UNIVERSITY OF CALIFORNIA, SAN DIEGO

Characterizing behavioral phenotypes of Dishevelled-2 knockout mice

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in

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by

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The Thesis of Areg Peltekian is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Characterizing behavioral phenotypes of Dishevelled-2 knockout mice

by

Areg Peltekian

Master of Science in Biology

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Professor Yimin Zou, Chair
Professor Jill Leutgeb, Co-Chair

The segment polarity protein Dishevelled-2 (Dvl2) is a component of the Dishevelled protein family that plays an important role in the Wnt developmental signal transduction pathways expressed within the central nervous system. It has an antagonistic function to other Dishevelled proteins in facilitating endocytosis of Frizzled3 Wnt

receptors, though the specific behavioral effects have yet to be properly elucidated. A recently described genomic disorder characterized by a microdeletion of the 17p13.1 region, which contains an overlapping region with Dvl2, correlated to a recognizable phenotype in human patients consisting of intellectual disability and delay in social interaction. We sought to utilize various behavioral assays to test several behaviors associated with these phenotypes in adult mice lacking Dvl2 to demonstrate if the disruptions of neural development can be replicated in rodents and to potentially implicate Dvl2 knockout with these developmental delays. The behavioral paradigms used include open field, novel object recognition, and three-chamber social interaction tests to assess locomotor activity, anxiety-like behavior, learning and memory, sociability, and social novelty. An exploratory drive towards the center of a new environment as well as less preference for novel objects in contrast to familiar ones was observed in Dvl2-deficient mice, revealing a noticeable impairment in anxiety-like behavior and in context-independent memory. Impairments in sociability and social novelty were also observed, though without completely removing functionality. Therefore, evidence is provided that a lack of Dvl2 is associated with anxiety, memory, and social behavior deficits.

I:

Introduction

To our current understanding, there are 19 Wnt secreted proteins that are highly conserved across invertebrate and vertebrate species and perform a multitude of functions, including the facilitation of axonal targeting and later stimulating the formation of synapses in the central nervous system through various signal transduction pathways (Salinas and Zou, 2008; Purro et al., 2008). The exact effect of Wnt on axon guidance and synaptogenesis depends on the specific Wnt analogues that constitute different signaling pathways, the best characterized pathways being the canonical Wnt pathway and the noncanonical planar cell polarity (PCP) and Wnt/Calcium pathways (Davis et al., 2008; Salinas, 2012).

One such Wnt analogue is Dishevelled, a cytoplasmic scaffolding protein family which plays a foundational role in branching off the Wnt signal into the canonical and noncanonical PCP and Wnt/Calcium pathways, facilitated by the binding of a Wnt ligand to a Frizzled (Fzd) receptor and followed by signal transduction through varying combinations of the highly conserved amino-terminal DIX domain, central PDZ domain, and carboxy-terminal DEP domain. In the case of the canonical pathway, activation of Fzd and a low-density lipoprotein receptor-related protein (LRP)5/6 coreceptor induces phosphorylation of Dishevelled through the DIX and PDZ domains and disrupts the protein complex comprised of axin, glycogen synthase kinase 3 (GSK3), GSK3-binding protein (GBP), and casein kinase 1 (CK1). This in turn stabilizes β-catenin, which ends up mediating transcription of Wnt target genes (Habas and Dawid, 2005).

The molecular mechanisms of Dishevelled-1 (Dvl1), one of three Dishevelled homologs in mammals, with other signaling components have been further elaborated upon. This includes the inhibition of Dvl1-mediated feedback inhibition by Vangl2 for sharpening of PCP signaling and growth cone turning (Shafer et al., 2011). Behavioral effects such as a lack of sociability have also been noted in Dvl1-null mice (Lijam et al., 1997; Long et al., 2004). However, extensive study has not likewise been placed on Dvl2, thus its molecular and behavioral effects remain poorly understood. Dvl2 has previously been described in the context of the Wnt PCP signaling pathway as reducing Frizzled3 phosphorylation to facilitate its endocytosis and in turn antagonizing Dvl1's function of hyper-phosphorylating Frizzled3 to prevent its endocytosis (Onishi et al., 2013), though further study is required to characterize the role of Dvl2 in different brain regions. More significantly, the assessment of behavioral tasks such as those previously performed on Dvl1-deficient mice have yet to be further compared in Dvl2 models.

Through array comparative genome hybridization, a diagnostic tool used for clinical reports of children with intellectual disabilities, a new microdeletion syndrome consisting of a distal 17p13.1 region deletion has been identified (Zeesman, 2012). The gene locus for Dvl2 is among one of these overlapping genes and all human patients display developmental delay, consisting of easily distracted attention and difficulties in social comprehension and interaction (Zeesman, 2012). It is with the goal of elaborating on these relatively few studies examining Dvl2's effect on social 4

interaction and other behaviors that we sought to provide a comprehensive characterization of various behavioral phenotypes in Dvl2 knockout mice.

