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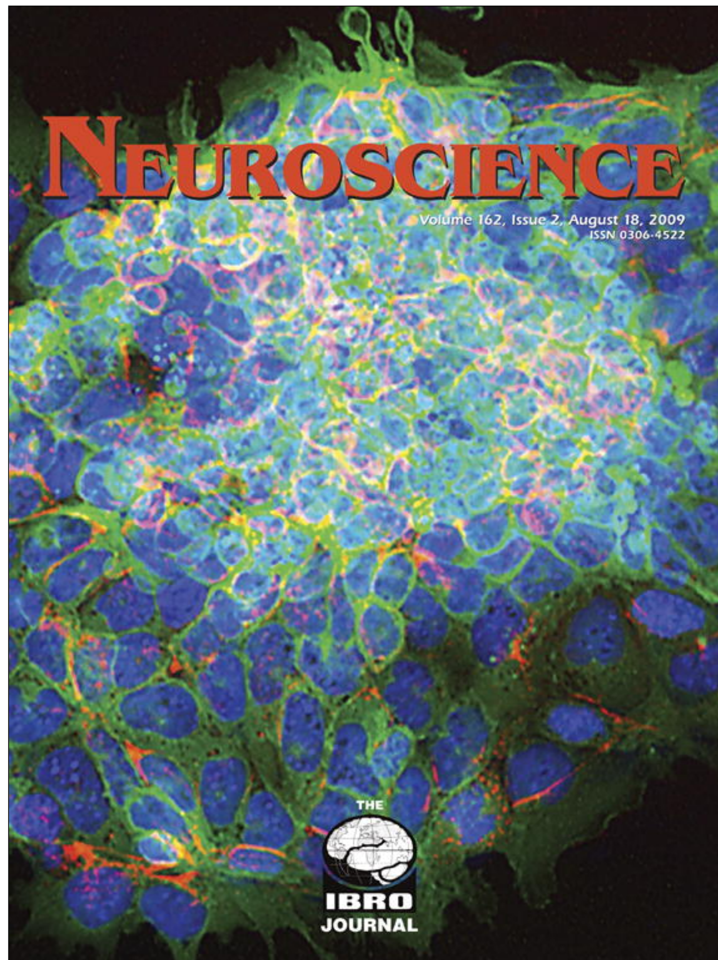

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DETECTION OF BEHAVIORAL ALTERATIONS AND LEARNING DEFICITS IN MICE LACKING SYNAPTOPHYSIN

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Abstract—The integral membrane protein synaptophysin is one of the most abundant polypeptide components of synaptic vesicles. It is not essential for neurotransmission despite its abundance but is believed to modulate the efficiency of the synaptic vesicle cycle. Detailed behavioral analyses were therefore performed on synaptophysin knockout mice to test whether synaptophysin affects higher brain functions. We find that these animals are more exploratory than their wild type counterparts examining novel objects more closely and intensely in an enriched open field arena. We also detect impairments in learning and memory, most notably reduced object novelty recognition and reduced spatial learning. These deficits are unlikely caused by impaired vision, since all electroretinographic parameters measured were indistinguishable from those in wild type controls although an inverse optomotor reaction was observed. Taken together, our observations demonstrate functional consequences of synaptophysin depletion in a living organism. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: transgenic mice, synaptophysin, behavior, learning, electroretinogram, optokinetics.

Synaptic vesicle (SV)–mediated transmitter release is the main mechanism of neuronal information transfer. SVs are characterized by a very specific polypeptide composition to facilitate this tightly-regulated process. Among SV membrane proteins, synaptophysin (syn) is not only the first one whose cDNA was cloned but is also, together with its binding partner synaptobrevin2, by far the most abundant polypeptide component (Hübner et al., 2002; Valtorta et al., 2004; Takamori et al., 2006). It was therefore quite surprising to find out that complete depletion of syn in mice is compatible with an apparently normal life (Eshkind and Leube, 1995; McMahon et al., 1996). One possible reason may be molecular redundancy allowing compensation by related SV proteins, most notably synaptoporin/syn II and the synaptogyrins. In support, detailed morphological analyses of photoreceptors that also lack synaptoporin revealed considerable morphological changes presumably due to the inability to fully compensate for the loss of syn in these neurons (Spiwoks-Becker et al., 2001). The observed SV depletion and relative increase in clathrin-coated vesicles point to a specific though not essential role of syn for the SV cycle. Also, double knockout (k. o.) mice lacking both syn and synaptogyrin1 display defects in synaptic plasticity (Janz et al., 1999). Furthermore, evidence has been presented implicating syn in plasticity-related changes after injury of hippocampal neurons (Sun et al., 2007) and in age-related cognitive impairment (Smith et al., 2000; King and Arendash, 2002). Smith and colleagues (2000) found an age-dependent reduction in hippocampal syn immunoreactivity, especially in the CA3 region that receives input from the entorhinal cortex and is part of a circuit critical for hippocampal learning. In contrast, King and Arendash (2002) detected increased syn immunoreactivity in an Alzheimer mouse model indicative of a pathophysiological compensatory process reflecting neurodegenerative plasticity. A similar syn increase was also reported after chronic stress in hippocampal neurons (Gao et al., 2006). A recent publication further described an age- but not gender-dependent increase of syn in mice after extensive behavioral characterization, possibly representing a mechanism whereby the aged brain tries to compensate for cognitive decline in a stress situation, i.e. the Morris water maze (Benice et al., 2006). Independent of stress, intact information transfer necessary for learning and memory appears to rely on syn. Thus, there is convincing evidence that tyrosine phosphorylation of syn is required for long-term potentiation (LTP), a cellular correlate of learning and memory (Zhao, 2000; Purcell and Carew, 2003; Evans and Cousin, 2005). A direct link to LTP may exist, since tyrosine phosphorylation of syn was found to be increased in parallel with glutamate release from synaptosomes that had been prepared from hippocampal slices previously stimulated by LTP (Mullany, 1998).

Taken together, syn is apparently not essential for the SV cycle per se but most likely has a function in determining synaptic strength thereby modulating the efficacy of learning and memory. As transgenic *in vivo* models are still best suited to investigate the impact of such plasticity-related processes, we decided to subject syn k. o. mice to behavioral tests focusing on novelty, anxiety and learning/memory.

