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VGLUT2-mediated glutamatergic neurotransmission during development of neuronal circuits

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Title (Swedish)

VGLUT2-medierad glutamaterg neurotransmission under utvecklingen av neuronala kretsar

Abstract

The phenotype of a VGLUT2-targeted mice strain with the new Emx1-VGLUT2 construct was examined with knockout techniques in order to draw conclusions of a potential schizophrenia-like phenotype. Methods of verification included *in situ* hybridisation, immunofluorescent analysis, tracing techniques and behaviour analysis. Results from this analysis will be compared to previous results with the CamKII-VGLUT2.

Keywords

VGLUT2, Emx1, glutamate, schizophrenia, vesicular glutamate transporters

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OUTLINE

VGLUT2-mediated glutamatergic neurotransmission during development of neuronal circuits

Assar Bergfors

Sammanfattning

I detta projekt studerades fenotypen hos en nyskapad knockout mus med bland annat vävnadstekniker för att sluta sig till om den liknade en schizofreniliknade musmodell som producerats tidigare av forskargruppen. Tidigare forskning har nämligen visat att den schizofreniliknande musmodellen fås om man tar bort en vesikulär glutamat transportör (VGLUT2) från att musen är 15 dagar gammal i hjärnområdena retrospleniala kortex (RSG), subiculum och amygdala. Det nya konstrukt som detta projekt baseras på använder den vävnadsspecifika promotorn Emx1 som aktiveras från att fostret är 9 dagar gammalt. När Emx1 aktiveras tas VGLUT2 bort från de vävnader som har denna promotor. Det som främst förväntades var att knockningen skulle skilja sig temporalt mellan de båda modellerna, men det visade sig att även regionerna som knockades skiljde sig åt. Tekniker som användes under analysen var *in situ* hybridisering, immunofluorescens, tracingtekniker och beteendetester.

I detta projekt observerades utslagningen av mRNA uttryck från VGLUT2 genen i hjärnområdena RSG, subiculum och amygdala vilket var väntat. Vad som var nytt var att förändring av mRNA uttryck observerades även i claustrum, piriform cortex, ventral endopiriform nucleus och några regioner av luktbulben. På grund av att dessa ytterligare områden inte tidigare varit påverkade analyserades de utförligt med avseende på möjlig funktion. Det gjordes även försök med tracingtekniker för att försöka verifiera hur nervcellerna från de påverkade områdena kommunicerar med andra nervceller och vad som i så fall händer nedströms från dessa områden. Slutligen gjordes ett beteendetest för att ytterligare förstå fenotypen hos den studerade knockoutmusen.

Examensarbete 20p i Molekylär bioteknik

Uppsala Universitet, oktober 2008

1. AIM

The aim with this project is to study the phenotype of a novel conditional VGLUT2-targeted mouse line, the Emx1-VGLUT2 mouse line.

This will be done by analyzing the histology and behaviour of Emx1-VGLUT2 mice, and by comparing the results between knockout and control mice.

2. Introduction

Glutamate is the most prominent excitatory neurotransmitter in the CNS and it is present in all neuronal circuits. Defects in signaling with glutamate gives substantial alterations of brain function. Some diseases earlier associated with dopamine such as schizophrenia, depression, Parkinson's and chronic pain are now viewed as possibly being caused by defective glutamate signaling [1-3]. The fact that these diseases have been associated with dopamine and not glutamate in the earlier research is mainly because there were some missing pieces in the dopamine-glutamate puzzle. A neuron's inherent ability to be glutamatergic has been hard to assess because of the fact that glutamate is present in all cells in the nervous system. This has previously focused the research in this field on downstream receptors. All of this changed with the identification of three new members in the solute carrier family (*slc17a6-8*, aka VGLUT1-3) named vesicular glutamate transporters, which gave the means to identify neurons signaling with glutamate [4-7].

The three vesicular glutamate transporters are VGLUT1, VGLUT2 and VGLUT3. They are active during different stages of development and have different areas of functionality. VGLUT2 can be detected from midgestation and is most prominent prior to birth, but subsequently gets down-regulated in most of the forebrain in favour of VGLUT1 which acts in a similar way and is most prominent around the second week of postnatal development. As the levels stabilize there is a complementary expression in the tissues that initially only expressed VGLUT2. VGLUT1 is up-regulated in the areas of cerebral cortex, hippocampus and cerebellar cortex but VGLUT2 is still dominant in the thalamus, brainstem and deep cerebellar nuclei in the adult brain [8]. VGLUT1 and VGLUT2 are viewed as the most reliable markers for glutamatergic neurons and are important in the study of excitatory neurons *in vivo*. VGLUT3 on the other hand is expressed in cells that do not use glutamate as their primary neurotransmitter, e.g. cholinergic neurons in the striatum. VGLUT3 is therefore not used as a cellular marker for glutamatergic neurons [9].

The omnipresence of glutamate in all cells has set an evolutionary direction towards compartmentalization in order to achieve signaling in neurons that use glutamate as their neurotransmitter. This compartmentalization primarily takes place in two steps.

First there is a division between extracellular and intracellular space where the glutamate transporters EAAT 3-4 create a gradient of concentration that is around 10^4. The second compartmentalization is done by the neuronal vesicles where the VGLUTs create a secondary gradient of concentration that is 10^1-10^2. These transporters and gradients are depicted in Figure 1. In order to achieve signaling with glutamatergic neurons the CNS need a constant production of these synaptic vesicles for the neurotransmission to occur. The vesicles are produced in the golgi system in vicinity of the nucleus and are subsequently transported along axons to the presynaptic terminal. Once there, VGLUTs are incorporated in the vesicle membranes, filled up with glutamate and ready for depolarization. Every excitatory neuron that uses glutamate as a mediator for signaling is specific for only one of the three types of VGLUTs [8].

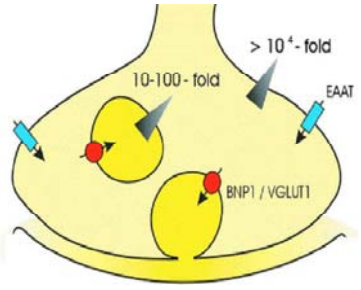


Figure 1. The synapse of a glutamatergic neuron showing VGLUT1 or VGLUT2 (red dots) and glutamate transporters EAAT 3-4 that maintain the gradients of concentration between external and internal environments. Illustration used with permission from Åsa Mackenzie.

2.1 Background

To study the role of VGLUT2-mediated signaling in the nervous system, a conditional VGLUT2 gene targeted mouse (VGLUT2Lox) was produced by the Kullander lab [10]. In the VGLUT2Lox mouse, exons 4-6 of the genomic VGLUT2 locus is surrounded by Lox sites (a schematic drawing of the construct is shown in Figure 3). In order to achieve region-specific deletion of VGLUT2 expression, the VGLUT2Lox mouse is crossed with a region-specific Cre mouse, in which the Cre recombinase is expressed under control of a region-specific promoter. In the initial study, the VGLUT2Lox mouse was crossed to the PGK-Cre mouse line, where the Cre driving deletion of VGLUT2 is ubiquitously expressed thus leading to a full knockout of VGLUT2. These mice suffered from respiratory abnormalities and died soon after birth. The analyses identified VGLUT2 as required for formation of the respiratory neurocircuitry and showed that VGLUT2 is essential for life [10]. While VGLUT2 is thus known to be essential for life-supporting functions, higher brain functions such as emotion, cognition and memory have been coupled to VGLUT1, which is heavily expressed in the forebrain, and not VGLUT2. For instance, it has been shown that a full knockout of VGLUT1 produced viable mice but with substantial neurodegradation leading to premature death [11-13].

However, as VGLUT2 is expressed in a few cell populations in the adult forebrain, Mackenzie and Kullander used the CamKII-Cre mouse line to delete VGLUT2 from postnatal day 15 (P15) in these cells in order to study whether VGLUT2 has a role in higher brain function. In CamKII-VGLUT2 mice, VGLUT2 was deleted in the retrosplenial group (RSG) and subiculum in the cortical-hippocampal region. This site-specific deletion also took place in the basomedial amygdala (BMA), medial amygdala (MeA), anterior cortical (ACo) and bed nucleus of the anterior olfactory tract (BAOT) nuclei of the amygdala. This deletion induced behavior characteristic in the mice that is associated with the disease schizophrenia in human patients. These symptoms included the positive, negative and cognitive defects that are the cornerstones of this diagnosis. To make a schizophrenia-like distinction of the mice, a rigorous phenotyping route was constructed containing eight steps with behavioural and physiological character. If the mice showed defects in at least one characteristic for each of the three symptoms that make out the cornerstones it was determined to have the phenotype [14].