Behavioral analysis of transgenic animals has become a standard procedure in linking specific genes to specific behaviors and have provided effective models for many human genetic diseases and neurodevelopmental disorders as well as a way of evaluating the general functions of genes expressed in the central nervous system and the way in which these functions affect behavioral variation (Young et al., 2008). While mice models with targeted genetic mutations may not perfectly encapsulate all the robust phenotypes that are a result of multiple genetic interactions underlying complex pathologies such as the microdeletion syndrome, describing behavioral deficits in models such as the Cre-mediated transgenic mice model used for this project can nonetheless provide a gateway to the study of genetic abnormalities in many disorders. Models for the participation of Dvl2 in complex behaviors through different developmental processes such as the canonical and noncanonical Wnt pathways may then be proposed.

Highly validated behavioral assays were sought in order to provide a quantifiable measure of impairments in memory and social behavior as a result of knocking out Dvl2 in mice: Novel object recognition serves as a test for recognition memory and can be used to investigate the influence of genetics on memory and the efficacy of memory-enhancing compounds (Langston and Wood, 2010). Similarly, the three-chamber social approach task has strong face validity to simple social approach

behaviors in humans frequently impaired in autism and other developmental disorders Hsiao et al., 2013). Also, as a means of providing a comprehensive set of behaviors characterized to assist in future projects, to, an open field test was used gauge changes in emotionality. Open field has been demonstrated to provide reliable measures of anxiety-like behavior in rodents and is commonly used to test the effects of pharmacological agents on the exploratory drive of the animal model based on locomotor motion and preference for the center of the field as opposed to the peripheral edges (Goma and Tobeña, 1978).

II:

Materials and Methods

Animals

The conditional Dvl2 knockout mice were generated through breeding of heterozygous constitutive Dvl2 knockout ($Dvl2^{fl/fl}$) mice with a single-neuron labeling inducible Cre-mediated knockout (SLICK) transgenic mouse line provided by Jackson Laboratory. SLICK-A is a tamoxifen-inducible technique involving the co-expression of Cre recombinase and Enhanced Yellow Fluorescent Protein driven by 2 bidirectional copies of the mouse thymus cell antigen 1 promoter – a pyramidal neuron marker as previously described (Young et al., 2008). This allows for conditional genetic manipulation with fluorescence imaging. Postnatal Cre recombination was induced in offspring through two 50µl intraperitoneal injections of 10 mg/ml tamoxifen at postnatal day 7 and 8 (WT: n = 13, SlickA+;$Dvl2^{fl/fl}$: n = 9). Animals were given free access to food and water and were reared on a 12-hour light-dark cycle. Genotypes of all mice were collected on postnatal day 21 using polymerase chain reaction and all behavioral studies were performed on eight-week-old adult mice. All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee at the University of California, San Diego.

Experiment 1: Open Field Exploration Test

The following behavioral paradigm was used to assess both locomotor and anxiety-related behavior in all rodents. Mice were individually placed in a 40x40x35 cm metal box with an empty arena to explore while the behavior was monitored and recorded for 15 minutes using a video camera placed on top of the arena. The

apparatus was illuminated in low light conditions and surrounded by white hospital screens to create a sense of symmetry. The mice were placed in the room 1 hour before the start of the experiment to habituate to the dimly lit environment. The open field arena was thoroughly cleaned with 70% Ethanol prior to the start of a new test to eliminate olfactory cues. The observers were blinded to the treatment of the mice. A MATLAB auto-phenotyping software (Patel, 2014) was utilized to track the trajectory of the mice movement recorded as total distance traveled (inches) and to analyze the time spent in the center of the arena (defined as the inner 35x35 cm area). The results for the latter were recorded in both 5 minute and 1 minute intervals.

Experiment 2: Novel Object Recognition Test

The following test was performed to evaluate spatial memory in mice. The same apparatus and environmental conditions were used for the novel object recognition test as that of the open field test. Both the methods and apparatus used were based on previously described protocols (Leger et al., 2013). Following the performance of the open field test, the experiment would begin with a familiarization session in the presence of two equal objects on the left and right sides of the arena for 10 minutes. After an intersession interval of 24 hours, the mice were placed back in the arena for a 10-minute test session in which one of the objects in which one of the objects is replaced with another unfamiliar object of different size and material properties. It is critical that the initial objects and the side in which the novel object is placed varies between the following mouse. The objects and arena were thoroughly

cleaned with 70% Ethanol prior to the start of a new session. The time spent by the mice interacting with the objects was recorded with a stopwatch. Interaction was defined as sniffing or touching the objects with the front paws or nose. The interaction bouts with the objects for each session were then analyzed. All trials were double-blinded.