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Abbreviations: ANOVA, analysis of variance; ERG, electroretinogram; k. o., knockout; LTP, long-term potentiation; MANOVA, multivariate analysis of variance; SV, synaptic vesicle; syn, synaptophysin.

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EXPERIMENTAL PROCEDURES

Mice

Preparation of a targeting construct, homologous recombination in embryonal stem cells of line R1 and generation of syn k. o. mice were described in detail by Eshkind and Leube (1995). The k. o. animals contain a neomycin-resistance-encoding insert in exon 2, which inactivates the X chromosomal syn gene. They do not synthesize detectable amounts of syn or syn fragments. They were inbred for several generations prior to outcrossing for over 10 generations with C57BL/6J (Harlan Winkelmann, Borchen, Germany). For the experiments, heterozygous syn$^{+/-}$ female animals were crossed with syn$^{+/0}$ C57BL/6J males. In this way, male littermates were generated carrying either a wild type or a mutant syn allele. The genotype of these males was determined by duplex PCR using primers 03–51 ACTTCCATCCCTATTTCCCA-CACC, 03–52 TTCCACCCACCAGTTCAGTAGGA, and 03–53 TCGCCTTCTTGACGAGTTCTTCTG, yielding a 236 bp fragment from the wild type allele and a ~500 bp fragment from the mutant allele.

Animals were housed four per cage (810 cm^2, type III) at 22 °C and 60% relative humidity. Food and water were provided *ad libitum* and a 12-h light/dark cycle was maintained (light on from 6:00 AM until 6:00 PM). Thirty-one male mice were tested from 3 months onward. Age-matched littermates were examined that were either wild type (controls, n=16), or mutant lacking syn (n=15). All procedures were carried out in accordance with the European Communities Council Directive regarding care and use of animals for experimental procedures and were approved by local authorities of the state of Rheinland-Pfalz. All efforts were made to minimize the number of animals used and their suffering.

Behavioral testing

The sequence of testing was in the following order for all animals:

- Days 1 and 2: elevated plus maze to measure anxiety-like behavior (day 1) and emotional memory (day 2).
- Day 3: open field to measure general activity and exploration.
- Day 4: enriched open field to measure exploration related to novel objects in the first trial and object recognition memory in the third trial.
- Day 5: water alley with visible platform to measure general swimming activity and visual performance.
- Days 8–11: Morris water maze test to measure spatial learning.
- Day 12: Morris water maze test to measure memory performance in a probe trial without platform.

All tests were carried out between 9:00 AM and 2:00 PM with artificial lighting provided indirectly by one 58 W neon bulb 2.5 m above the test arenas. Mice were housed in the test room to minimize the influence of habituation stress two weeks before tests started. After each trial, test arenas and objects were carefully cleaned.

Elevated plus maze

Anxiety-related behavior was investigated with the elevated plus-maze. The maze is made of dark-gray plastic. It consists of two open arms (each 42.5×15 cm) and two enclosed arms (each 42.5×15×14 cm). The arms extend from a central platform (15×15 cm). Parameters recorded for the elevated plus maze were entries into closed arms (n), entries into open arms (n), time spent in open arms (% of total test time) and latency (s) to enter an open arm (Erb et al., 2001; Schmitt et al., 2006). In an additional plus-maze trial 24 h later emotional memory was investigated similar to anxiety-like behavior the day before (File et al., 1993; Schmitt et al., 2006).

Open field activity

General activity of mice was assessed during a 10-min session in an open field paradigm. The test arena consists of dark-gray plastic and measures 100×100×35 cm. The following parameters were recorded during each trial automatically: total distance moved (cm), resting time (% of total recording time not moving), time spent along the walled parts of the maze (10 cm corridor; % of total time) and entries into the central part of the maze (30 cm diameter; number, n) (Schmitt and Hiemke, 1998; Schmitt et al., 2006).

Enriched open field

Exploration activity was investigated during a 5 min session using the same apparatus as before but in an enriched setup. The arena was enriched with two scoops of clay: one larger blue scoop (10 cm; object A) and one smaller white scoop (8.5 cm; object B). For evaluation of exploration the mean distance to objects (cm) and mean time spent with objects (s) were recorded.

Object novelty recognition

The novel object memory was assessed by the ability to recognize a novel object in the environment which was the same as in the enriched setup before. The test not only covers hippocampal integrity (Ennaceur and Aggleton, 1997). Procedure: Animals were tested after 3 min for a second time with the same object arrangement as in the enriched open field test before. After an additional 3 min time interval one of the objects in the arena was replaced by a new metal cylinder (10 cm×4 cm) and a third exploration test followed. Object recognition was assessed by measuring the time spent with the novel object and is expressed as percent of time spent with the objects the trial before.

Water alley

Water-related activity was assessed independently of learning in a straight alley (85 cm×15 cm) providing a visible platform (10 cm diameter 1 cm above surface) at one end of the alley to escape from the water. Animals were given two habituation trials and one test trial. Latency (s) to climb the platform and velocity (cm/s) were measured.

Morris water maze

Spatial learning and memory were investigated by the Morris water maze hidden platform task using the same maze and protocol as described (Postina et al., 2004; Schmitt et al., 2006). The platform stayed in the same quadrant for all trials and the animals were released from four different positions at the pool perimeter. Mice performed four trials per day on four consecutive days with a maximum length of 60 s and an inter-trial interval of 90 s. Mice were allowed to stay on the platform for 10 s. On the fifth water maze day a probe trial (60 s) without platform was performed. Learning was assessed by measuring the latency to find the platform and the distance swum. For characterization of memory performance the time spent in each quadrant was analyzed.

Monitoring of behavior

A computerized video system registered moving-path and duration in all different tests automatically. The hardware consisted of an IBM-type AT computer (Dell®, 466Dl, Frankfurt/a.M., Germany) combined with a video digitizer and a CCD video camera (Panasonic®, CCTV Camera WVBP330/GE, Sushou, China). The software used for data acquisition and analysis was EthoVision® release 2.1 (Noldus Information Technology, Utrecht, The Netherlands).