In the human population, schizophrenia affects about 1% and usually has an onset in early adulthood [1], [15-16]. The positive, negative and cognitive defects are behaviorally expressed as paranoia and hallucination, blunted affect and social withdrawal and finally defects in cognitive processing. A complete neurobiological understanding of schizophrenia has however still not been made because of the complexity of the disease. According to the main theory, an increase in dopamine signaling leads to the disease. This is based on observations of neuroleptics that block dopamine-signaling receptors leading to a positive therapeutic effect on the disease, and also on the fact that abusive drugs which mimic the symptoms of schizophrenia increase the dopaminergic signaling. There is however another theory that explains the disease as caused by decreased glutamate signaling. The indications point towards the direction that both dopamine and glutamate play important roles in the acquirement of schizophrenia. Drug development drawn from the hypoglutamatergic theory is however not well explored due to lack of animal models and molecular knowledge. A schematic picture of regions and pathways suggested to be involved in schizophrenia is depicted below in Figure 2.

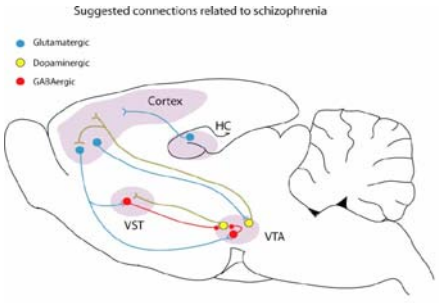


Figure 2. Proposed model of glutamate and dopamine signaling pathways putatively involved in schizophrenia. Illustration used with permission from Åsa Mackenzie.

The CamKII-VGLUT2 mouse is a strong support for the hypoglutamate theory because it shows the connection between the hypoglutamate state and behavioural phenotypes related to schizophrenia. This conclusion could be drawn with the collected data from the rigorous phenotyping route. The eight steps in this route are multivariate concentric square field (MCSF), elevated plusmaze, forced swim test, social dominance test, pre pulse inhibition (PPI), morris water maze, electrophysiology and micro positron emission tomography (PET) analysis.

As schizophrenia is believed to contain a developmental component and VGLUT2 is expressed from midgestation in the mouse embryo, the Mackenzie/Kullander lab crossed the VGLUT2Lox mouse with the Emx1-Cre mouse to analyze deletion of VGLUT2 expression from midgestation. The Emx1 promoter is active from embryonic day 9 (E9) and the Emx1 gene is expressed in many different types of cells including radial glia cells, Cajal-Retzius cells, astrocytes, oligodendrocytes and glutamatergic neurons of the forebrain. Expression of Emx1 has also been documented in some of the cranial nerves (VII-X) that connect the brain directly to the sensory input from the ears, eyes and other sensory systems [17].

By producing the Emx1-VGLUT2 mice it is possible to analyze the impact on the formation and function of the neuronal circuitry involved in cognitive, emotional and social functions during embryonal development where VGLUT2 seems to play a major role. In my exam project, I have analyzed the extent of the Emx1-Cre-driven deletion of VGLUT2 expression in the Emx1-VGLUT2 mice and also the projections of cells that express Emx1-VGLUT2. I have been involved in the breeding of Emx1-VGLUT2 mice for behavioral analyses and I have participated in conducting the social dominance test. As the group is interested in further analyzing the phenotype of the CamKII-VGLUT2 mice, I have performed in situ hybridization analysis of genes which may act as future promoters for Cre. I have also used tracing analyses to detect projecting pathways. The experiments will be described and discussed below.

3. Material and methods

3.1 The mice

A male mouse with the EMX1-Cre driving promotor is bred with a female mouse containing the VGLUT2-Lox construct, as seen in Figure 3. A certain percentage of the pups will have both the VGLUT2-Lox and EMX1-Cre constructs inserted resulting in region-specific deletion of VGLUT2. All handling with the animals were executed according to the standard procedures for animal regulations.

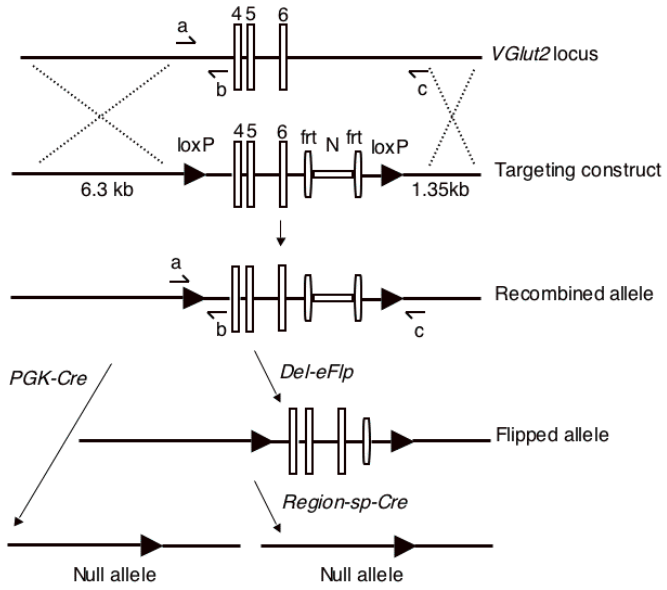


Figure 3. VGLUT2 expressing exons (4, 5 and 6) are surrounded by the lox sites, which are marked as black triangles each consisting of a 34bp sequence. The neomycin resistance gene (N), which is used for selection in bacterial cells and in embryonic stem cells is surrounded by FRT sites each consisting of a 22bp sequence. The N cassette is excised by del-flp. Illustration used with permission from Åsa Mackenzie.

In order to determine the genotype of the mice a piece of the tail is collected, put in an eppendorf tube and incubated in 96°C in 75µl Tail buffert I (250 mM NaOH and 2 mM EDTA) for 45 min. Subsequently the tube is put on ice for 5 min before adding 75µl Tail buffert II (400 mM Tris-HCl pH 8). The next step in analysis is genotyping with PCR and separation of DNA fragments with regard to size on an ethidium bromide-containing agarose gel.

3.2 PCR

The different PCRs conducted in this project were done on a PTC-200 PCR-machine. They test for the presence of EMX1-Cre and whether the VGLUT2 alleles are surrounded by Lox sites (in which case no recombination has occurred) or whether only one Lox site remains, which means that exons 4-6 have been deleted. The xlx-PCR is used to verify if the alleles have been deleted. The primer sites related to the VGLUT2-Lox are depicted in Figure 3.

Tau^{mGFP} (SAM) is a transgenic mouse line that is used as a reporter for Cre activity and it is genotyped using LacZ-specific primers. Presence of Cre will delete the stop codon in the tau transgene, which leads to transcription of the GFP and LacZ genes. This construct is depicted in Figure 4.

Tau - ATG -►STOP►- ATG - GFP - ATG - NLS-LacZ

Figure 4. Tau^{mGFP} used in immunofluorescense analysis. Transcription will give tau-coupled gfp and nls-coupled LacZ that aggregates in fibres and the cell nuclei respectively.

When performing a PCR analysis, the reagents are first mixed in an eppendorf tube on a bed of ice. Finally the DNA preparation is added to the PCR tubes and gravitated to the bottom by low rpm centrifugation. The suiting PCR program is initiated. PCR mixtures and related programs are given below in Table 1. Primer sequences are given in Table 2.