Experiment 3: Three-Chamber Social Interaction

The following test was used to assay sociability in mice. The same environmental conditions were used for the novel object recognition test as that of the previous behavior tests. The apparatus is a transparent Plexiglass box with three chambers separated by two doorways. The experiment consists of four 10-minute phases based on previous protocols (Yang et al., 2011). The first phase involves habituating the subject mice to the starting center chamber with the doors closed. The second phase involves habituating the mice in all three chambers through removing the doors and is the stage in which time spent in the left and right chambers is quantified. The third phase serves as a test for sociability: A cylindrical cage is placed in the left and right chambers, one of which is empty and the other which contained an untested wild-type mouse (Stranger 1). It is critical that the chamber in which the novel mouse is placed varies between the following mouse and that the subject mouse is confined to the center chamber before the novel mouse and object are placed in the apparatus. The novel object is positioned before the novel mouse to prevent contaminating the novel object with animal odors. The fourth and final phase serves as

a test for social novelty: a second mouse is placed in the previously empty cage (Stranger 2). The time spent by the mice interacting with the objects was recorded for the third and fourth phases with a stopwatch and analyzed. The chambers and cages were thoroughly cleaned with 70% Ethanol at the end of the fourth stage. All trials were double-blinded.

Statistical analysis

T-tests were performed for analyses of total distance travelled for open field tests. Time spent in the center of the open field arena with both 1 and 5 minute intervals was analyzed using a 2-way analysis of variance (ANOVA) with repeated measures for each genotype. As for time spent interacting with objects in novel object recognition and three-chamber social interaction tests, paired t-tests were performed for all sessions. In all figures, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. All statistical data were analyzed using GraphPad Prism 6.0. Averages are reported as mean ± SEM.

III:

Results

Experiment 1: Open Field

The heatmaps and trajectory of the mice within the open field chamber in (Fig. 1A) were generated with the video recordings of the open field test and allowed the following quantifications: Dvl2 knockout mice demonstrated no significant difference ($F(12, 8) = 1.015$, $p = 0.5932$) in terms of total path length travelled for the 15-minute test sessions (Fig. 1B; $mean_{WT} = 2100.33 \pm 115$ inches, $mean_{Dvl2KO} = 2196.09 \pm 137.3$ inches).

Time spent in the center of the field significantly increased with the knockout mice when compared to the WT mice ($F(8,12) = 7.145$, $p = 0.025$), corresponding to a 62% increase of the seconds inside the center (Fig. 2A; $mean_{WT} = 233.8 \pm 16$ seconds, $mean_{Dvl2KO} = 382.11 \pm 52.98$ seconds). This is further clarified by analyzing the time spent in the center within every 1 and 5 minutes. The exploration time of the knockout mice in the center maintains the significant increase compared to the control group for every 5 minutes (Fig. 2B; $F(1, 19) = 8.6$, $p = 0.0057$), with total time length for the 5-10 min and 10-15 min interval remaining roughly the same since the mice habituate to the novel stimulus during the first 5 minutes of the test. The exploration time during every 1 minute further confirms the difference (Fig. 2C; $F(1,300) = 60.47$, $p = 0.0053$) as well as demonstrates the more rapid increase of time spent in the center of the knockout mice in contrast to the control group during the first 5 minutes until a baseline level of exploration is reached for the final 10 minutes of the test.

Experiment 2: Novel Object Recognition

The control group spent a similar amount of time exploring left and right objects during the familiarization session of the novel object recognition task (Fig. 3A (left); $t(12) = 2.165$, $p = 0.0512$). The knockout mice likewise contained no significant difference in interaction time between the two equal objects in the field (Fig. 3A (right); $t(8) = 0.2017$, $p = 0.8452$). As for the test session performed 24 h after the familiarization session, the paired t-test analysis showed that the WT mice explored the novel object significantly more (65.4%) than the familiar objects (Fig. 3B (left); $mean_{novel} = 43.45 \pm 2.75$ seconds, $mean_{familiar} = 26.27 \pm 3.12$ seconds, $t(12) = 4.071$, $p = 0.0016$). The knockout mice performance, however, showed no discrimination between the novel and familiar object, irrespective of which objects were used for each session, and the interaction with objects did not differ from chance (Fig. 3B (right); $t(8) = 0.4111$, $p = 0.6918$). Therefore, only mice in the control group exhibited preferential exploration of the novel object.