Electroretinographic analysis

Electroretinograms (ERGs) were performed according to previously described procedures (Seeliger et al., 2001). The ERG

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equipment consisted of a Ganzfeld bowl, a direct current amplifier, and a PC-based control and recording unit (Multiliner Vision; VIASYS Healthcare GmbH, Hoechberg, Germany). Mice were dark-adapted overnight and anesthetized with ketamine (66.7 mg/kg body weight) and xylazine (11.7 mg/kg body weight). The pupils were dilated and single flash ERG responses were obtained under dark-adapted (scotopic) and light-adapted (photopic) conditions. Light adaptation was accomplished with a background illumination of 30 candela (cd) per square meter starting 10 min before recording. Single white-flash stimulus intensity ranged from −4 to 1.5 log s cd/m^2 under scotopic and from −2 to 1.5 log s cd/m^2 under photopic conditions, divided into 10 and eight steps, respectively. Ten responses were averaged with an inter-stimulus interval (ISI) of either 5 s (for −4, −3, −2, −1.5, −1, and −0.5 log s cd/m^2) or 17 s (for 0, 0.5, 1, and 1.5 log s cd/m^2). For additional photopic bright flash experiments, a Mecablitz 60CT4 flashgun (Metz, Zirndorf, Germany) added to the Ganzfeld bowl was used. The range of intensities used in the protocol was 2.0–4.0 log s cd/m^2 in steps of 0.5 logarithmic units. We also obtained responses to trains of flashes (flicker) for both a fixed intensity (scotopic: −2 log s cd/m^2, and photopic: 0.5 log s cd/m^2) with 12 frequencies (0.5, 1, 2, 3, 5, 7, 10, 12, 15, 18, 20 and 30 Hz), and a fixed frequency (6 Hz) with a range of intensities (−5.0 to 1.2 log s cd/m^2 in steps of 0.2 logarithmic units).

Optomotor experiment

Spatial acuity was measured in an optomotor experiment as previously described by Schmucker et al. (2005). Mice were individually placed in a clear transparent acrylic glass cylinder that was placed in the middle of a rotating drum in which the mice were freely moving. The drum provided the mouse with a drifting vertical square-wave pattern as it rotated in the vertical axis. Spatial frequency was set to 0.1 cycles/°. The drum was turned by an electric DC motor (Conrad Electronics, Hirschau, Germany). The direction of rotation could be changed by reversing the polarity of the voltage. The best optomotor responses were obtained for an angular speed of the stripe pattern between 50 and 60°/s. Because the Perspex cylinder containing the mouse was closed, it was unlikely that the mouse was stimulated by air currents that might have been generated by the rotating drum. The illumination in the drum was 400 lx.

Programming algorithms and measured parameters

It was impossible to judge by eye whether the mouse followed a stripe pattern or not, since presumed phases of tracking were interrupted by movements in the opposite direction or by complete loss of interest, as the mouse often engaged in long periods of cleaning behavior. It was therefore necessary to automate the movement analysis. To this end, the mouse was imaged with a simple infrared-sensitive monochrome miniature surveillance video camera (PAL format, 768×576 pixels; Conrad Electronics, Hischau, Germany) that was equipped with a lens with a focal length of 5 mm to achieve a large field of view. The camera was mounted in the center of the top cover of the acrylic glass cylinder. Image processing software written by one of the authors (F.S.) tracked the body center and measured the angular orientation of the snout–tail axis over time with a temporal resolution of 25 Hz. After 20 s, the average angular movement of the mouse relative to the center of the drum (referred to as "running speed") and the average angular movement of the snout–tail axis (referred to as "orientation speed") were automatically determined by the program and a one-sample *t*-test was performed to find out whether there was a significant trend of the mouse to move in the direction of the drifting stripe pattern. Because tracking the activity of the mouse was essential for gaining statistically reliable data, the locomotor activity was also recorded. The average absolute angular position change from one frame to the next was taken as a measure of activity. The procedure was repeated five times for each direction of rotation of the drum.

Measurement procedures

Angular running speed, angular orientation speed, and locomotor activity were recorded for each direction of rotation (Schmucker et al., 2005).

Statistical analysis of behavioral investigations

A one-way analysis of variance (ANOVA) was performed for differences between wild type and syn k. o. Multivariate analysis of variance (two-way MANOVA) was performed for learning and memory. Probe trial scores within experimental groups were evaluated by a one way ANOVA. Post hoc comparisons of the genotype were based on the Scheffé test. Differences were considered as significant for $P \leq 0.05$.

Optomotor response

The response of the mouse to different stripe patterns was defined as the difference of its angular movement preference when the drum was rotating clockwise versus counter clockwise. This difference was analyzed both for the angular running speed and angular body orientation speed. The more this value differed from zero or the more it differed from the condition when no visual stimulation occurred, the more important the visual input was to the behavior of the mouse. Mean responses and standard deviations were plotted against spatial frequency. Responses of wild type and k. o. mice were compared by one-way ANOVA. Post hoc analysis (the Dunnett test) was performed on factors that were found to be significant in the ANOVA with $P \leq 0.05$. Statistical tests were performed with the help of a computer (JMP, ver. 4 software; SAS Institute, Cary, NC, USA).

RESULTS

Syn. k. o. mice tend to be more exploratory

Behavioral experiments typically measure motor responses to sensory information. Therefore, results of experiments examining neurobiological processes such as learning and memory cannot be properly interpreted without evaluating motor function. Thus, locomotor activity was examined in syn k. o. mice in comparison to wild type control mice in several behavioral tasks. No differences could be detected in any of the different paradigms (Table 1). Parameters analyzed were *distance moved* ($F_{(1;29)}$=3.382; n.s.) and *resting time* ($F_{(1;29)}$=4.113; n.s.) in the open field, *distance moved* ($F_{(1;29)}$=3.045; n.s.) also in the enriched open field, *total number of entries* ($F_{(1;29)}$=0.572; n.s.) and *number of entries in closed arm* ($F_{(1;29)}$=0.121; n.s.) in the elevated plus maze, *latency to climb the visible platform* ($F_{(1;29)}$=1.181; n.s.) and *swim speed* ($F_{(1;29)}$=0.666; n.s.) in the water alley.