PCR components		PCR program		
Cre PCR	*Volume (µl)*	*Step*	*Min*	*Temp* (°C)
10 X Buffer	2	1	5:00	95
MGCl2	2	2	0:30	95
dNTP	1	3	0:30	55
Primer 393	0.5	4	0:40	72
Primer 394	0.5	5	Go to step 2	30 times
Klas Taq	1	6	6:00	72
MilliQ	12	7	0:20	20
DNA	1	8	End	
Total	20			

PCR components		PCR program		
xlx PCR	*Volume (µl)*	*Step*	*Min*	*Temp* (°C)
10 X Buffer	2	1	2:00	95
MGCl2	2.4	2	0:30	95
dNTP	1	3	1:20	60
Primer 284	0.8	4	1:15	72
Primer 286	0.8	5	Go to step 2	30 times
Klas Taq	1	6	6:00	72
MilliQ	11.5	7	2:00	20
DNA	0.5	8	End	
Total	20			

PCR components		PCR program		
wt/lx PCR	*Volume (µl)*	*Step*	*Min*	*Temp* (°C)
Hifi	6.25	1	2:00	94
Primer 283	0.4	2	0:30	94
Primer 284	0.4	3	0:30	60
MilliQ	5.4	4	2:00	68
DNA	1	5	Go to step 2	30 times
Total	13.45	6	7:00	68
		7	20:00	20
		8	End	

PCR components		PCR program		
SAM PCR	*Volume (µl)*	*Step*	*Min*	*Temp* (°C)
Hifi	6.25	1	2:00	94
Primer 79	0.4	2	0:50	94
Primer 80	0.4	3	1:00	60
MilliQ	5.4	4	1:00	68
DNA	1	5	Go to step 2	35 times
Total	13.45	6	6:00	72
		7	on	4
		8	End	

Table 1. Components and programs for the PCRs used. Primers were supplied by the lab and diluted 1:10 to larger batches before usage to give a final concentration of 1x. The KlasTaq was produced by the lab.

Primer	Sequence
OKK 79	CAGGACAGTCGTTTGCCGTCTGA
OKK 80	CAGAGGATGATGCTCGTGACGG
OKK 283	GCAATCACATTTCACTGTTC
OKK 284	CTGTCCACCTTTGTATCCCA
OKK 286	CACACCCACTCCACTTGAGG
OKK 393	ACGAGTGATGAGGTTCGCAAGA
OKK 394	ACCGACGATGAAGCATGTTTAG

Table 2. Primer sequences for the PRCs.

After completion of the PCR program, loading buffer is added to the amplified DNA and the resulting mixture is loaded on an agarose gel with either 1 or 2% concentration depending on the size of the fragments analyzed. A 2% gel is used to get sufficient resolution on the wt/lx PCR with two fragments of 150bp and 184bp size. The remaining PCRs are run on a 1% gel. After separation of the bands is achieved, the gel is placed on a UV table and a picture is saved for future reference. The result is compiled in tables and concluding genotypes are determined. The goal is to have at least one full knockout and one control from the same breeding cage but it is also useful to have additional knockouts as well as control brains. The next step is either to perfuse the mouse and collect the brain or the entire head for small pups, alternatively to save animals for behavioral analyses. Perfusion was done according to animal regulations and protocols. Postfixation is done in 4% formaldehyde solution (Histolab), for a duration ranging from 3h up to a couple of days. Further analysis of mRNA and protein levels was done with *in situ* hybridization on vibratome or cryo sections and immunoflorescent analysis on cryosections. Further analysis on projecting pathways for neurons was done with tracing techniques.

3.3 Cryosectioning

In order to perform cryo sectioning, the mouse brain or head is casted in Tissue Tek (polyvinylalcohol <11%, carbowax<5%, non-reactive ingredients<85%) and left in -80°C freezer over night. Before sectioning there is a temperature stabilizing period to -20°C of thirty minutes in the Leica Cryocut-1800 sectioning apparatus. The brain is glued with Tissue Tek to a small metallic plate and left to aggregate in the apparatus. Sectioning was done in a rostrocaudal coronal fashion. The angle of the cutting knife is calibrated with regard to the tissue in order to get symmetrical sections. Width was set to 12µm and completed sections were thaw-mounted onto SuperfrostPlus slides (Thermo Sci, Menzel-Gläser) and left to dry in room temperature during the remaining of the sectioning. After sectioning is completed the glass plates were either analyzed by *in situ* hybridization, immunofluorescence or stored in -80°C for further usage.

3.4 Vibratome sectioning

An important thing to consider when analysis with *in situ* hybridization is to be performed is to keep all equipment and solutions free from RNases. Because of this, all of the equipment is cleansed with RNA Zap before usage and autoclaved solutions based on DEPC-H_2O were used. Vibratome sectioning is performed on the fixed brain by first encapsulating it in 4% agarose gel, cast in a 6 well plate and left to coagulate on ice.

The agarose surrounding the brain is trimmed and the remaining cube is fixed to a small magnetic plate with superglue. The plate is attached to the vibratome by magnetic force and the sample is covered in PBS (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4, dilution with DEPC-H_2O, pH 7.4 adjusted with HCl). Sections were of 60µm

width, detached from the surrounding agarose with a brush and moved to a 6 well plate with 3 ml of RNase free PBS (DEPC) in every well. Sections were dehydrated by successive washes in 25%, 50% and 75% in methanol with RNase free PBT (PBS-DEPC, 0.1% Tween-20) for 10 min at each step. Final storage was made in 100% methanol in -20°C freezer.

3.5 Detection of mRNA levels with *in situ* hybridization (floating sections)

Keeping the sections free from RNases before the hybridization step is crucial and all solutions and equipment used were RNase free until this step was completed. For removal and addition of liquid a 1000 µl pipette was used. All steps were performed at room temperature on shaker if not otherwise stated. First, vibratome sections required for the analysis was put in new 6 well plates with a brush and rehydrated in first 75% then 50% and 25% methanol in PBT (PBS-DEPC, 0.1% Tween-20) for 10 min in each step. Sections were washed for 3x5 min in PBT, bleached in 500µl/well of 6% H_2O_2 diluted in PBT for 15 min before washing with PBT for 3x5 min. Subsequent treatment with 0.5% Triton X followed for 5 min before washing 2x5 min with PBT, digestion with 500µl/well of a mixture containing 0.15µl/ml proteinase K (PBT with 10mMTris-Cl pH 7.5, 20 mM $CaCl_2$, 50% glycerol) and PBT for 15 min before 3x5 min washes in PBT. Following this procedure, sections are post fixed in 4% formaldehyde (Histolab) for 20-30 min before washing 3x5 min in PBT and incubation in hybridization buffer with 500µl/well for 2h in incubation oven at 55°C. The next steps is a preparation of the probe by dilution to 1 ng/µl, heat denaturation in hybridization buffer at 80°C for 5 min, cooled on ice for 5 min and finally prewarmed to 55°C. Hybridization buffer was carefully removed from the wells before adding 300-500µl/well of the mixture containing the denatured probe. The well plate was sealed with tape to avoid evaporation before incubation at 55°C over night.

The next morning successive washes were performed 3x30 min with 1ml/well of wash buffer II (PBT with 50% formamide, 2xSSC pH 4.5, 0.1% Tween-20), 3x30 min with 1ml/well wash buffer III (PBT with 50% formamide, 0.2xSSC pH 4.5, 0.1% Tween-20). All washes were performed with liquids and incubation oven at 55°C. The following washes in TBST (TBS + 0.1% Tween-20) were performed for 2x5 min before incubating in 1x blocking reagent in TBST with 700µl/well for 2h. In the next step anti-DIG antibody was diluted 1:5000 in blocking solution before being added at 500µl/well and incubated over night at 4°C after sealing the plate with parafilm to avoid evaporation.

The next morning starts with placing BM purple AP substrate in room temperature, washing the sections with 500µl/well of TBST containing 2µl/ml levamisol for 5x10 min and with 500µl/well of NTMT containing 2µl/ml levamisol for 1x10 min. Applying of the room temperate BM purple AP on the sections and development continues until sufficient staining has been achieved. When stained, the sections are

washed 3x10 min in PBS before post fixed in 4% formaldehyde (Histolab) for 15 min, both times with 1 ml/well. After fixation the sections are washed a final time 3x5 min in PBS before being mounted to glass slides, preserved with addition of PBS, cover slides and fixed with nail polish.

3.6 Detection of protein levels with immunofluorescent analysis (cryo sections)

It is important not to touch the sections during the analysis and keep all tools and liquids free from contamination. The first thing after completing the cryo sectioning is to let the slides dry for 30-60 min and to draw a line around the sections with a hydrophobic Pap-pen. After this, washing for 3x5 min with PBS is initiated in a glass cyvette before incubation in a humidity chamber with PBS mixed with 0.3% Triton X and 1.5% goat serum for 30 min. Next step is incubation over night in humidity chamber. The chamber consist of a plastic lid and bottom with plastic pipette tips creating an elevated level for the slides to lay on, the bottom is also covered in paper towels soaked in PBS. To this 100µl/slide is added of PBS mixed with 0.3% Triton X and the primary antibodies rabbit ß-gal in concentration 1:2000 and chicken GFP in concentration 1:1000.

