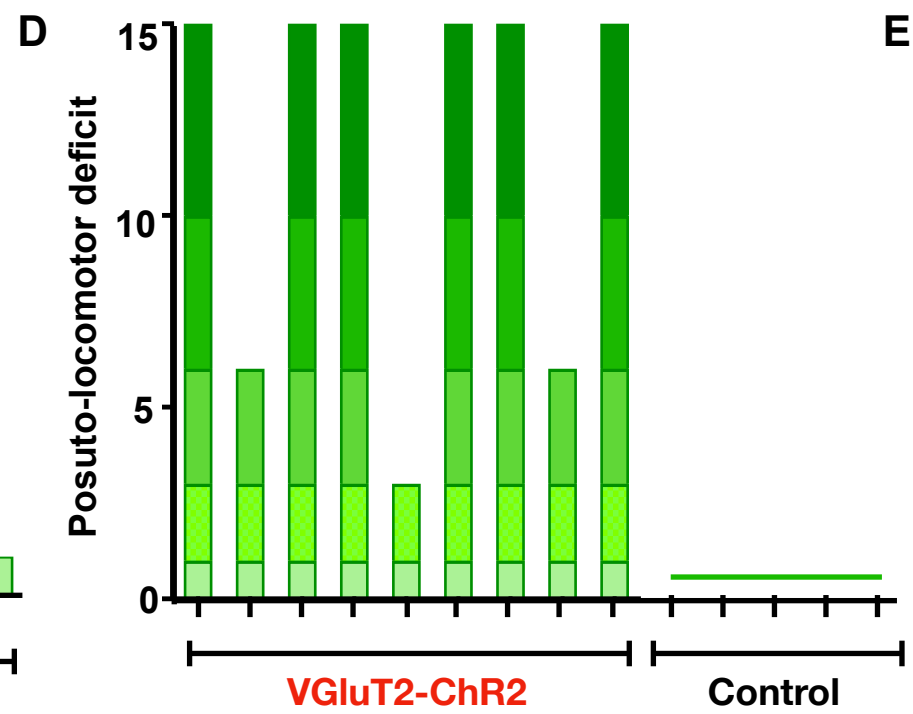
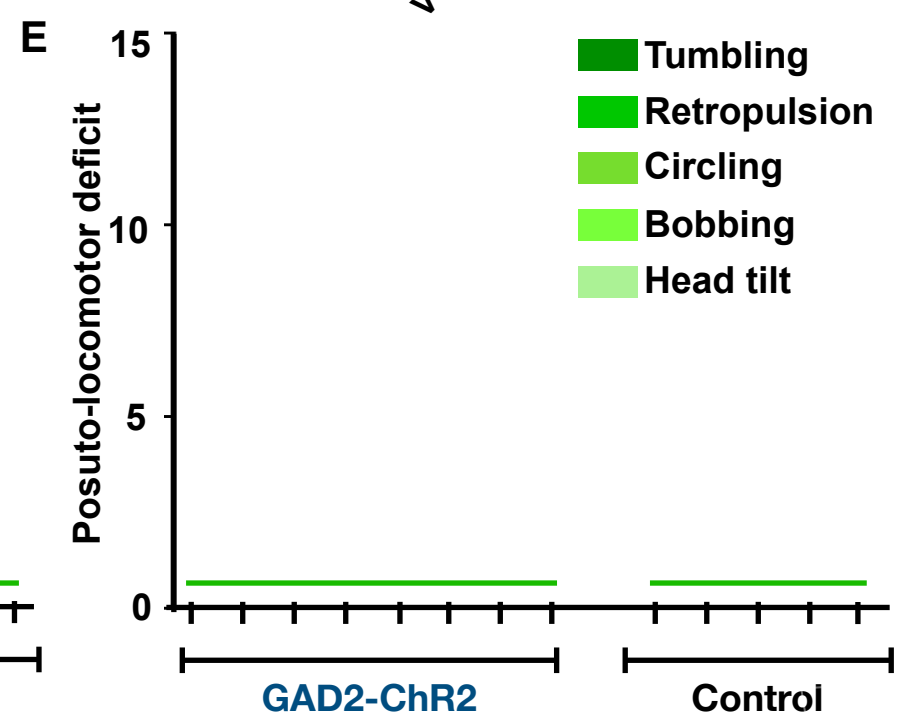