Experiment 3: Three-Chamber Social Interaction

The second habituation phase of the social interaction test, in which the mice explores the left and right chambers from the starting center chamber, was the first to be recorded. A paired t-test revealed no preference to explore one chamber over the other for both the control group (Fig. 4A; $t(12) = 0.0743$, $p = 0.9420$) and knockout mice ($t(8) = 0.7793$, $p = 0.4582$). For the third phase which assessed sociability, WT mice preferentially explored the chamber containing the cage with the novel mouse

significantly more than the chamber on the opposite side containing the empty cage (Fig 4B; $mean_{mouse}$ = 124.2 ± 9.39 seconds, $mean_{object}$ = 89.95 ± 7.82 seconds, t(12) = 6.721, p < 0.0001). The knockout mice also preferentially interacted with the novel mouse rather than the novel object, though the increased time spent with the mouse was less pronounced ($mean_{mouse}$ = 85.43 ± 6.04 seconds, $mean_{object}$ = 64.47 ± 7.01 seconds, t(8) = 3.887, p = 0.0046). The fourth and final phase assessing preference for social novelty showed results similar to the previous phase: WT mice spent more time with the novel mouse than the familiar mouse (Fig 4C; $mean_{novel}$ = 89.72 ± 8.44 seconds, $mean_{familiar}$ = 63.69 ± 9.193 seconds, t(12) = 5.08, p = 0.0003), while Dvl2 knockout mice displayed a slight deficit in terms of preference for the novel mouse versus the familiar one ($mean_{novel}$ = 62.5 ± 8.67 seconds, $mean_{familiar}$ = 43.31 ± 7.07 seconds, t(8) = 4.094, p = 0.0035).

IV:

Discussion

Comprehensive behavioral analysis of Dvl2-deficient mice suggested that a genetic effect of Dvl2 on locomotor activity could not be inferred, based on the lack of phenotypic differences regarding the total distance travelled by Dvl2 knockout mice compared to WT mice. The distinction between Dvl2 knockout and WT mice becomes evident when contrasting the significantly higher exploratory time Dvl2 knockout mice spent in the center of the open field compared to the control group. Significant outliers for the knockout mice suggest a potential inability of tamoxifen to induce Cre-LoxP recombination and generate conditional Dvl2 knockout. Western blot analysis might be necessary in the future to determine the expression of Dvl2 proteins in multiple brain regions and confirm the success of the Cre recombinase system.

While the increase in exploratory behavior is noteworthy, it isn't sufficient to indicate that the increased time spent exploring the center as opposed to the periphery is due to less anxiety and fearful aversion to threatening stimulus or a stronger exploratory drive. Analysis of the exploratory time in the center classified into 5 minute intervals elaborates upon this by accentuating the increase of exploration time in the 5-10 min interval compared to the 0-5 min interval and the minimal increase in the 10-15 min interval compared to the 5-10 min interval. Habituation of the mice for both genotypes, therefore, occurs primarily in the first 5 minutes of the open field test. Exploration time of the field categorized into 1 minute intervals highlights the fact that the knockout mice require less time to habituate to the arena and reach a baseline level of time spent in the center for every minute than the WT mice. This suggests limited anxiety for the knockout in contrast to the WT mice and an overall larger drive to

explore the field, further indicating that Dvl2 might be involved in regulating anxiety-like behavior in novel environments.

No significant difference between the Dvl2 and WT mice was observed during the familiarization sessions of the novel object recognition test, indicating an equal amount of time spent across both initial objects throughout the 10 minute session and showing no preference for the left or right objects irrespective of genotype. The test session commenced after a 24-hour intersession following the familiarization session and the knockout mice displayed a similar lack of preference between the two objects despite one being novel, in contrast to the WT mice spending more time exploring the new object than the familiar one. Inability to properly distinguish between the two objects provides the case for a memory deficit. This suggests that Dvl2 knockout impairs recognition memory performance in healthy adult mice.

As for the social interaction test results, no significant difference in chamber exploration time during the second phase confirmed that both mice groups lacked side chamber preferences and were unaffected by outside external environmental cues. The third phase to test sociability followed right afterwards. WT mice displayed a much more significant increase in in time spent with the novel mouse rather than the empty cage compared to the Dvl2 knockout mice, though both results are significant. Similar results are observed for the fourth phase testing preference for social novelty: the time spent by both groups around the novel mouse cage was significantly higher than the time spent around the familiar mouse cage, but to a smaller degree for the knockout

mice. It is therefore suggested that Dvl2 knockout mice displayed deficits in sociability and social novelty, though these behaviors were not fully impaired.