Exploratory locomotion was examined in the open field set up. *Time spent along the walled parts* and *entries into the center part* were analyzed. Both parameters were not significantly different between genotypes ($F_{(1;29)}$=0.681 n.s., $F_{(1;29)}$=1.178 n.s., respectively). Therefore, the influence of a novel environment was evaluated in the enriched open field. Syn k. o. mice explored the objects more closely ($F_{(1;29)}$=22.261; $P \leq 0.001$; *distance to objects*) and more intensely ($F_{(1;29)}$=17.896; $P < 0.001$; *time spent with*

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Table 1. Behavioral activity characterization

	Wild type (mean±SEM)	Syn k. o. (mean±SEM)	Post hoc *P*-value
A open field			
DM (cm)	4712±146	5135±180	n.s.
Resting (% time)	64.4±1.9	69.6±1.7	n.s.
B enriched open field			
DM (cm)	4516±143	4909±1760	n.s.
C elevated plus maze			
Total arm entries (*n*)	22.0±1.2	23.7±1.1	n.s.
Closed arm entries (*n*)	16.4±1.2	16.9±1.0	n.s.
D Water alley			
Latency (s)	6.1±0.9	7.7±1.1	n.s.
Velocity (cm/s)	16.4±1.4	14.9±1.0	n.s.

Measurements of locomotor activity in the open field, enriched open field, elevated plus maze and water alley. Parameters determined are DM (distance moved), resting time, arm entries, latency and velocity. Note that no differences are detected between age- and sex-matched wild type (n=16) and syn k. o. littermates (n=15; $P \leq 0.05$).

objects) than wild type mice (Table 2). To further examine anxiety-like behavior, open arm behavior was evaluated in the elevated plus maze test. In this assay no phenotypic differences were noted between syn k. o. and wild type mice (Table 3) as assessed by the percentage of *open arm entries* ($F_{(1;29)}$=0.542; n.s.), the percentage of *time spent on the open arm* ($F_{(1;29)}$=0.175; n.s.), and the *latency to enter an open arm* ($F_{(1;29)}$=1.439 n.s.).

Learning and memory capabilities are compromised in syn k. o. mice

Individuals engage many times each day in discriminating familiarity and memorizing new spatial conditions. Impaired recognition is therefore a considerable handicap and tests of learning and memory are fundamental in phenotypic characterization of transgenic individuals. In the novel object preference test significant differences were observed between wild type and syn k. o. mice, which presented reduced object novelty recognition (Fig. 1; $F_{(1;29)}$=5.409; $P \leq 0.05$). In addition, the ability of spatial learning and recalling spatial information was impaired in syn k. o. mice in the Morris water maze (Fig. 2). The first parameter analyzed was the *distance swum to find the platform*. Two-way ANOVA revealed significant differences for factor A time ($F_{(3;27)}$=51.190; $P \leq 0.001$), factor B genotype ($F_{(1;29)}$=10.301; $P \leq 0.01$), and also for the A×B interaction ($F_{(3;27)}$=5.248; $P \leq 0.01$) (Fig. 2a). The second parameter was the *latency to find the platform*. Two-way ANOVA revealed significant differences for factor A *time* ($F_{(3,27)}$=34.248; $P \leq 0.001$) and factor B *genotype* ($F_{(1,29)}$=8.617; $P \leq 0.01$) but not for time×genotype interaction ($F_{(3,27)}$=2.367; P=0.076 n.s.) (Fig. 2b). Remarkably, syn k. o. exhibited significantly prolonged latency times.

Table 2. Explorative activity characterization

	Wild type (mean±SEM)	Syn. k. o. (mean±SEM)	Post hoc *P*-value
A open field			
Time (%) spent along the walled parts	69.1±1.7	67.9±2.0	n.s. ($P \geq 0.05$)
Number of entries in center	16.5±1.2	18.4±1.4	n.s. ($P \geq 0.05$)
B enriched open field			
Distance to object (cm)	22.0±0.5	18.9±0.4	$P \leq 0.0001$
Time (s) spent with objects	3.5±0.5	8.7±1.1	$P \leq 0.0001$

Assessment of exploration in the open field and enriched open field of age- and sex-matched wild type (n=16) and syn k. o. littermates (n=15). Time spent along the walled parts is expressed as % of total time. Note the more intense exploration of the syn k. o. animals in the enriched open field.

Table 3. Anxiety-like behavior characterization

Elevated plus maze (first trial)	Wild type (mean±SEM)	Syn. k. o. (mean±SEM)	Post hoc *P*-value
% Open arm entries	25.6±3.8	28.8±1.9	n.s.
% Time spent in open arm	9.0±3.7	10.7±1.3	n.s.
Latency to enter open arm (s)	26.7±13.0	10.0±4.0	n.s.

Determination of anxiety-related parameters in the elevated plus maze (first trial; significance level $P \leq 0.05$). No differences ($P \leq 0.05$) are noticeable between age- and sex-matched wild type (n=16) and syn k. o. littermates (n=15).

Next, recall of spatial information was tested on the fifth day by removing the platform. Two-way ANOVA results on the time spent in the different quadrants failed to demonstrate significant differences: factor A *time spent in quadrants* (treated as repeated measure; $F_{(3;27)}$=18.419, $P \leq 0.001$), factor B *genotype* ($F_{(1;29)}$=1.226; n.s.), and A×B interaction ($F_{(3;27)}$=1.408; n.s.). Because of the reduced learning abilities of syn k. o. mice we tested whether each group per se was able to memorize the platform location. ANOVA results on time spent in quadrant revealed a significant effect for the goal quadrant in the case of wild type ($F_{(3;45)}$=10.559; $P \leq 0.001$) but not syn k. o. mice ($F_{(3;42)}$=2.761; n.s.) (Fig. 3). It is of note that swim speed in the Morris maze did not differ from that in the water alley test with visible platform (n.s., data not shown). Finally, emotional-related components of memory were tested by a second elevated plus maze trial revealing no significant differences between wild type and syn k. o. animals (Tables 4 and 5).