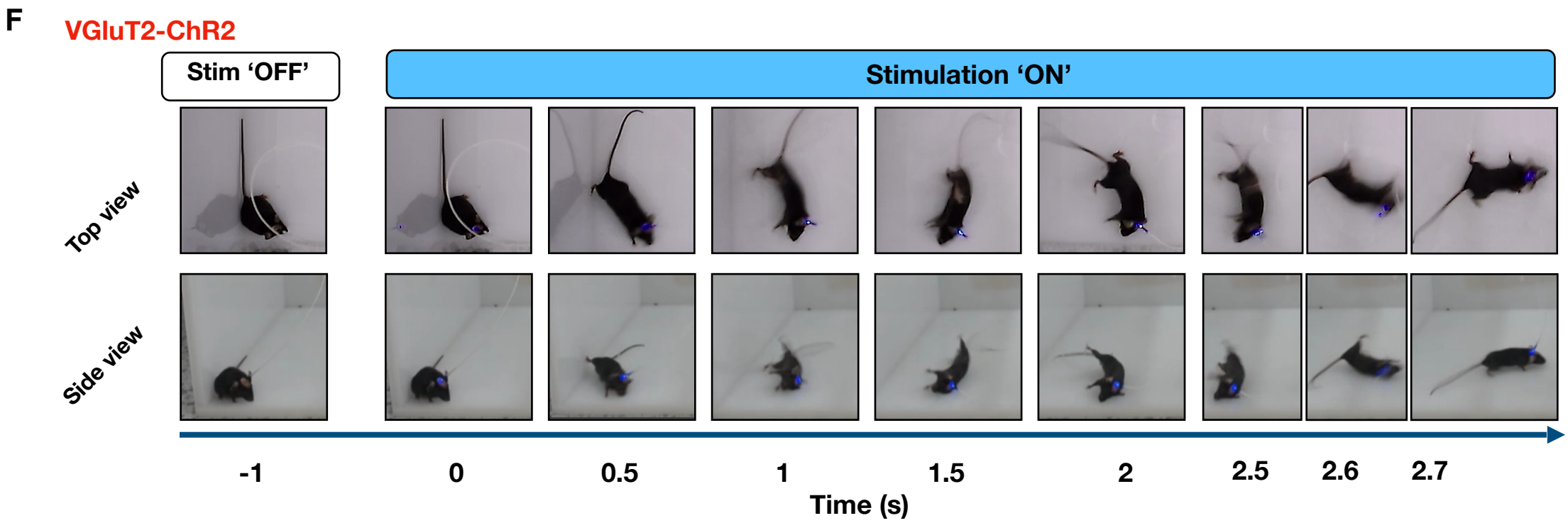
*
*
Base line
Day 1
Day 2
Day 3
Week 1
Week 3
VGluT2-cre
GAD2-cre