Next day, slides are washed 3x10 min in PBS before a second incubation for at least two hours in humidity chamber with 100-200µl/slide of PBS mixed with 0.3% Triton X and the fluorescent secondary antibodies anti-ß-gal (red) and anti-GFP chicken (green) diluted 1:300. During incubation with the secondary fluorescent antibodies and in the following steps it is important to keep all slides protected from light. Aluminum foil was used for this protection. After incubation the slides were washed for 2x10 min in PBS before washing 10 min in PBS with 1:1000 concentration of the nuclear stain DAPI (1µg/ml). Finally the incubation is washed once in PBS for 10 min before mounting with anti-fade medium and cover slides before sealing with nail polish. Slides were stored in darkness or documented with the microscope.

3.7 Detection of projecting pathways

Projection studies were done in two trials with whole or parts of the fixated brain. The projecting component is red crystals (DiI) that are inserted to specific parts of the brain. The dye travels with 0.3 mm per day which makes it possible to calculate an estimation of the incubation time needed for a specific projecting fiber. The first trial was conducted on fixed wildtype brains sectioned in a coronal fashion with the aid of a matrix form and razor blade to display the ventral tegmental area (VTA). Crystals were then applied with tweezers or a thin needle to the site of interest before incubation in 4% formaldehyde (Histolab) at 37°C for 2-3 weeks. After incubation the brains were cast in 4% agarose gel and prepared for 60µm vibratome sections. This was conducted under low illuminated conditions to preserve the light sensitive crystals and separated sections were put in a 6 well plate on a bed of ice with 3ml/well of PBS with 2% FA (Histolab) for further

fixation. After completion, sections were applied to glass slides with addition of anti-fading mounting medium for preservative effect and finally sealed with cover glasses and nail polish.

In the second trial, the fixed wildtype brains were first sectioned in a coronal fashion with the matrix form but this time to display the striatum, RSG, subiculum and amygdala areas. After this step, the brains halves were exposed to the floating section in situ hybridization protocol with some small modifications. These were as follows: Variation of proteinase K in PBT on the different half-brains with concentrations 0.10, 0.15, 0.20 and 0.25µl/ml in the digestion step. Different amount and concentration of added probe and finally, the total amount of solution in all steps were adjusted to cover the half-brains. Time of development was 4.5 h. After the developed brains were washed the final 3x5 min in PBS, crystals were applied with tweezers and a thin needle to the areas where development had occured. The brain halves were incubated in 4% formaldehyde (Histolab) at 37°C for 5-8 weeks. After incubation the brains were cast in 4% agarose gel and prepared for 60µm vibratome sections. For the remaining steps the previous protocol was followed.

3.8 Social dominance test

The social dominance test was conducted in collaboration with Karin Nordenankar, a PhD student in the lab, in a setting where no perfusion or killing of mice had occurred previous to the test. The setup contains a tube constructed by plastic sheets of regular paper size (A4) and was assembled with tape on the longest side to create the longest possible tube length (25cm), the diameter was set to 3 cm. One knockout versus a control mouse was tested at each trial with one of the test leaders being unaware of the genotype, to prohibit biased decisions in the assertion of dominance. This was performed in the following procedure. One mouse at a time was collected from the breeding cage and left to rest on the sleeve of the test leader's protection coat. Both mice were then inserted to either end of the test tube and released simultaneously. After one of the mice had been pushed by the other mouse or spontaneously backed out it was determined as socially submissive while the other one was determined to be socially dominant. This procedure was performed twice for every pair of participating mice. After testing was completed the data was summarized by my collaborator, to determine possible significant trends between the wildtype and knockout mice.

4. Results

Expression of VGLUT2 mRNA was analyzed with *in situ* hybridization and qualitatively determined with regard to level of staining. The analysis was simultaneously performed on one knock-out mouse and one control mouse to guarantee equal conditions. In these mice, a total deletion of VGLUT2 mRNA was found in the cortex, retrosplenial group and subiculum. The nuclei of the amygdala were also analyzed but with insufficient clarity in the VGLUT2 mRNA expression, due to lack of sections in the expressed areas. The observed trend was however that the anterior cortical (ACo) and basomedial amygdala (BMA) displayed partial deletion while no deletion was found in the medial amygdala (MeA) and bed nucleus of the anterior olfactory tract (BAOT) shown in Figure 5. Further analysis showed total deletion in the mitral cell layer and partial deletion in the periglomerular layer of the olfactory bulb. In addition to these areas it was also shown that claustrum, ventral endopiriform nucleus and piriform cortex had partial deletion shown in Figure 6. These results are displayed in below.

4.1 Expression in RSG, subiculum and amygdale

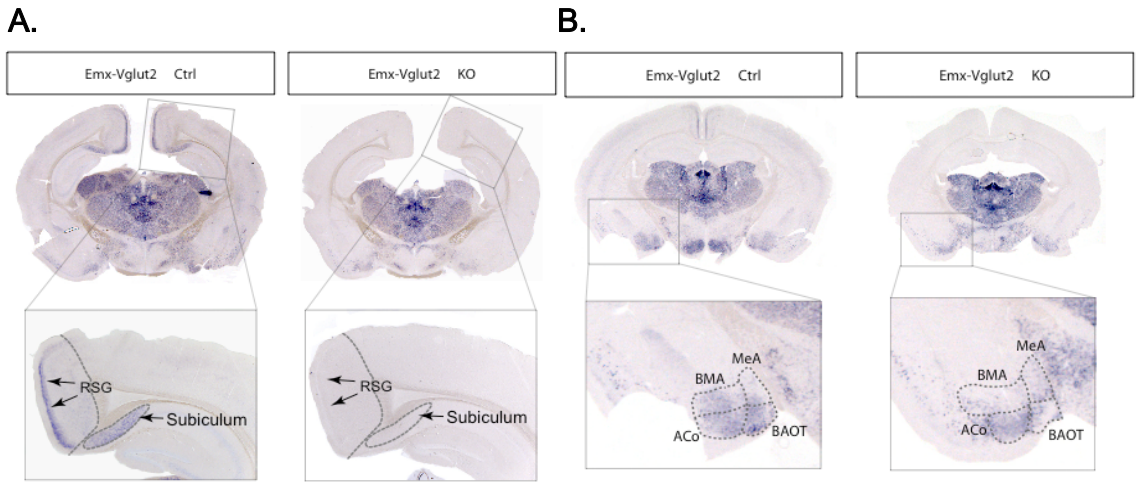
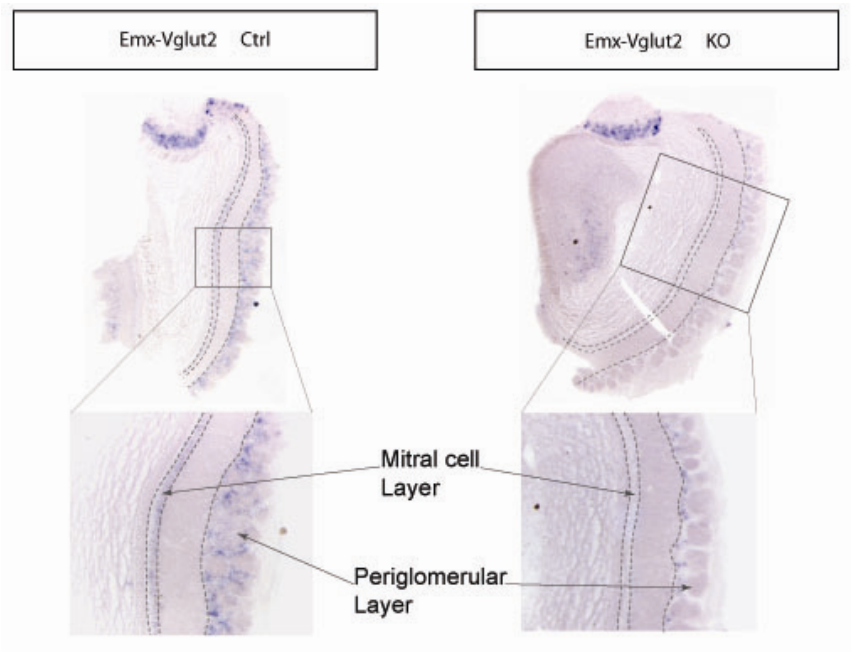