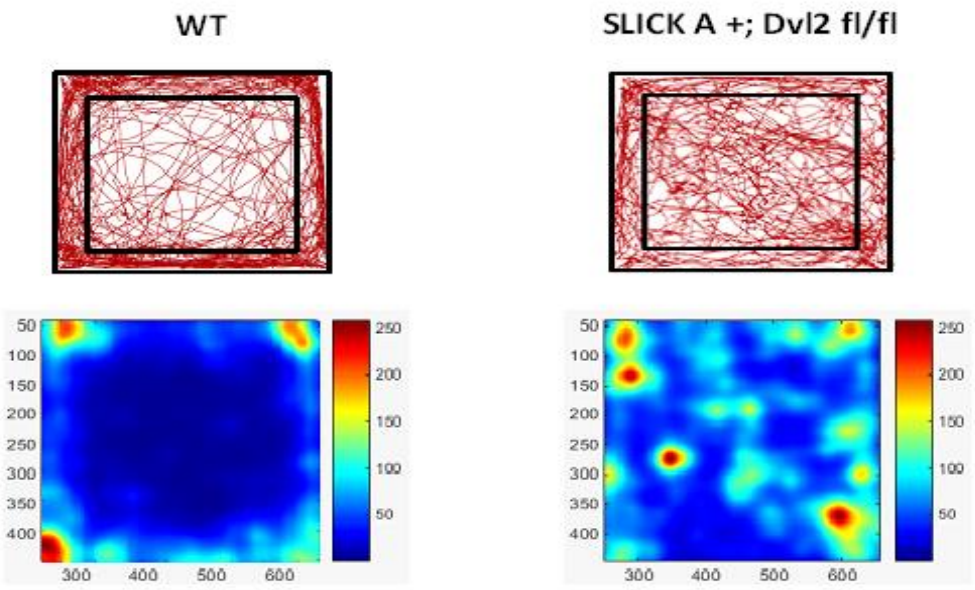
Overall, the activity exhibited by Dvl2 knockout mice in the behavioral paradigms used for this project makes it evident that Dvl2 plays a role in regulation of anxiety-like behavior, recognition memory, and social interaction. Nonetheless, further behavioral paradigms designed to assess the same behaviors as the preceding assays need to be used to clarify these findings. Previous analyses have revealed that similar anxiety and locomotion-related factors were produced by the commonly used elevated plus maze and light/dark box tests as that of open field, as all three are based on the free exploration of aversive environments (Ramos et al., 2008). In terms of learning and memory, the radial arm maze task and Morris water maze have been most extensively used to investigate specific aspects of spatial working and reference memory. Morris water maze in particular has an advantage over memory tasks like novel object recognition by being free from errors of omission or abortive choices in the sense that the mice will always make an attempt to find the platform on every trial (Wenk, 2004). Utilizing these complementary tests alongside open field and novel object recognition can allow for different aspects of emotionality in Dvl2 knockout mice to be assessed simultaneously.

These paradigms may also be modified to make new relevant assessments regarding of these behaviors in different contexts. Recognition memory is typically attributed to either context-dependent recollection of the objects during the learning

event or context-independent familiarity with the individual objects (Langston and Wood, 2010). The role of contextual information on object recognition was not evaluated with the parameters used for the novel object recognition test, as the environmental cues remained constant and the context was not changed for both sessions. Further study may evaluate the recognition process further through varying this intersession interval and the field apparatus to create different context and analyzing the changes in recognition during the test session. The same can be done for the open field and three-chamber social interaction tests: locomotor activity, exploratory behavior, and a preference to interact with other mice may be compared in Dvl2 knockout mice by monitoring activity in a novel context, such as the open field apparatus, to a familiar context such as the activities in a home cage for consecutive days.

Figure 1. Video analysis of total ambulation and locomotor activity in Dvl2 knockout mice. A) Representative red traces of locomotor pattern activity (above) and heatmap (below) of open field arena for wild-type and Dvl2 knockout mice. Each panel shows traces of one representative mouse after 15 minutes of tracking. B) Total distance travelled in the arena during the total 15 minutes of tracking. Mean and standard error of mean bars are shown. WT: n = 13 mice. SLICK A+;$Dvl2^{fl/fl}$: n = 9 mice.

A


WT
SLICK A +; Dvl2 fl/fl
50
100
150
200
250
300
350
400
300
400
500
600
250
200
150
100
50

B

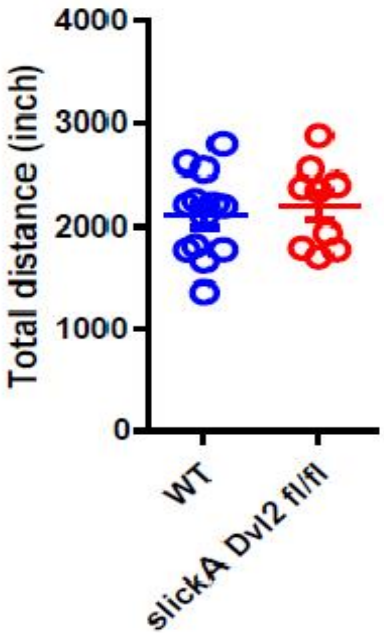

Total distance (inch)
4000
3000
2000
1000
0
WT
slickA Dvl2 fl/fl

Figure 2. Results of open field exploration with analysis of varying time intervals. A) Time spent in the virtual black square in the center area depicted in Fig. 1A in seconds during the total 15 minutes of tracking. B) Time spent in the virtual black square in the center area in seconds during 5 minute intervals of the 15 minutes of tracking. C) Time spent in the virtual black square in the center area in seconds during 1 minute intervals of the 15 minutes of tracking. Mean and standard error of mean bars are shown.