C
Posuto-locomotor deficit
WT lesioned


D
Posuto-locomotor deficit
VGluT2-ChR2
Control


E
Posuto-locomotor deficit
Tumbling
Retropulsion
Circling
Bobbing
Head tilt
GAD2-ChR2
Control


F
VGluT2-ChR2
Stim 'OFF'
Stimulation 'ON'
Top view
Side view
-1
0
0.5
1
1.5
2
2.5
2.6
2.7
Time (s)

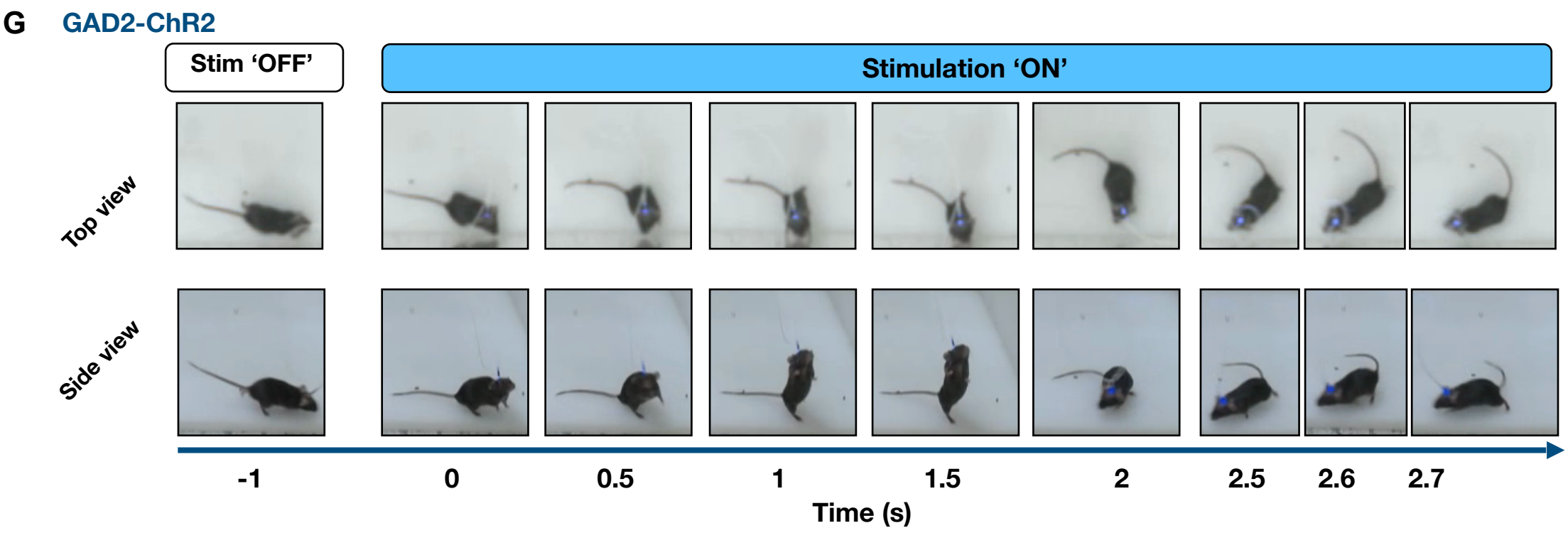
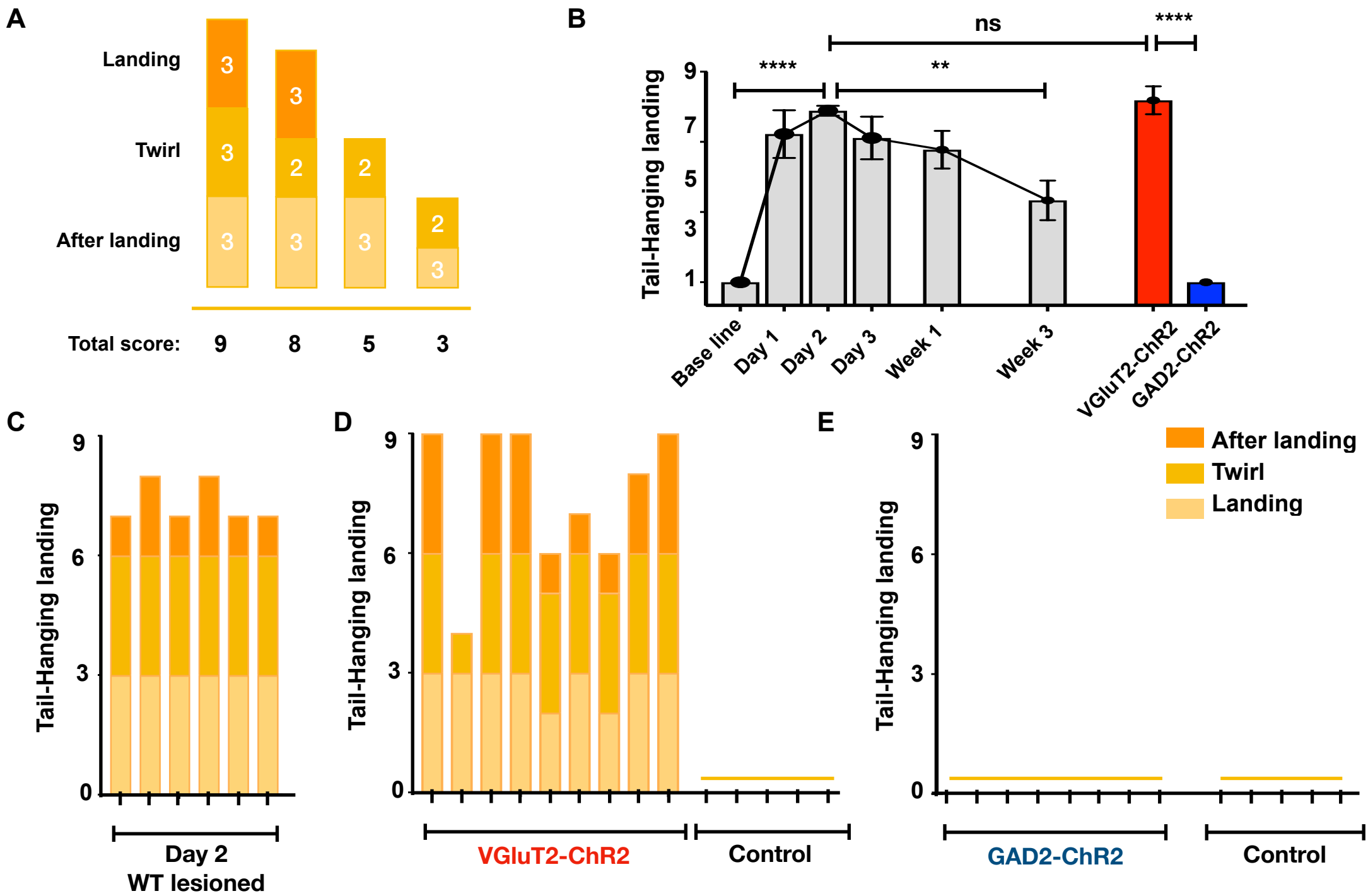