Figure 5. In situ hybridization analysis on 60 µm vibratome sections with BM purple AP staining displaying expression of VGLUT2 mRNA (purple staining) from wildtype and knockout mice. The four pictures to the left (**A**) *show the retrosplenial group (RSG) and subiculum with magnifications. The four pictures to the right* (**B**) *show four regions of the amygdala. These are the anterior cortical (ACo), basomedial amygdala (BMA), medial amygdala (MeA) and bed nucleus of the anterior olfactory tract (BAOT).*

4.2 Expression in olfactory bulb, claustrum, VeN and piriform cortex

Olfactory bulb



Claustrum, VeN and Piriform cortex

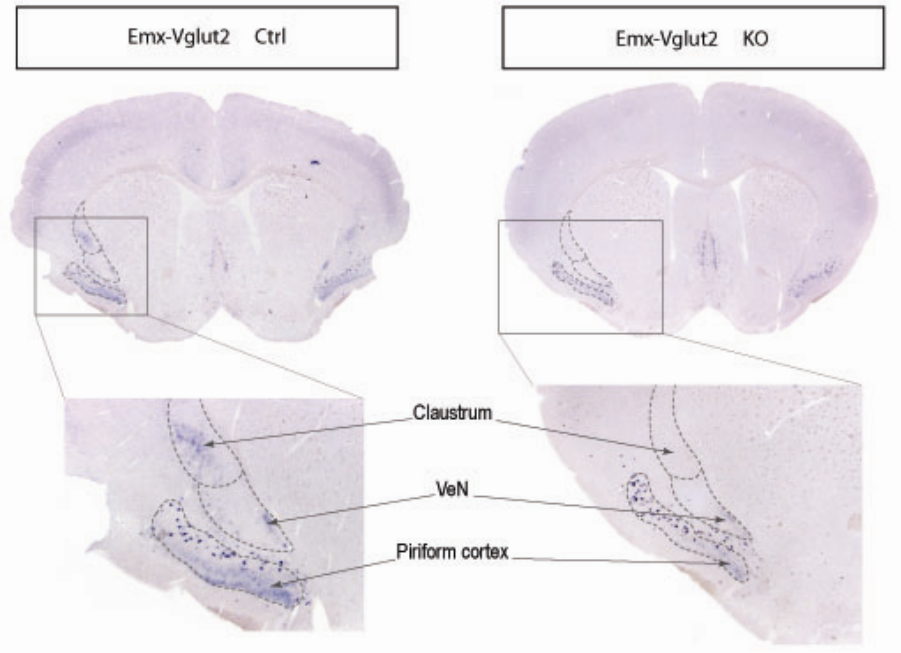


Figure 6. In situ hybridization analysis on 60 µm vibratome sections with BM purple AP staining displaying expression of VGLUT2 mRNA (purple staining). Top four pictures show the mitral and periglomerular cell layer of the olfactory bulb. Lower pictures display the claustrum, ventral endopiriform nucleus (VeN) and pirifom cortex.

4.3 Summary of VGLUT2 expression

In addition to the expression profiles described above, I also made a summary of the VGLUT2 mRNA expression in a large number of areas of the brain. This summary is shown in Table 3. Amount of expression is rated as present (+ sign), absent (– sign) or at low levels (–* sign).

Structure	Ctrl	KO	Structure	Ctrl	KO
PALLIUM			OLFACTORY BULB		
neocortex	+	-	mitral cell layer	+	-
RSG	+	-	external plexiform	-	-
subiculum	+	-	internal plexiform	-	-
hippocampus	-	-	periglomerular layer	+	-*
septohippocampus	-	-	granule cell	-	-
ventral endopiriform	+	-*	eppendymal layer	-	-
piriform cortex	+	-*	THALAMUS		
claustrum	+	-*	habenula	+	+
BORDER REGIONS			anterodorsal	+	+
lateral septum	-	-	laterodorsal	+	+
medial septum	-	-	ventrolateral	+	+
rostroventral septum	-	-	reticular	+	+
endopiriform nucleus	-	-	ventromedial	+	+
lateral amygdala	-	-	paraventricular	+	+
BMA	+	-*	centromedial	+	+
MeA	+	+	mediodorsolateral	+	+
BAOT	+	+	V/D LGN	+	+
ACo	+	-*	posterior	+	+
			subthalamic	+	+
SUBPALLIUM			HYPOTHALAMUS		
olfactory tuberculum	+	+	dorsomedial	+	+
striatum	-	-	posterior	+	+
ventral pallidum	-	-	lateral	+	+
nucleus accumbens	**ND**	**ND**	ventromedial	+	+
bed nucleus stria	**ND**	**ND**	MIDBRAIN/HINDBRAIN		
lateral olfactory tract	**ND**	**ND**	substrantia nigra	-	-
NON NEURONAL			ventral tegmental area	-	-
corpus callosum	-	-	superior colliculus	+	+
fimbria	-	-	deep gray of SC	+	+
external capsule	-	-	periaqueductal gray	+	+
fornix	**ND**	**ND**	deep mesenphalic	+	+
internal capsule	-	-	raphe nucleus	**ND**	**ND**
anterior commisure	-	-	pontine	**ND**	**ND**
anterior commisure	-	-	vestibular	**ND**	**ND**
			cerebellum	-	-

Table 3. VGLUT2 mRNA expression in areas of the brain known to express EMX1. Plus signs indicate presence of expression and a minus sign is none or low amounts of expression. ND is not determined.*

4.4 Conservation of histology and expression of VIAAT and Nurr1

To verify conservation of histology in the VGLUT2 knockout, *in situ* hybridization analysis with the Nurr1-probe was performed. To further verify if the vesicular inhibitory amino acid transporter (VIAAT) was affected in the knockout, *in situ* hybridization with a VIAAT-probe was conducted and the result is shown in Figure 7.

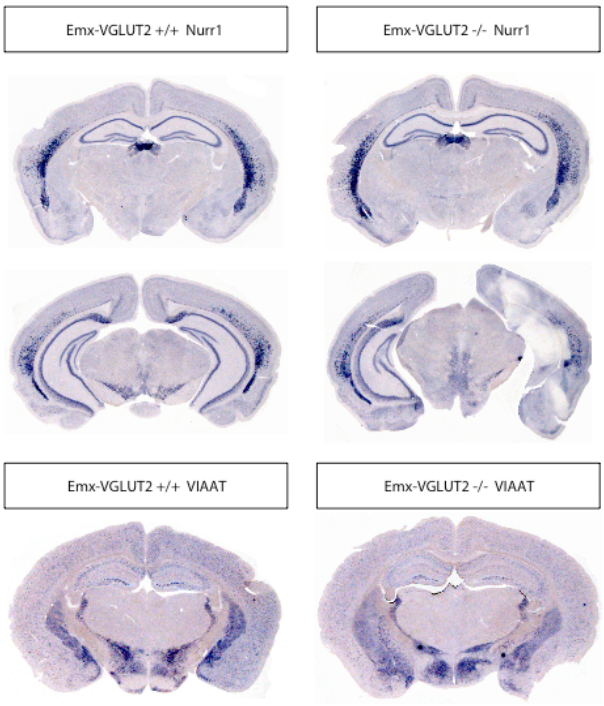


Figure 7. In situ hybridization analysis on 60 µm vibratome sections with BM purple AP staining displaying presence of Nurr1 and VIAAT mRNA (purple staining).

4.5 Detection of protein levels with immunofluorescent analysis

In order to see which nuclei that showed Cre-activity, an immunofluorescent analysis was made on 12 µm cryo sections from mice with the Tau^{mGFP} (SAM) construct. Expression of the nuclear red fluorescing LacZ and the green fluorescing GFP are activated in the cells expressing Cre. This is due to the inherent construction of the Tau^{mGFP} construct which allow detection of cells that express Cre by immunohistochemistry with beta-gal and GFP antibodies, respectively. This is depicted in Figure 8. The result is red nuclear staining and green staining of the projecting axons from these nuclei. Blue general nuclear staining is a result of the DAPI addition in the protocol.