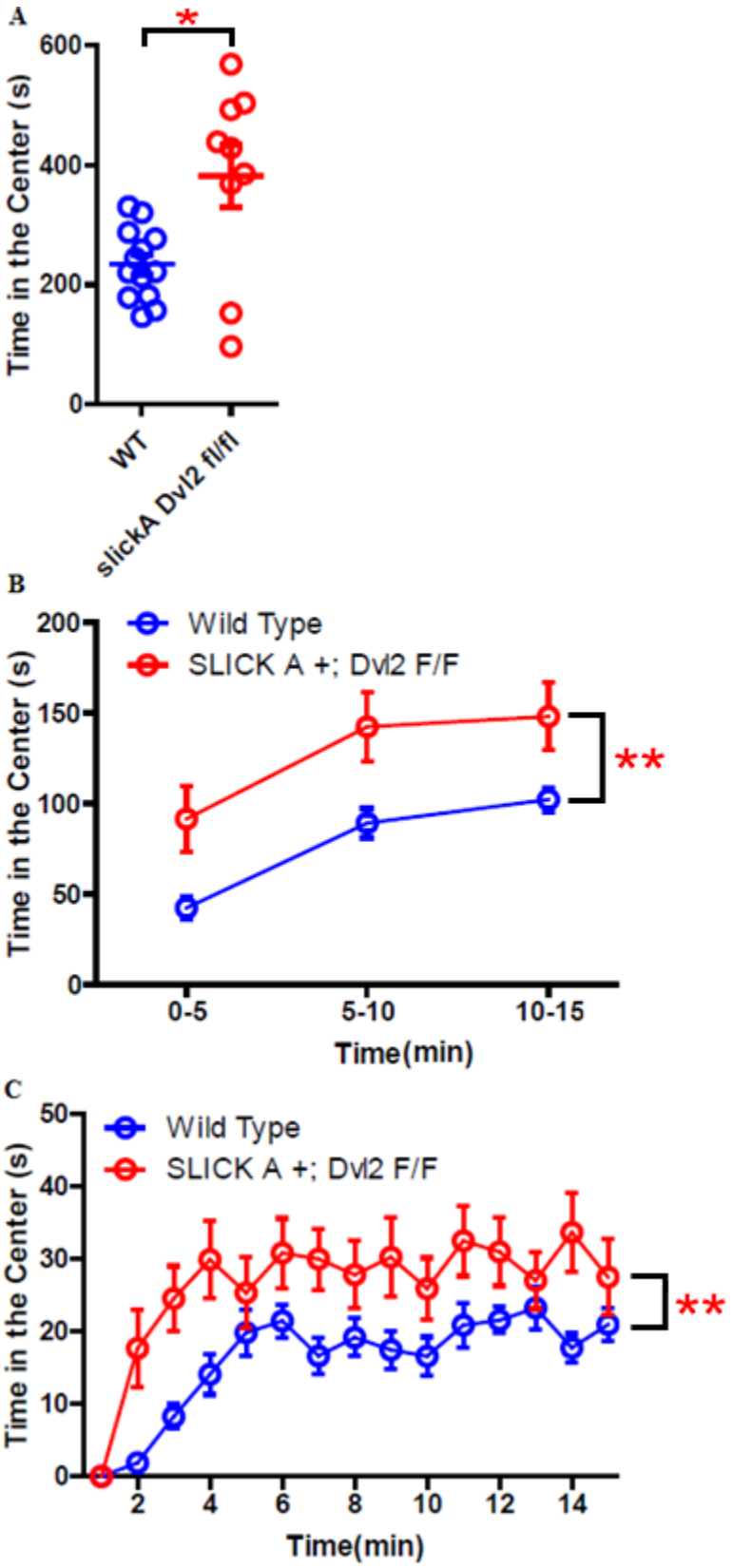

A
*
600
400
200
0
Time in the Center (s)
WT
slickA Dvl2 fl/fl
B
Wild Type
SLICK A +; Dvl2 F/F
200
150
100
50
0
Time in the Center (s)
**
0-5
5-10
10-15
Time(min)
C
Wild Type
SLICK A +; Dvl2 F/F
50
40
30
20
10
0
Time in the Center (s)
**
2
4
6
8
10
12
14
Time(min)

Figure 3. Results of interaction bouts in familiarization and test sessions of novel object recognition task. A) Exploration times of left and right equal objects during initial familiarization session for WT (left) and Dvl2 knockout mice (right) during the total 10 minutes of tracking. B) Exploration times of novel and familiar objects test session 24 h after familiarization session for WT (left) and Dvl2 knockout mice (right) during the total 10 minutes of tracking.