G
GAD2-ChR2
Stim 'OFF'
Stimulation 'ON'
Top view
Side view
-1
0
0.5
1
1.5
2
2.5
2.6
2.7
Time (s)

Fig. 3


A
Landing
Twirl
After landing
Total score: 9 8 5 3
B
ns
****
****
**
Tail-Hanging landing
Base line
Day 1
Day 2
Day 3
Week 1
Week 3
VGluT2-ChR2
GAD2-ChR2
C
Tail-Hanging landing
Day 2
WT lesioned
D
Tail-Hanging landing
VGluT2-ChR2
Control
E
Tail-Hanging landing
GAD2-ChR2
Control
After landing
Twirl
Landing

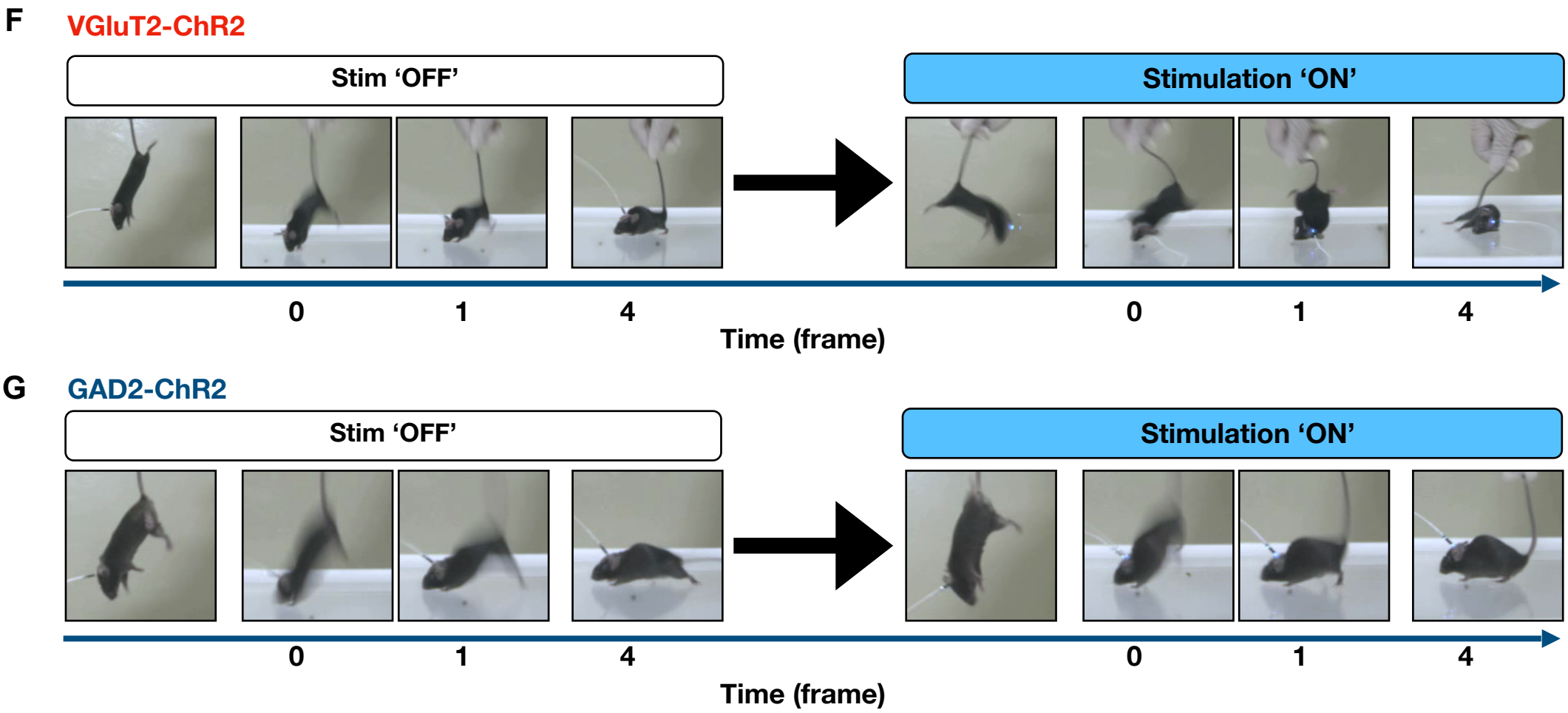
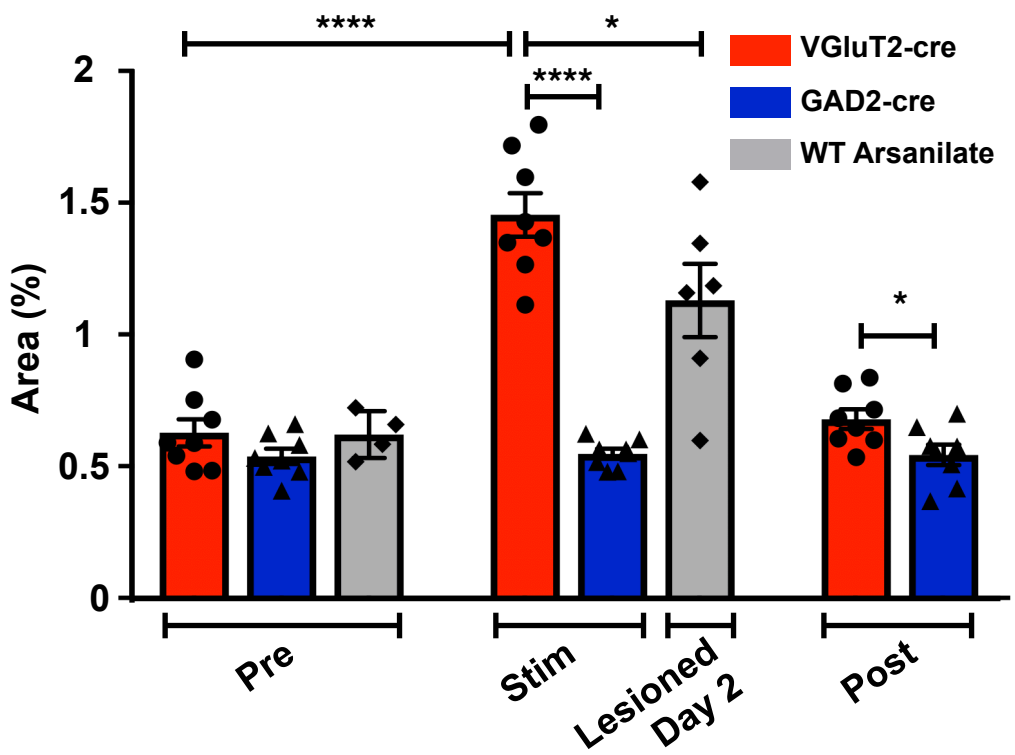