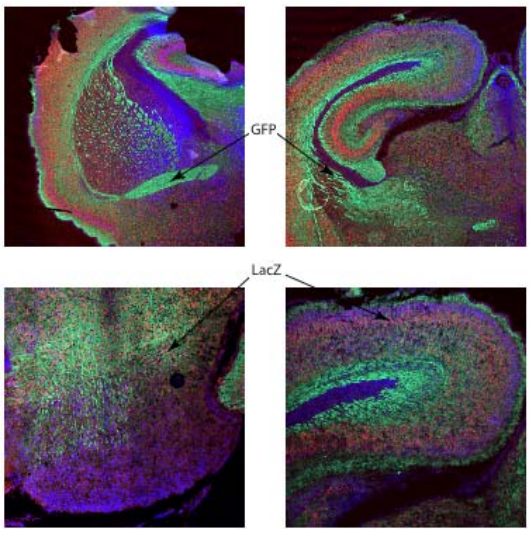


Figure 8. Immunofluorescense on 12µm cryosections with the Tau^{mGFP}(SAM) and EMX1-Cre present in the mouse. Tau-GFP(green) shows projections from Cre active nuclei, nls-LacZ (red) shows nuclei expressing Cre and DAPI(blue) is used as a general nuclear staining.

4.6 Half-brain in situ hybridization and tracing experiments

In the initial trial to evaluate the tracing method and time of incubation, experiments were conducted on *wildtype* brains by insertion of the DiI crystals in the ventral tegmental area (VTA). Time of incubation was 2-3 weeks based on an initial estimation of the projecting path length of the axons and the fact that the crystals travel 0.3 mm per day. These projections are shown in Figure 9.

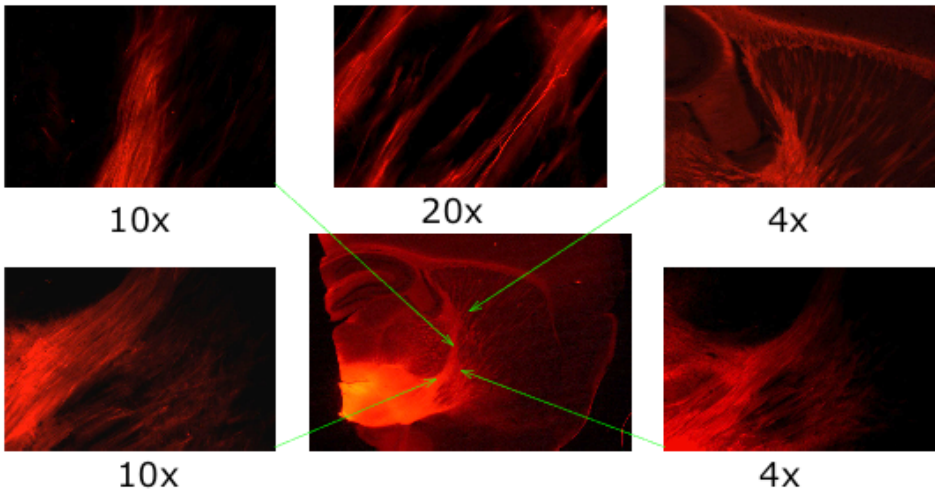


Figure 9. Insertion of DiI crystals in the ventral tegmental area (VTA). There are numerous axons projecting from this site and they are colored red.

The second trial used a modification of the floating section *in situ* hybridization protocol to directly stain the presence of VGLUT2 in half-brains. This was used as guidance when inserting the DiI crystals in one of the areas, RSG, subiculum and amygdala. The second trial used an incubation time of 5 weeks and remaining brains are being analyzed at the moment. These results are shown in Figure 10.

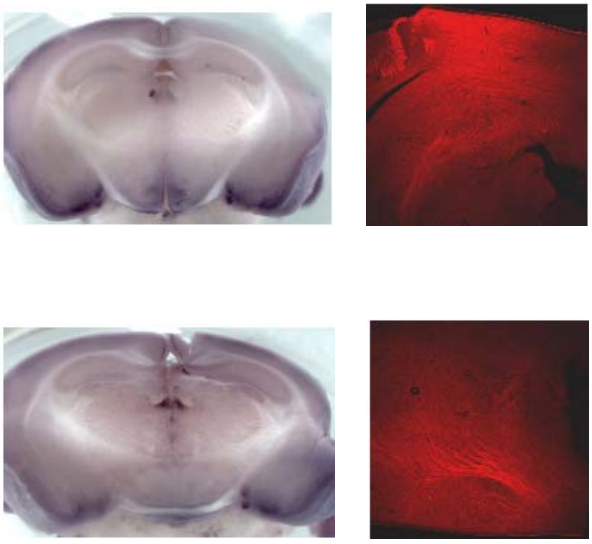


Figure 10. Half-brain in situ hybridization with VGLUT2 staining in the RSG, subiculum and amygdala. Below are initial results of projecting pathways from insertion in the RSG region of the cortex.

4.7 Investigation of satb2 and neurotensin probes

To achieve a more specific binding to single areas within or consisting of the RSG, subiculum and amygdala there was an evaluation of new probes specific for one or more of these areas. The selection of probes was made by my supervisor and applied in an ordinary *in situ* hybridization protocol on 60µm floating sections. Satb2 primarily target the cortex including the RSG while neurotensin target the subiculum, subthalamic nucleus, substantia nigra and the ventral tegmental area (VTA) among other areas. These stainings are shown in Figure 11.

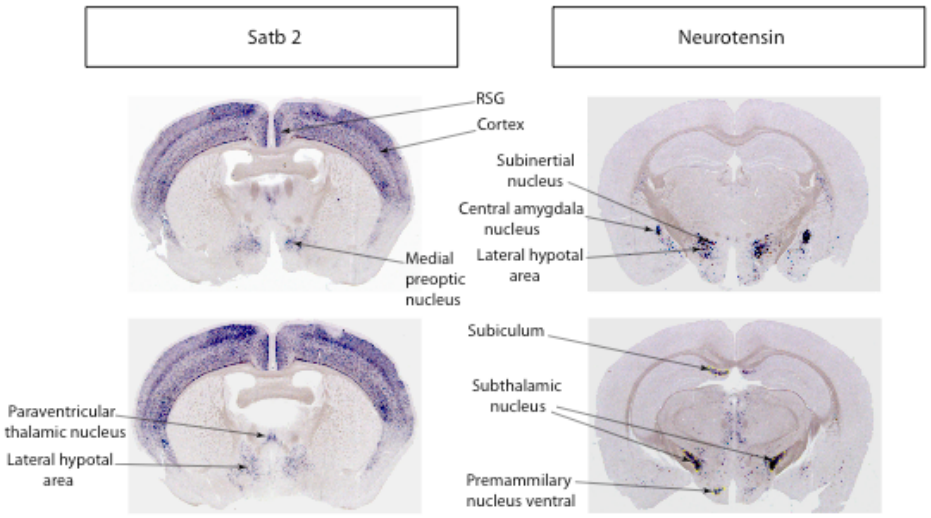


Figure 11. In situ hybridization analysis on 60 µm vibratome sections with BM purple AP staining displaying areas of expression for satb2 and neurotensin mRNA (purple staining).

4.8 Social dominance test

The result of the social dominance test was non-significant for both the females and males as separate groups as well as the entire test sample. These results are summarized in the tables and diagrams below. This experiment was conducted with support from Karin Nordenankar, a Ph.D student in the lab.

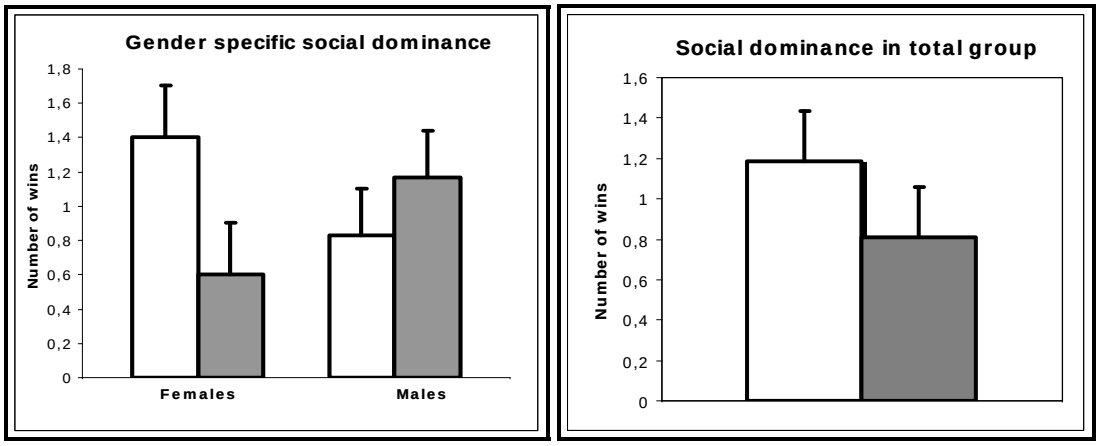


Figure 12. The diagram to the left show the social dominance for the female and males as separate groups. Grey bars indicate knockout mice and white indicate control mice. Collected data for males and females grouped together is shown to the right, again with grey bar for knockout and white for control mice. Illustration used with permission from Karin Nordenankar.