ERGs do not reveal abnormalities in syn k. o. animals

Considering the previously described anatomical alterations in retinal neurons of syn k. o. mice (Spiwoks-Becker et al., 2001) and the observed protracted learning capability of syn k. o. mice, we wanted to exclude that visual impairment accounts for the behavioral abnormalities of the syn mutants. Therefore, extensive ERG analyses were performed in a subset of mice some of which had been subjected to behavioral testing before. As reported previously (Spiwoks-Becker et al., 2001), scotopic and photopic single flash ERG responses in syn k. o. mice were com-

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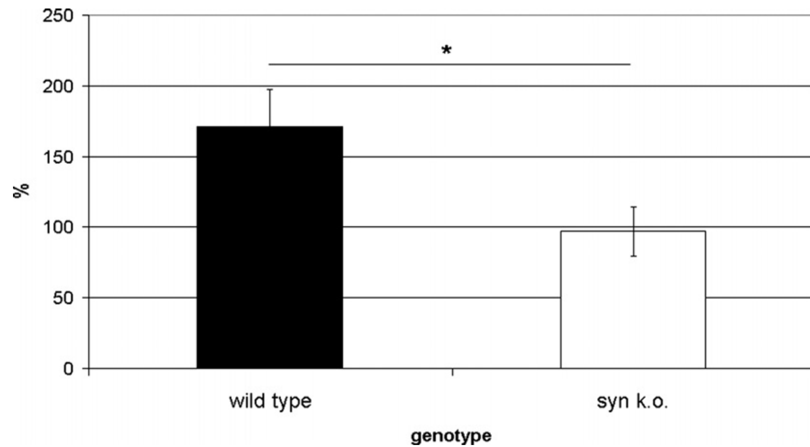

Fig. 1. Object novelty recognition. The histogram shows the object novelty index (see Experimental Procedures; mean±SEM) determined for age- and sex-matched wild type ($n=16$) and syn k. o. littermates ($n=15$). Note the significant differences (* $P \leq 0.05$ in post hoc Scheffé test) between both groups indicative of a memory deficit of the syn k. o. animals.

parable to those in wild type mice in the intensity range up to 1.5 log s cd/m^2 (Fig. 4a). To evaluate the cone system performance further, ERGs were recorded increasing the light intensity up to 4.0 log s cd/m^2 (Fig. 4b). Response

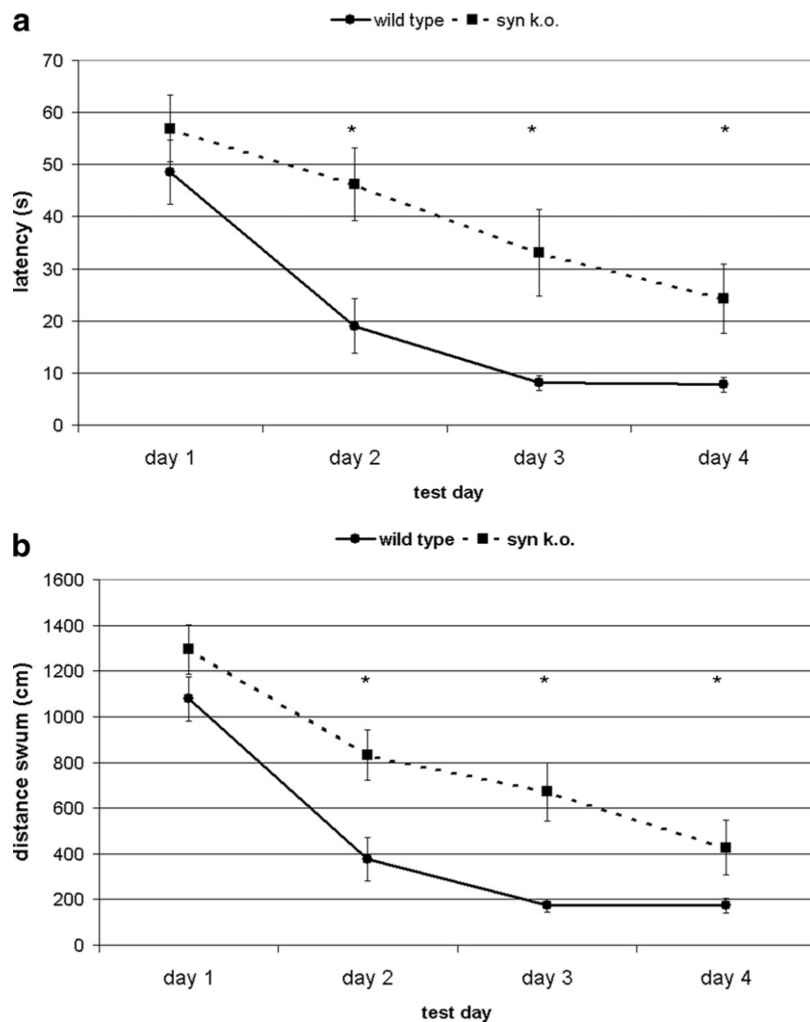

Fig. 2. Morris water-maze hidden platform task learning behavior. The distance swum (a) and latency (b) to reach the platform were determined on 4 consecutive days for age- and sex-matched wild type ($n=16$) and syn k. o. littermates ($n=15$). Although both groups improved significantly over time, wild type mice outperformed the syn k. o. on days 2–4 (* $P \leq 0.05$ in post hoc Scheffé test).