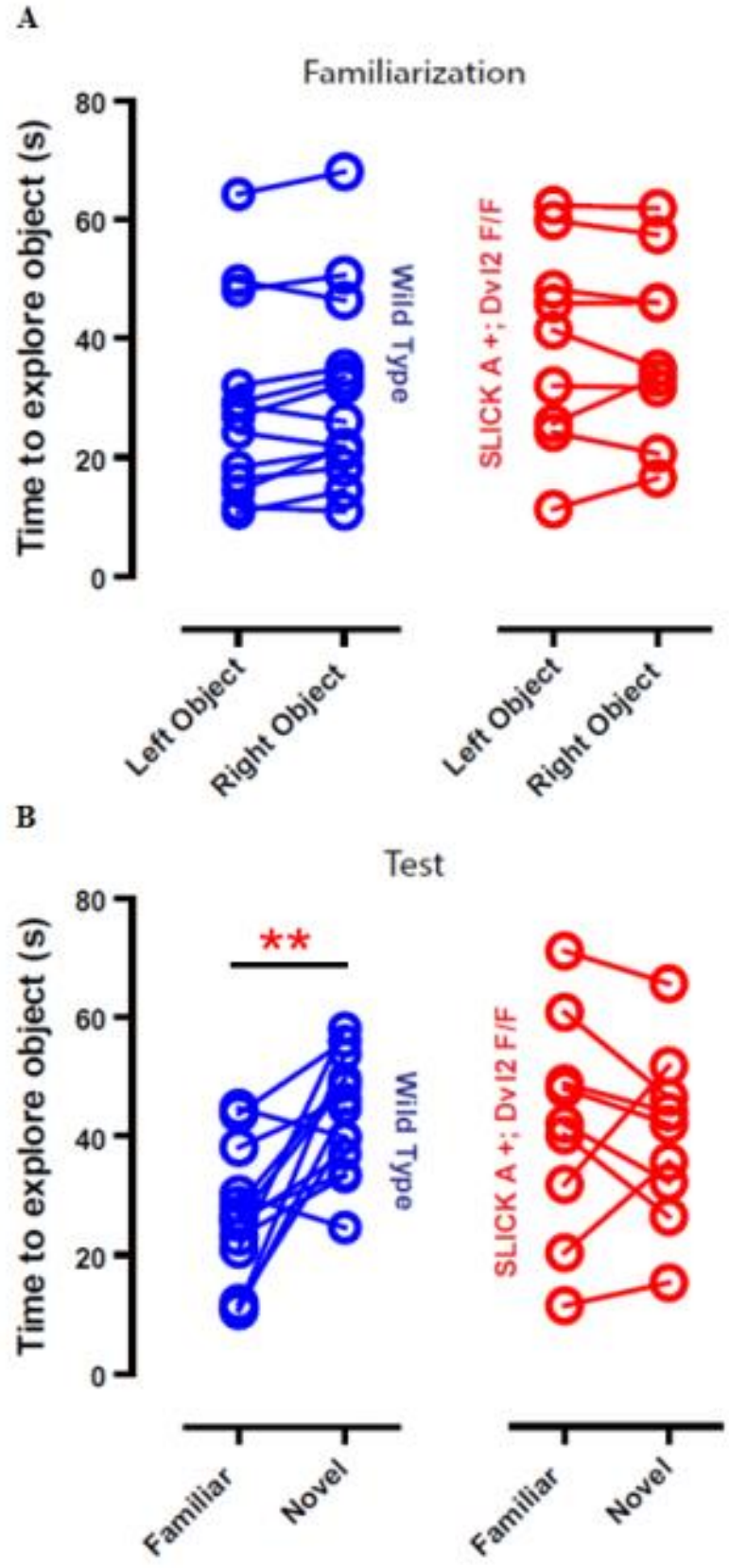
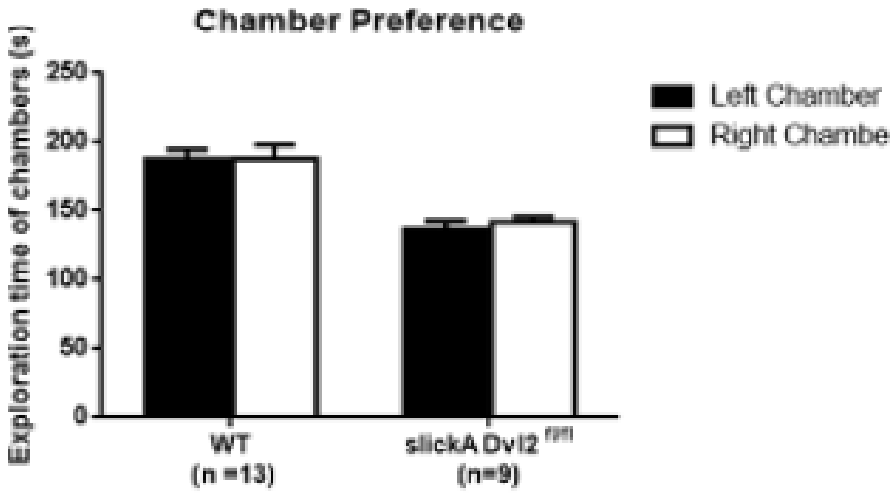
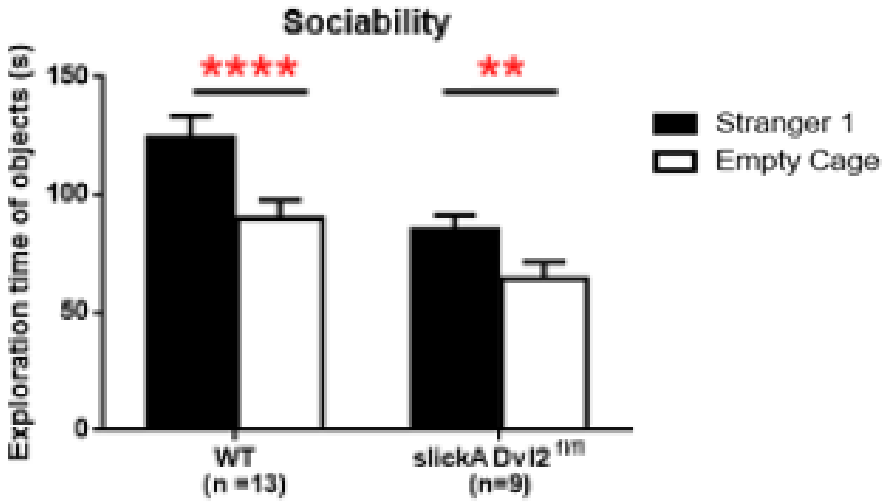