Entity	*Females*	*Males*	*Total group*
Mean CTRL	*1,4*	*0,75*	*1,1875*
SEM CTRL	*0,3055*	*0,25*	*0,2454*
SD CTRL	*0,9661*	*0,8660*	*0,9501*
p-value	***0,0806***	***0,2703***	***0,2883***
Mean KO	*0,6*	*1,1667*	*0,8125*
SEM KO	*0,3055*	*0,2706*	*0,2454*
SD KO	*0,9661*	*0,9374*	*0,9715*

Table 3. Statistical calculations of the social dominance experiment. The lowest level of significance in a two-tailed p-test is a value below o,o5 which none of the groups displayed. The tests were based on a sample of 17 females with 7 being controls and the remaining knockouts. On the male side there were 10 controls and 12 knockouts. The SEM-value is standard error of the mean, SD-value divided by Square root of these elements and constitute the bars in the diagram of picture 12.

5. Discussion

With the Emx1-VGLUT2 project there have been some clarifications on the role that VGLUT2 plays in the developing mouse brain due to the early onset (E9) of the Emx1-Cre promotor. This has previously not been possible. The significance of glutamate signaling in connection to schizophrenia has also been investigated further and the possibility of a schizophrenic-like phenotype for the Emx1-VGLUT2 has been evaluated and will be characterized further in the future.

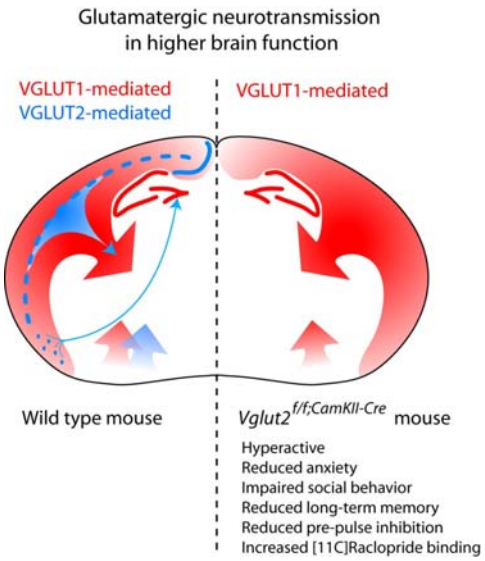


Figure 13. Summary of the CamKII-VGLUT2 phenotype. Arrows represent projecting neurons from areas in the cortex, hippocampus and amygdala. Left side is the wildtype mouse and right side is the CamKII-VGLUT2 knockout. Illustration used with permission from Åsa Mackenzie.

Figure 13 shows a complete summary of the CamKII phenotype including projections from cortical areas and amygdala. The specific physiological characteristics were hyperactivity, reduced anxiety, impaired social behavior, reduced long-term memory, reduced pre-pulse inhibition and increased [11C]Raclopride binding, which suggests altered dopaminergic activity.

These results can in a sense be explained by the inherent functions of the deleted areas. For instance RSG, have important contributions to spatial navigation and learning. Lesion studies on this region have shown that rats with RSG removed are worse at completing a water maze task, which measure long term memory [18-19]. This correlation was shown in the CamKII-VGLUT2 mouse which had deletions in the RSG region. Emx1-VGLUT2 has not yet been investigated in this matter but has shown the same deletion in the RSG, although the temporal difference between them might also be important. The subiculum is on the other hand an important region for interactive

signaling between the cortex and hippocampus. The functional separation is in a dorsal and ventral part and the dorsal is mainly concerned with processing of information regarding space, movement and spatial memory. The ventral part on the other hand is principally an interface between the hippocampal formation and the hypothalamo pituary adrenocortical (HPA) axis. This also relates to spatial memory and the water maze task and both CamKII-VGLUT2 and Emx1-VGLUT2 had total deletion in this region.

When comparing the CamKII-VGLUT2 and Emx1-VGLUT2 mice in the amygdala region there are important similarities but also differences. CamKII-VGLUT2 had total deletion in the BMA, MeA, BAOT and ACo while Emx1-VGLUT2 showed partial deletion in the BMA and ACo while BAOT and MeA were seemingly unaffected. Amygdala consists of 10 nuclei and is connected to responses in emotion, anxiety and aggression. The anatomical region responsible for these emotional responses is called the basolateral complex. This region projects unconscious and conscious emotions to the hypothalamus, gyrus singuli and orbitofrontal cortex. Lesions or other similar damages on the amygdala can result in a lack of recognition of facial expression and also an inability to use both biological and learned responses such as conditional responses.

Sufficient behavioural analyses had not yet been performed on the Emx1-VGLUT2 mice, at the time point when I completed my part of the project, to draw any conclusions whether the Emx1-VGLUT2 also has schizophrenia (SZ)-like behavior, but the expression pattern detected still raises interesting questions. For instance whether certain areas in the amygdala are more or less important for expressing a SZ phenotype and if the Emx1-VGLUT2 possibly will show a milder form of SZ-like behavior. This can in that case give important information in the different aspects of the disease, both temporally and functionally in the sense that some of the characteristic might be fulfilled while others are missing. This could potentially give rise to greater understanding of the genetic factors coupled to the disease and also possibly new ways of identifying it in an early age.

Further on, differences between the CamKII-VGLUT2 and Emx1-VGLUT2 mice were shown in the additional areas claustrum, VeN and piriform cortex. Emx1-VGLUT2 had near total deletion in the claustrum and partial deletion in the VeN and piriform cortex while CamKII-VGLUT2 was unaffected in these areas.

The claustrum is most prominent in the in-and-out traffic of the cortical areas relating to touch, vision and auditory functions, but it is still unclear which role it has in this signaling. There is however speculations of which behavioral role claustrum can have and the hypothesis is that it has a more underlying function in our perception of consciousness in mammals [20]. This description most certainly relates to the disease of SZ but has not been verified thoroughly in the Emx1-VGLUT2.

The piriform cortex is sometimes called olfactory cortex because it process signals from the mitral cells in the olfactory bulb. It is however also connected to the entorhinal cortex and amygdala of the limbic system, mediodorsal and midline amygdaloid nuclei of the thalamus and the ventral part of the nucleus accumbens. Conclusively it has to do with functions relating to emotional and olfactory processing. It remains to see if deletion of VGLUT2 in this region is modulating the inherent functions. Further on there was also partial deletion in the VeN that is closely related to the piriform cortex in both function and axonal projections. The VeN project heavily to the entorhinal cortex, insular cortex, orbital cortex and all cortical amydaloid areas. Lighter projections are directed to perihinal cortex, olfactory tubercle and subdivisions of the hippocampal areas. The differences in efferent signals between the piriform cortex and VeN are foremost in two areas, the medial cortical amygdaloid nucleus that receive a big input from VeN but not from the piriform cortex and the hippocalpal formation that also only receive projections from the VeN but not from the piriform cortex [21]. In relation to these areas there have been a hypothesis regarding the induction of epileptic seizures that originate from these regions. The overall function of these areas in olfaction and memory consolidation could have an effect on the behavior of the Emx1-VGLUT2 mice, which future experiments will test.