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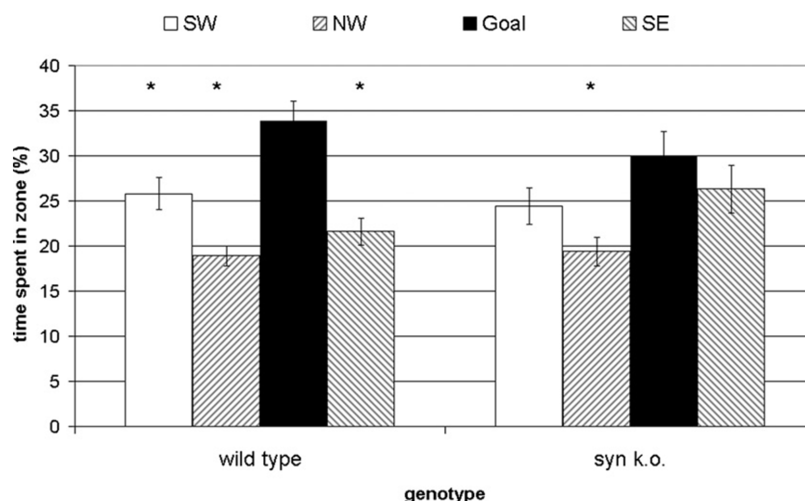

Fig. 3. Morris water-maze probe trial. The histogram shows the time spent in the different quadrants (mean±SEM; * $P \leq 0.05$ in post hoc Scheffé test compared to goal-quadrant) of age- and sex-matched wild type ($n=16$) and syn k. o. littermates ($n=15$). Note that wild type animals were able to recall the platform location whereas syn k. o. were not. Abbreviations: NW, northwest; SE, southeast; SW, southwest.

amplitudes resulting from photopic bright flash recordings are hard to measure exactly due to an initial flash artefact, but the statistical evaluation of the responses in syn k. o. mice and wild type mice did not reveal any difference (Fig. 4c). In addition, trains of flashes (flicker) were used to assess dynamic properties of the rod system (Fig. 4d, f), those of the cone system (Fig. 4e, f), and sensitivities of rods and cones (Fig. 4g, h). No substantial alterations were found in all three flicker examinations.

Optomotor reactions

To further examine the optic system, optokinetic analyses were done on a subset of 10 mice that had been behaviorally examined. These experiments showed that the average locomotor activity as defined by the average absolute angular position change from one frame to the next was not significantly different between both genotypes (wild type 0.332 versus syn k. o. 0.325). However, the average running speed revealed significant differences (Fig. 5) because wild type mice turned clockwise whereas syn k. o. mice turned counterclockwise with respect to the stimulation. Angular orientation speed also appeared to be different but failed statistical significance.

Table 4. Emotional memory characterization

Elevated plus maze (second trial)	Wild type (mean±SEM)	Syn. k. o. (mean±SEM)	ANOVA $F_{(1,28)}$
% Open arm entries	12.1±2.4	15.8±3.2	0.825; n.s.
% Time spent in open arm	5.2±1.7	7.6±1.6	1.089; n.s.

Examination of emotional memory in the second trial of the elevated plus maze test comparing age- and sex-matched wild type ($n=16$) and syn k. o. littermates ($n=15$). Group comparison by ANOVA shows no differences ($P \leq 0.05$).

DISCUSSION

Syn immunoreactivity serves as one of the core markers for synaptic integrity. Yet, the specific role of syn in synaptic transmission is hardly understood. Generation of syn k. o. mice was thought to provide answers but turned out to be much more complicated than expected: complete loss of syn does not affect viability (Eshkind and Leube, 1995) and crude behavioral analyses did not reveal an obvious phenotype (Eshkind and Leube, 1995; McMahon et al., 1996; Spiwoks-Becker et al., 2001). Even the observed reduction of SVs in retinal photoreceptors does not seem to affect neuronal transmission as assessed by ERG (Spiwoks-Becker et al., 2001). We therefore subjected the syn k. o. mice to further in-depth behavioral and visual/physiological analyses. We were able to detect altered exploration of novel objects. This difference is probably not caused by altered anxiety and impaired visual functions, since elevated plus maze assays and ERG analyses did not reveal any abnormalities in syn k. o. mice. In addition, we did not find any indication that altered habituation during object exploration is responsible for the phenotype (data not shown). Furthermore, syn k. o. mice had a significant deficit in object recognition despite their enhanced object exploration. In addition, syn k. o. mice showed

Table 5. Statistical data for emotional memory

Elevated plus maze (second trial)	MANOVA factor *time* (trials) $F_{(1,28)}$	MANOVA interaction *time*×*group* $F_{(1,28)}$
% Open arm entries	30.493; $P \leq 0.001$	0.128; n.s.
% Time spent in open arm	3.025; n.s.	0.72; n.s.

MANOVA results comparing the first and second elevated plus maze trial of age- and sex-matched wild type ($n=16$) and syn k. o. littermates ($n=15$). The significant difference in the percentage of open arm entries of both groups demonstrates intact emotional memory, although the percent of time spent in the open arm was not different in either group ($P \leq 0.05$; for genotype comparison see Table 4).