F
VGluT2-ChR2
Stim 'OFF'
Stimulation 'ON'
0
1
4
0
1
4
Time (frame)
G
GAD2-ChR2
Stim 'OFF'
Stimulation 'ON'
0
1
4
0
1
4
Time (frame)

Fig. 4

A



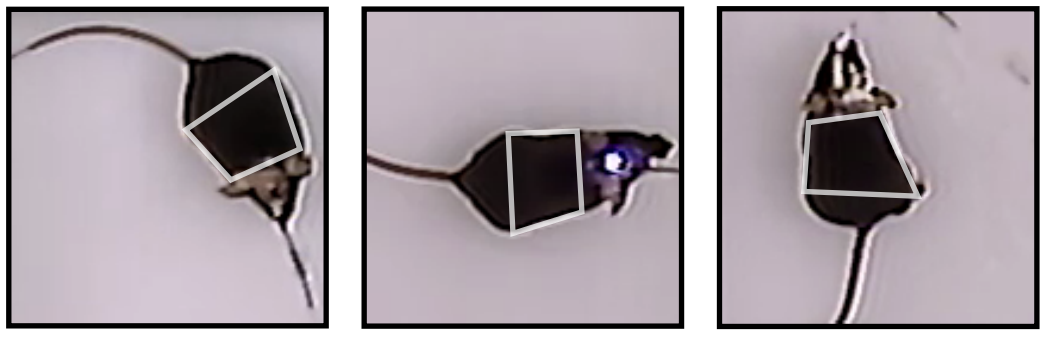
B

VGluT2-cre

Pre-stim | Stim | 3 min post-stim

C

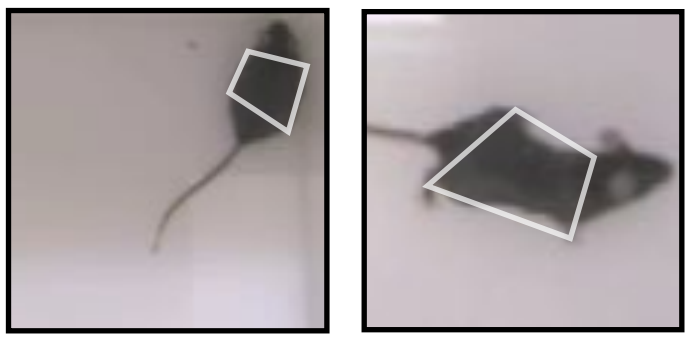
GAD2-cre



Pre-stim | Stim | 3 min post-stim

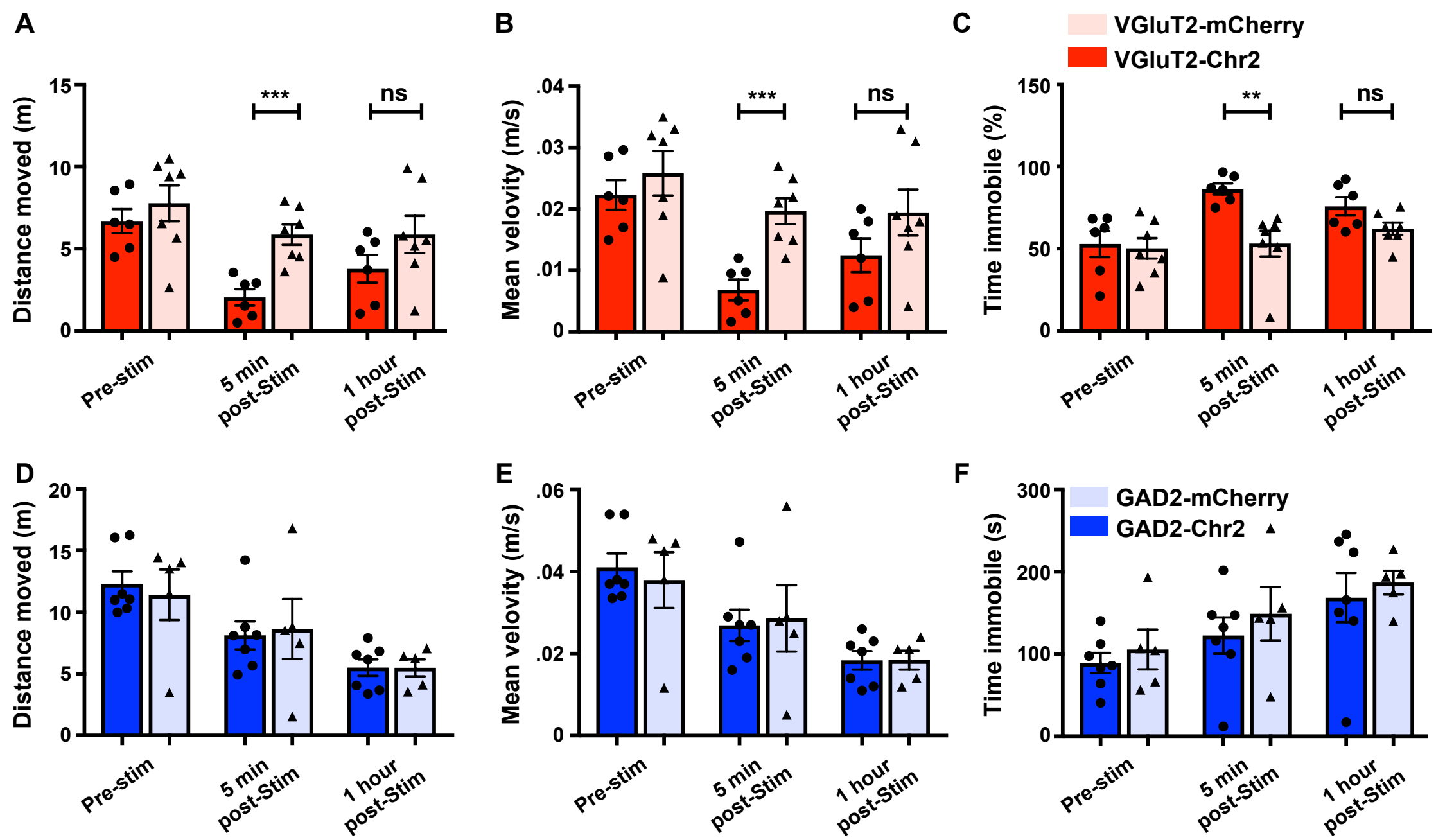
D

WT-lesioned



Pre-stim | Day 2

Fig. 5


A
B
C
D
E
F
VGluT2-mCherry
VGluT2-Chr2
GAD2-mCherry
GAD2-Chr2
Distance moved (m)
Mean velovity (m/s)
Time immobile (%)
Time immobile (s)
***
**
ns
Pre-stim
5 min post-Stim
1 hour post-Stim