Expression in the olfactory bulb was not sufficiently characterized in the CamKII-VGLUT2 mouse and no concrete conclusions can be drawn concerning a potential difference between the strains in this area. The deletion in the periglomerular and mitral cells in the Emx1-VGLUT2 is however very interesting because of the connection between olfaction and behavior. In the olfactory system, odors are transduced to synaptic signals by the olfactory receptor neurons (ORN) and axons projecting from this area make up the olfactory nerve that terminates in the olfactory bulb. The first integrative cell layer is the periglomerular that is responsible for the initial integration of information. Two important molecules that are involved in transferring information from this region are dopamine and GABA. The partial deletion of VGLUT2 in the periglomerular layer could hypothetically indicate that some of the transduction of odors is lost or modified which relates to our hypothesis of glutamate and dopamine signaling which suggests that, at least in the striatum, down-regulation of glutamate signaling results in up-regulation of dopamine signaling. This relates to downstream processing as well, for instance in the mitral cell layer that had total deletion of VGLUT2 expression. Mitral and tufted cells are the output neurons of the olfactory bulb that communicate with other olfactory related areas. They use glutamate, aspartate, GABA, N-acetylaspartyl and corticotropin releasing factor (CRF) as neurotransmitters. The output signal goes to various areas such as olfactory peduncle (AON), piriform cortex (PC), olfactory tubercle (OT), entorhinal cortex (Ent) and some of the amygdaloid nuclei [22].

A functional olfaction is important for many physiological and behavioral responses including neuroendocrine regulations, emotional responses and aggression. The impact of the deletions has not yet been fully characterized and the initial evaluation, between knockouts and wildtype of the Emx1-VGLUT2 on the social dominance test, did not show significant results as opposed to the previous CamKII-VGLUT2. In the social dominance test, a big factor is recognizing the more dominant of the two mice. This process of selection is a combination of many factors including interpretation of dominant scents and can be a reason for the non-significant results of this test.

In situ hybridization and immunofluorescense analysis

The various probes in the *in situ* hybridizations were done according to the presented protocols in all cases but the half-brain test where there were some variations of parameters. Those were in the amount of reagents put in the 6-plate wells, and the time of incubation.

The initial results from the half-brain *in situ* hybridization/tracing did not show the expected level of tracing with the DiI crystals from the RSG region. There is a possibility that the treatment of doing *in situ* hybridization is somehow destroying the axons or the lipid walls that DiI crystals are dependent on. For instance the variation of H_2O_2 might somehow break down the surface membranes where tracing is initiated. This could modify the efficiency of the tracing if the crystals are more widely diffused when incubated.

In situ hybridization with the Nurr1 and VIAAT probes was done to test for synergetic effects of the VGLUT2 targeted deletion. Nurr1 and VIAAT *in situ* hybridizations are performed in exactly the same manner as the VGLUT2 with the only difference being in selection of the binding probe and incubation time. Nurr1 is an intracellular transcription factor that plays a significant role in maintenance of the dopaminergic system and in our experiments function as a marker for histological differences between the controls and knockouts of VGLUT2, due to its abundant expression in the brain. VIAAT on the other hand is expressed in inhibitory neurons signalling with the neurotransmitters glycine and GABA. The expression profile of Nurr1 is unchanged between knockout and controls meaning that the Emx1-VGLUT2 does not change the gross histology of the brain. This is an important result because you want to rule out the possibility that the observed behavior is coupled to changes in the dopaminergic system and deletion of any anatomical regions. It was also found that the VGLUT2 knockout does not change the presence of the vesicular inhibitory amino acid transporter mRNA, which is a good indication that GABAergic and glycinergic neurons are not affected by the Emx1-VGLUT2.

In the immunofluorescent analysis on Emx1-VGLUT2/Tau^{mGFP} mice, expression of Cre removes VGLUT2 exons 4-6 but it also removes the stop codon in the Tau^{mGFP} that gives an expression of nls-associated LacZ and tau-associated GFP. Projecting fibers turn green as a result of immunohistocemical analysis of the GFP. In the results, I have shown that there are numerous Cre-containing nuclei in the cortex and other areas that seem to project to lower levels in the cortical regions and further down to the striatum and also in the anterior commisure anterior and anterior commisure posterior. The anterior region is a communicator between the olfactory regions, the posterior part is more connected to the frontal cortex with connections to behavior. These results imply that there is Cre-activity in the olfactory regions, possibly in the olfactory bulb which was also shown with *in situ* hybridization.

Tracing experiments

The initial tracing experiments with DiI crystals exemplified some of the projecting pathways that can be seen with the immunofluorescent analysis. This type of analysis is primarily done to verify where the final projection site is localized which was our intention with the following experiments using *in situ* hybridized half-brains. Insertion of DiI crystals were done in various regions where VGLUT2 in deleted in the Emx1-VGLUT2 and CamKII-VGLUT2 mice, such as RSG, subiculum and amygdala. There was not sufficient time in my project to analyze all these tracing experiments but when analyzing one initial hindbrain with insertion to the RSG region there was no significant projecting paths to be found. This could be due to the harsh *in situ* treatment or site of insertion. No conclusion could be drawn from this analysis but future experiments will hopefully unravel the paths of projection from the affected areas in the Emx1-VGLUT2 mouse.

Behaviour

The result of the social dominance test is a clear distinction from the earlier studies done on CamKII-VGLUT2 where a significant difference in the level of social dominance was shown towards wildtype mice. This can be the result of different factors such as the incomplete deletion in the amygdala nuclei or other deletions in for instance the mitral and periglomerular olfactory regions. Some factors that can be attributed to the olfactory region are the ability to sense male and female scents, including the pheromones. More testing is however needed in order to make any distinct assessment on possible schizophrenia-like or other characteristic behavior changes.

6. Aknowledgements

I would like to thank my supervisor Åsa Mackenzie for support and valuable input to the project, Karin Nordenankar for excellent and patient introduction to the various techniques used in the project and for assistance with the statistical evaluations in the social dominance test. I also would like to thank the rest of the Kullander lab and surrounding labs for good times and great discussions and for the very giving questions during the first presentation at the institution, which gave valuable feedback for writing the final discussion and lifting up important issues in the presentation.

7. References

1. Chen, J., B.K. Lipska, and D.R. Weinberger, Biol Psychiatry, 2006. **59**(12): p. 1180-8.
2. Marino, M.J., O. Valenti, and P.J. Conn, Drugs Aging, 2003. **20**(5): p. 377-97.
3. Nestler, E.J., et al., Neuron, 2002. **34**(1): p. 13-25.
4. Herzog, E., et al., J Neurosci, 2001. **21**(22): p. RC181.
5. Takamori, S., et al., Nature, 2000. **407**(6801): p. 189-94.
6. Bellocchio, E.E., et al., Science, 2000. **289**(5481): p. 957-60.
7. Fremeau, R.T., Jr., et al., Neuron, 2001. **31**(2): p. 247-60.
8. Fremeau, R.T., Jr., and K. Kam, Scienceexpress, 2004. p. 1-10.
9. Takamori, S., and P. Malherbe, EMBO, 2002. **3**(8): p. 798-803.
10. Wallén-Mackenzie, Å., et al., J Neurosci, 2006. **26**(47): p. 12294-12307.
11. Schuske, K., and E.M., Jorgensen, Science 2004. **304**: p. 1750-52.
12. Fremeau, R.T., Jr., et al., Science, 2004. **304**(1815): (10.1126/science.1097468).
13. Wojcik, S.M., et al., Proc. Natl. Acad. Sci. U.S.A., 2004. **101**(7158) [Medline].
14. Wallen-Mackenzie, Å., et al., 2008, manuscript in preparation.
15. Laruelle, M., L. Kegeles, and A. Abi-Dargham, Ann N Y Academy of Science, 2003. **1003**: p. 138-58.
16. Sawa, A., and S.H. Snyder, Science, 2002. **296**(5568): p. 692-5.
17. Gorski, J.A., and T. Talley., J Neurosci, 2002. **22**(15): p. 6309-14.
18. Donald, P.C., et al., Behavioral Brain Research, 2006. **170**: p. 316-25.
19. Harker, K.T., and I.Q. Wishaw., J Neurosci, 2002. **22**(3) p. 1155-64.
20. Raman, F.E., et al., Brain Research, 2007. **1159**: p. 94-111.
21. Behan, M., et al., J Comparable Neurology 1999. **408**: p. 532-48.
22. Shipley, T.S., and M. Ennis, J Neurobiology 1995. **30**(1): p. 123-176.
23. Purves, D., et al., 2001, Neuroscience 2nd Edition, Sinauer Associates Inc. Publishers, Sunderland, Massachusetts.