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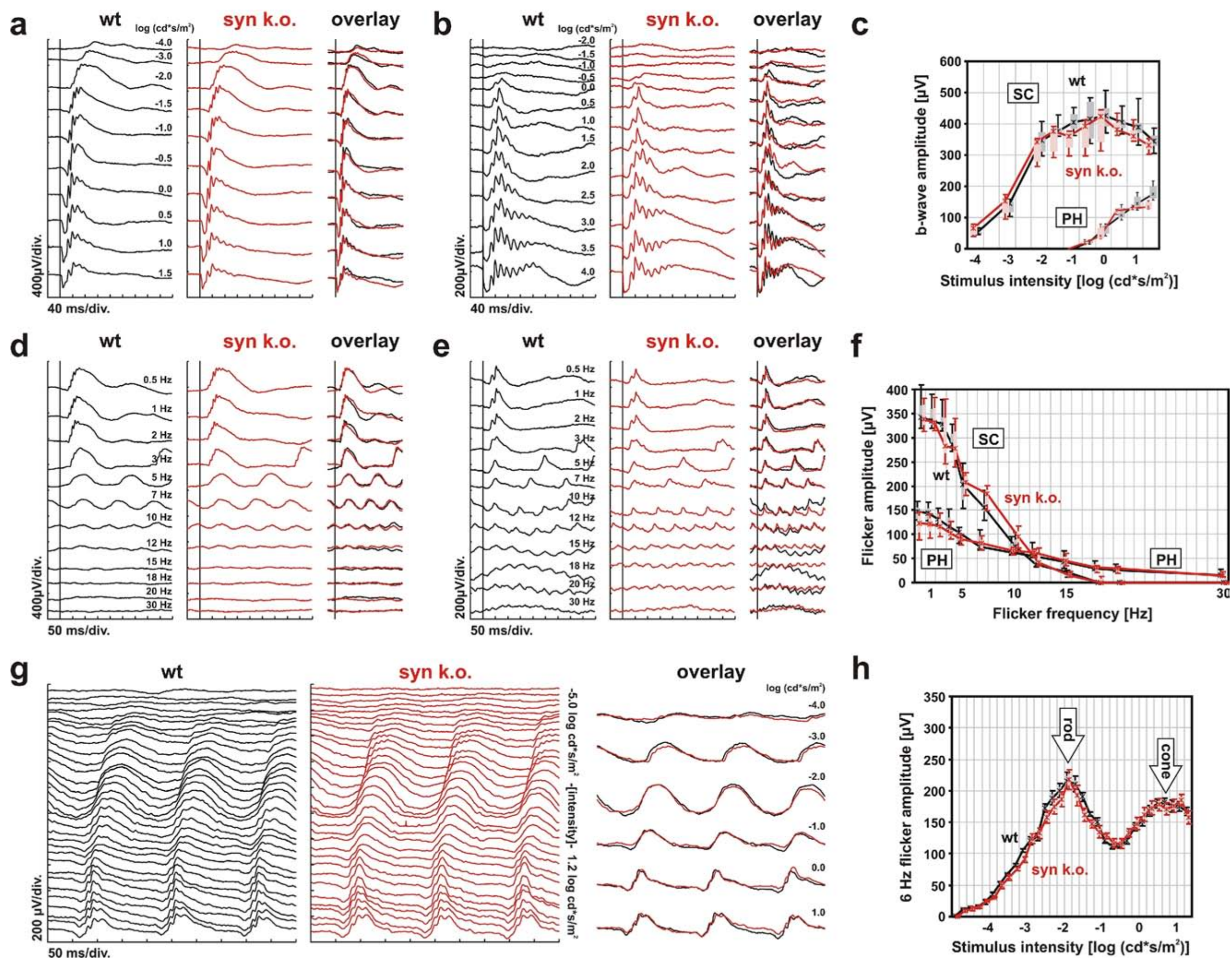

Fig. 4. ERG. (a, b) Comparisons of representative single flash ERG recordings performed in dark-adapted (a) and light-adapted (b) wild type and syn k. o. mice. (c) The statistical evaluation (box-and-whisker plot) of dark-adapted (scotopic; SC) and light-adapted (photopic; PH) single flash ERG b-wave amplitudes in wild type (black; $n=3$) and syn k. o. (red; $n=3$) mice. Boxes indicate the 25% and 75% quantile range, whiskers indicate the 5% and 95% quantiles, and the asterisks indicate the median of the data. Note that response amplitudes resulting from photopic bright flash experiments in the higher intensity range above 1.5 log s cd/m^2 are difficult to measure due to an initial flash artifact. However, no differences are noted between wild type and syn k. o. mice. (d–f) Comparisons of representative records of scotopic flicker frequency series at the intensity of −2.0 log s cd/m^2 (d) and photopic flicker frequency series at the intensity of 0.5 log s cd/m^2 (e) between wild type and syn k. o. mice. Statistical evaluation (box-and-whisker plot as above) of scotopic and photopic flicker amplitudes in wild type (black) and syn k. o. (red) mice (f) revealing no differences. (g, h) Comparisons of representative 6 Hz flicker ERG recordings of wild type and syn k. o. mice (h; statistical evaluation and color code as above). Neither shift nor reduction of both rod and cone peaks is detected. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

retarded learning in the Morris water maze with slightly impaired memory performance. Taken together, syn deficiency leads to subtle deficits in behavioral tasks that test hippocampal integrity. In light of these findings, the reported alterations of syn in hippocampal neurons in various situations such as nerve injury, increased age, Alzheimer's disease, chronic restraint stress and repeated water maze testing (Smith et al., 2000; King and Arendash, 2002; Benice et al., 2006; Gao et al., 2006; Sun et al., 2007) become meaningful and further support a central function of syn in synaptic plasticity. The dependency of the syn-synaptobrevin 2 complex on neuronal maturation and activity provides a possible molecular mechanism for such functions (Becher et al., 1999; Pennuto et al., 2002; Khvotchev and Sudhof, 2004; Reisinger et al., 2004).

Since performance in the ethological tasks examined in this study is dependent, at least in part, on the integrity of the visual system, retinal functions were analyzed by comprehensive ERG. Extending earlier results (Spiwoks-Becker et al., 2001), we still could not find any indication of differences that would explain the observed behavioral abnormalities concerning novelty, learning and memory. Yet, differences in optokinetic response were noted which might have an effect on behavior of the syn k. o. mice. It was recently shown that rod integrity is necessary for spatial acuity as mice lacking rod activity either due to a lack of rhodopsin or a specific cation channel were impaired in optokinetic responses (Schmucker et al., 2005). While these rod-impaired mice showed a reduced response, syn k. o. mice responded quite well but presented

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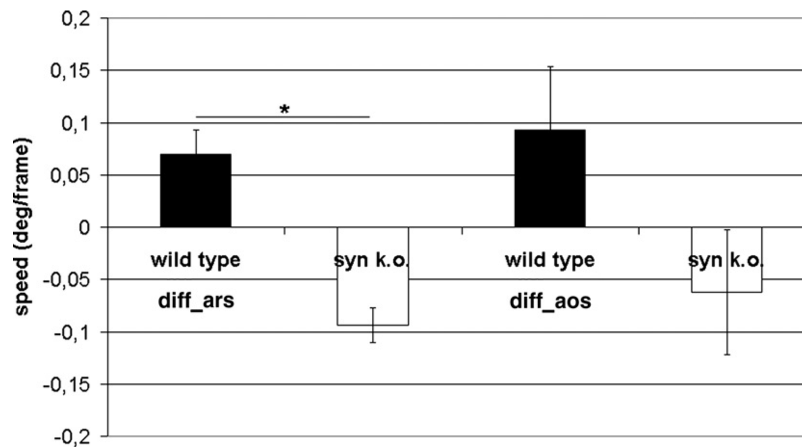