A
Familiarization
Time to explore object (s)
80
60
40
20
0
Wild Type
SLICK A +; Dvl2 F/F
Left Object
Right Object
Left Object
Right Object
B
Test
Time to explore object (s)
80
60
40
20
0
**
Wild Type
SLICK A +; Dvl2 F/F
Familiar
Novel
Familiar
Novel

Figure 4. Results of chamber preference, sociability, and social novelty tests of the three-chamber social interaction test. A) Chamber preference: Time spent in the left and right chambers adjacent to the starting center chamber in seconds during the total 10 minutes of tracking. B) Sociability: Time spent interacting with the left and right chambers adjacent to the starting center chamber in seconds during the total 10 minutes of tracking. C) Social novelty: Time spent in the left and right chambers adjacent to the starting center chamber in seconds during the total 10 minutes of tracking. Mean and standard error of mean bars are shown.

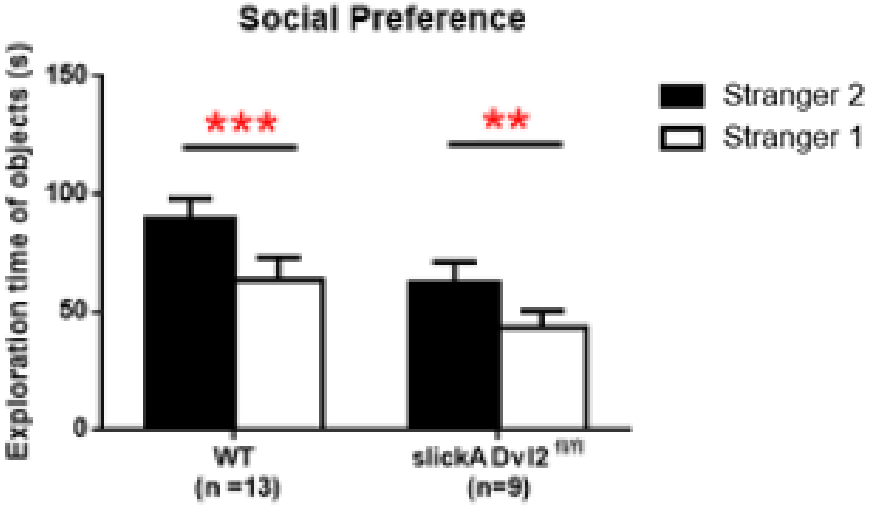
A


Chamber Preference
Exploration time of chambers (s)
250
200
150
100
50
0
Left Chamber
Right Chambe
WT
(n =13)
slickA Dvl2 fl/fl
(n=9)

B


Sociability
Exploration time of objects (s)
150
100
50
0
****
**
Stranger 1
Empty Cage
WT
(n =13)
slickA Dvl2 fl/fl
(n=9)

C


Social Preference
Exploration time of objects (s)
150
100
50
0
***
**
Stranger 2
Stranger 1
WT
(n =13)
slickA Dvl2 fl/fl
(n=9)

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