Fig. 5. Optomotor reactions. The histogram depicts the average running speed (ars; mean±SEM) and average orientation speed (aos; mean±SEM) determined in optokinetic drum assays of wild type ($n=5$) and syn k. o. mice ($n=5$). Note the opposite direction of running resulting in significant group difference in ars (* $P\leq0.05$).

a reversed optokinetic response compared to their wild type counterparts (Schmucker et al., 2005). Hence, processing of flow field motion and triggering of optokinetic nystagmus are still intact; the reversal of the direction of compensatory body movements suggests a misrouting of the nerve fibers in the optic chiasm. Such a phenomenon was previously observed in zebra fish mutants (Huang and Neuhauss, 2008). In that instance, however, reversed optokinetic nystagmus was coupled to oculomotor instability resulting in a curious swimming behavior described as circling (Huang and Neuhauss, 2008). Although the neuronal correlate of the reversed optokinetic response is not known in zebra fish, the authors made a strong argument for aberrant ipsilateral projections. The most drastic case of aberrant ipsilateral projections is a chiasmatism which has been reported for some black Belgian sheepdogs whose optokinetic response, however, was not altered (Dell'Osso and Williams, 1995). In some human albinos reversed optokinetic response has been noted (Collewijn et al., 1985). Behavioral abnormalities, however, have not been reported either in the Belgian sheepdogs or the human albinos. We think, however, that the different optokinetic response in syn k. o. does not explain the observed impairments in learning and memory, since all ERG parameters (i.e. responses to light- and dark-adapted single flash, flicker frequencies and amplitudes) and basic locomotion in open field or elevated plus maze were normal. Furthermore, visually-deprived rats immediately enter the center field of an open field (Göb et al., 1987). This is in contrast to the syn k. o. mice for which we did not find any differences in latency to enter the center field in comparison to wild type (data not shown). Also in contrast to our syn k. o. mice, FVB/N mice with a genetic visual deficit due to retina degeneration were unable to learn the water maze task (Royle et al., 1999; D'Hooge and De Deyn, 2001). These observations argue against a significant visual impairment in syn k. o. mice and suggest that their optokinetic abnormalities do not account for the behavioral deficits described above. Clearly, further analyses of optic tract anatomy are needed to understand the pathology of the reversed optokinetic response.

Coincident with impaired learning and memory, decreased levels of synaptic proteins have been reported in several investigations (Royle et al., 1999; Smith et al., 2000; Wu et al., 2008). Whether these differences reflect a reduced synaptic connectivity which is known to lead to deficits in learning and memory (Sun et al., 2007) or whether it is the cause of deficits is presently unclear. Investigations using syn immunoreactivity as a marker demonstrate that its reduction is paralleled by decreased connectivity, subsequent functional deficits in synaptic transmission and eventual cell loss (Sun et al., 2007). With respect to learning, Frick and Fernandez (2003) reported that increased syn correlates with improved spatial memory in the water maze (Frick and Fernandez, 2003) corresponding to the present phenotype of syn k. o. mice. In addition, findings in aged male rats indicate that environmental enrichment can significantly increase pre-SV number in the frontal cortex (Nakamura et al., 1999) and syn expression in the hippocampus as well as several cortical regions (Saito et al., 1994). Thus, modulation of syn may play a role in enrichment-induced alterations in memory. Recently, Holahan and colleagues (2006) were able to demonstrate in rats that water maze learning in the hidden platform design induces expansion of presynaptic terminal fields in hippocampal mossy fibers. The authors were able to show that this learning-induced plasticity is reflected by an increase in several presynaptic markers which also included syn suggesting that it is involved in learning and memory-dependent neuronal plasticity (Holahan et al., 2006). In syn k. o. animals compensatory mechanisms may be used to fulfill requirements of synaptic transmission in newly formed synapses. While these mechanisms will eventually lead to the same outcome they may be less efficient and take longer to achieve full functionality. In accordance, the phenotype characterized here shows deficits in learning and memory that appear to result from a delayed or retarded learning since the syn k. o. mice were

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able to learn over time but achieved the level of wild type mice only two days later. Since the Morris water maze is an aversively motivated task (D'Hooge and De Deyn, 2001) altered stress response might affect behavioral performance as well. Recently, the expression of proteins involved in SV exocytosis in the hippocampus was shown to correlate to the efficiency of neurotransmitter release after chronic stress (Gao et al., 2006). Thus, synaptobrevin and also syn were increased in conjunction with pronounced morphological changes. The importance of syn for neuronal stress-response is also supported by the finding that chronic corticotrophin administration leads to increased syn levels (Grillo et al., 2005). On the other hand, 4 h of chronic immobilization stress for three consecutive days has been associated with decreased syn immunoreactivity in the CA1 subfield of the hippocampus (Xu et al., 2004), and another study reported a reduction in syn mRNA expression in the CA1 and CA3 fields and the dentate gyrus of animals subjected to acute or chronic exposure to this type of stress for 1 h on 5 consecutive days (Thome et al., 2001). The latter study also reported on increased synaptotagmin expression in the same regions. Generally, immobilization stress is considered to be more severe than restraint stress, with more profound effects on hippocampal plasticity occurring in a much shorter period of time (Vyas et al., 2002). Independent of the duration and intensity of stress, however, the response appears to be altered in syn null mice to such an extent that learning abilities under stress conditions like the Morris water maze are compromised.

On a molecular level, the demonstration of the association of syn with multiple components of the SV cycle provides a basis for its modulating role in higher-order brain functions (Felkl and Leube, 2008). An attractive idea is that it is part of a membrane platform that acts as a facilitator of various steps of the SV cycle and thereby affects the neurotransmission efficacy. Regulation could be accomplished by phosphorylation of syn's multiple tyrosine residues (Hübner et al., 2002; Evans and Cousin, 2005; Felkl and Leube, 2008). The current identification of phenotypic changes related to the absence of syn therefore provides a big step forward to identify and characterize the precise functions of this abundant SV protein.

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